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THE INFLUENCE OF SOCIODEMOGRAPHIC FACTORS, PERINATAL CHARACTERISTICS, SELF-ESTEEM, AND MOTHER'S SOCIOCULTURAL ATTITUDES TOWARDS PHYSICAL APPEARANCE ON FIRSTBORN BREASTFEEDING RATE AND DURATION IN THE REPUBLIC OF CROATIA

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ABSTRACT

Aim: Breast milk is the only form of nutrition that provides optimal conditions for growth and development of an infant, while it also provides a close connection between a mother and her child. Due to the activities of multiple organizations which promote breastfeeding and education of mothers, the rate of breastfeeding is increasing in Croatia. The aim of this study was to determine the influence of sociodemographic characteristics of the mother and perinatal particularities to breastfeeding and the length of breastfeeding of the first child.
Materials and methods: For the purpose of this study, an on-line questionnaire that consisted of 48 questions was designed and subsequently completed by 571 mothers from all parts of Croatia.
Results: The first child was breastfed by 526/571 (92.12%) examinees. The results of the research demonstrated that the place of residence did not significantly affect the rate and duration of breastfeeding (p=0.054). A positive correlation between their monthly income (p=0.004), level of education (p=0.016), and the rate and duration of breastfeeding was established. The decision to breastfeed was influenced by complications during pregnancy (p=0.034), but the same connection was not established for the duration of breastfeeding. Mothers who attribute greater importance to physical appearance have breastfed their child for a shorter period of time (p=0.012). No statistically significant connection was established between the levels of self-respect of examinees and breastfeeding (p=0.737).
Conclusion: The level of education and monthly income have a significant influence on both the decision to breastfeed and on the duration of breastfeeding of the first child. Therefore, educational programmes should be intensified among young mothers and among those with a lower level of education. Furthermore, the results of this research have demonstrated a negative influence that standards of modern society impose on the physical appearance of women, which, as a result, affects their decision to breastfeed.

Keywords: breastfeeding, sociodemographic characteristics, self-respect, physical appearance

INTRODUCTION

It is an unquestionable scientific truth that breast milk the is best food for infants because it satisfies all of their needs and a natural diet should always be encouraged (Tješić-Drinković et al., 2014). It contains many immune factors that protect the child from infection, including probiotic strains of bacteria. The chemical composition of breast milk provides the infant with the optimal conditions for growth and development. From the very beginning, it allows an emotional connection between the mother and the child, and aside from being the healthiest way to feed a new-born it is also the cheapest way, which is not negligible for the majority of the population. It reduces the risk of atopy, obesity later in life, and the development of some chronic diseases such as diabetes (Arenz et al., 1999). It also programs the systems in the body that help regulate blood pressure. In addition to the benefits for the child, breastfeeding is also beneficial for the mother. Women who breastfeed have a lower risk of breast cancer and some forms of ovarian cancer, as well as osteoporosis later in life (Lancet, 2002, Čatipović, 2013). It enhances uterine contractions, leading to rare postpartum bleeding. Breastfeeding helps the body to return to its weight before pregnancy, by reducing the build-up of fatty tissue (Čatipović, 2013).

The World Health Organization recommends exclusive breastfeeding for the first six months, meaning breastfeeding without adding liquids and other foods (Hörnell, 2013.). At the moment of introduction of complementary foods, breastfeeding should be continued for as long as the mother and child want to. It is essential that trained staff in the hospital help mothers initiate breastfeeding and provide support through continuous monitoring (Čatipović, 2013). "Maternity Hospital - Friends of Children" (Baby Friendly Hospital Initiative - BFHI) is an initiative which was launched in 1991 by The World Health Organization and UNICEF in order to protect, support, and promote breastfeeding, which is still carried out (Grgurić, 1996; Grgurić, 2014). In 1993, UNICEF and the Ministry of Health initiated a programme titled Promoting Breastfeeding in the Republic of Croatia and within five years the implementation of the programme resulted in the total of 34 maternity wards in Croatia from 15 hospitals achieving the title of a "Maternity Hospital - Friends of Children", and Croatia had achieved the third place in Europe, behind Sweden and Norway, for the number of hospitals called "Maternity Hospital - Friends of Children" (Zakanj, 2001). The Committee on the Rights of the Child and the National Plan for the Rights and Interests of the Child recommended that the Republic of Croatia, as a Member State, pays more attention to the effective promotion of proper breastfeeding practices in line with international standards. Today, 30 out of 31 maternity wards bear the title "Maternity Hospital - Friends of Children", resulting in the growing number of breastfed infants in maternity wards, and in an increasing number of exclusively breastfed children for the first months of life. All Croatian maternity wards have a common room for the mother and the child after birth (Rooming-in), which was not the case in Croatian maternity wards before the implementation of programmes for promoting breastfeeding in Croatia (WHO, 2002).

The aim of this study was to determine the influence of sociodemographic characteristics of the mother and perinatal particularities to breastfeeding and the length of breastfeeding of the first child.

MATERIALS AND METHODS

Participants

The study included mothers from all over Croatia. A total of 571 mothers participated and data on the age and the place of residence at the time they gave birth to their first child was collected. Most subjects were from the Zagreb area, 212 (37.1 %), then from Slavonia, 179 subjects (31.3 %), from Dalmatia 88 (15.4 %), and 54 women (9.5 %) were from Istria and Kvarner, 27 from Zagorje (4.7 %), and the least from Lika and Banovina, only 11 subjects (1.9 %).

Methodology

The survey was conducted from 27 to 30 October 2015. We used a questionnaire for data collection, an Internet-type server created in Google Chrome - Google Drive. This type of survey made it possible to access a large number of women, provided them with anonymity and ensured data protection, with the option of excluding participants from the survey without affecting the further course of the study. The survey was divided into several Facebook pages and groups, which are thematically related to parenting and gather a large number of young mothers. The survey was voluntary with the possibility of interruption at any time. In case of cancellation of further fulfilment, the data would remain recorded. After a sufficient amount of data has been collected, the survey has been removed from the Internet.

Questionnaire

The survey consisted of four groups of questions. The first group consisted of four questions on the general socio-demographic characteristics at the time of birth of their first child, such as age, place of residence, completed qualifications, and the monthly income of the mother at the time of the birth of her first child.
The second group consisted of 11 questions about the child's age at the time of filling out the survey, the decision to breastfeed, and the length of breastfeeding, the reasons not to breastfeed, or the reasons for weaning, and the data on the possible introduction of an infant formula. The questions about the reasons for not breastfeeding or weaning offered predefined responses, including the response "other". After that, there were questions about perinatal characteristics, such as pregnancy complications and premature birth.
The third group was the Rosenberg's Self-Esteem Scale (Rosenberg, 1965; Mirjanić, 2011). The Self-Esteem Scale consists of 10 statements that assess the level of self-esteem. Agreement with the statement is expressed according to a scale consisting of four degrees of agreement (fully agree, partly agree, partly disagree, completely disagree). The minimum number of points that can be achieved is zero, and the maximum is 30. The range from 15 to 25 is considered to be normal; people with less than 15 points are assessed as those with low self-esteem, and those with the score of more than 25 as people with high self-esteem.
The fourth group of questions was a scale of sociocultural attitudes about outward appearances. The authors of the scale of sociocultural attitudes about outward appearances,

„Sociocultural Attitudes Toward Appearance Questionnaire", are Heinberg, Thompson, and Stormer (Heinberg et al., 1995; Matić, 2008). It consists of 44 claims, and because of the similarity among the claims, 10 of them were used in this study, 6 of which are related to awareness and 4 to the internalization of social norms. The answers are scored from 1 to 5, so the minimum number of points is10 and the maximum is 50.

Data processing

Data collected by the survey was processed in SPSS / PC. We used descriptive statistical measures such as mean, standard deviation (SD), and range (minimum and maximum value). To calculate statistically significant difference, we used the Student's t-test and the ANOVA test, and to see the correlation we used the Pearson correlation coefficient. A P-value less than 0.05 was considered statistically significant.

RESULTS

Of the 571 total respondents, 526 of them, or 92.12%, breastfed their first child, of which 32.89% used additional infant formula. The average length of breastfeeding was 12.49 ± 9.10 months. The minimum length of breastfeeding was one month and the maximum was 48 months.

The average age at the time of birth of the first child for our respondents was 25.95 ± 4.35. The youngest primipara was 13, while the oldest was 40 years old. The average age of women who breastfed was 26.09 ± 4.30, and for those who did not breastfeed it was 24.42 ± 4.78, with an established statistically significant difference ($t = 3.163$, $p = 0.002$; $p < 0.05$). A positive correlation between the mothers' age and the length of breastfeeding was also established ($r = 0.182$; $p = 0.000$; $p < 0.05$).

No connection was established between regional affiliation and breastfeeding. However, due to the non-representative sample of certain regional parts of Croatia, such results cannot be considered relevant. The influence of the monthly income on the rate of breastfeeding was not determined, but we demonstrated a significant positive correlation ($F = 3.091$; $p = 0.004$; $p < 0.05$) between the duration of breastfeeding and the monthly income (Table 1). There was also a statistically significant positive correlation between higher levels of education and breastfeeding rates ($t = 2.935$, $p = 0.016$; $p < 0.05$), as well as between a higher level of education and the length of breastfeeding of the first child ($F = 3.476$, $p = 0.016$; $p < 0.05$). (Table 2 and 3).

The influence of complications during pregnancy on the decision to breastfeed was statistically significant ($t = 2.134$, $p = 0.034$; $p < 0.05$) (Table 4), while their influence on the duration of breastfeeding was not established ($t = -0.243$; $p = 0.808$, $p > 0.05$).

The scale of sociocultural attitudes about outward appearance has a range of 10 to 50, which incidentally were the minimum and maximum score achieved among our respondents. The average score on the scale for our respondents was 23.94 ± 9.23. In the group of women who breastfed their first child, the average score was 24.41 ± 9.32, and in the group of women who did not breastfeed their first child it was 20.8 0 ± 7.45. The difference between these two groups was statistically significant ($t = 2.8\,81$, $p = 0.006$; $p < 0.05$).

Looking at the correlation between the length of breastfeeding and the results of the scale of sociocultural attitudes on the outward appearance, we found a small negative

correlation, with the Pearson coefficient of -0.118, and a coefficient of correlation of 0.012 significance ($p<0.05$), indicating that the respondents with a higher acceptance and internalization of sociocultural attitudes about outward appearances were breastfeeding for a shorter period of time.

Table 1. Relation between the duration of breastfeeding and the monthly income

Monthly income	Duration of breastfeeding (months)		p
	Average	Standard deviation	
Less than 550 euro	12.55	10.68	0.004
From 550 to 1075 euro	11.31	8.20	
From 1075 to 1600 euro	14.38	9.31	
From 1600 to 2100 euro	14.55	8.55	
More than 2100 euro	11.06	7.26	

Table 2. Relation between the share of women who breastfed and the level of education

Level of education at the time of giving birth to the first child	Mothers who breastfed		Mothers who did not breastfeed		p
	N	%	N	%	
Elementary	4	80.00	1	20.00	0.016
High school	236	89.15	32	10.85	
Bachelor's degree	77	96.25	3	3.75	
Master's degree	182	95.29	9	4.71	

Table 3. Relation between the duration of breastfeeding and the mothers' level of education

Level of education at the time of giving birth to the first child	Duration of breastfeeding (months)		p
	Average	Standard deviation	
Elementary	2.75	1.26	0.016
High school	11.30	8.97	
Bachelor's degree	11.92	8.58	
Master's degree	14.91	9.13	

Table 4. Relation between complications during pregnancy and the share of women who breastfed

Were there any complications during pregnancy?	Women who breastfed		Women who did not breastfeed		p
	N	%	N	%	0.034
Yes	112	86.82	17	13.18	
No	414	936. 6	28	63. 4	

DISCUSSION

The sociodemographic profile of mothers who breastfeed in Croatia is consistent with that of developed, industrialized countries. According to a research conducted in 2011 among 500 Croatian mothers, the share of women who breastfeed, as well as the length of breastfeeding, is proportional to the women's age and increases as their age increases (Berović, 2003).

The literature often mentions the impact of housing on the decision about breastfeeding and the duration of breastfeeding in the context of the difference between urban settlements and the rural location (Hoyer, 1994). A research conducted in Slovenia determined a difference in the length of breastfeeding, but not between the proportion of lactating women in urban and rural areas. In our study, we compared the difference between the share of nursing mothers and the length of breastfeeding in different regions of the Republic of Croatia, not taking into account the structure of the settlement where the mother lives. In line with our set hypothesis, we did not establish a statistically significant difference in the rate of breastfeeding among regions. Our results are opposite to a research from 1996, according to which the mothers in Istria, Croatian Primorje, and Gorski Kotar breastfed for a significantly longer period than mothers in Slavonia and Zagreb (Zakanj, 2000). The same survey indicates that the length of breastfeeding in 1996 was significantly higher in areas of Croatia that were not affected by the war, as opposed to those that were. The average length of breastfeeding in areas affected by war was 2.7 ± 2.1 months, while in the parts not affected by war it was 3.7 ± 3.1 months, which is certainly considerably less than the average length of breastfeeding demonstrated in our study (12.49 ± 9.0 months), with a note that our study only investigated the duration of breastfeeding of the firstborn child (Zakanj, 2000).

Furthermore, the monthly income of the family has proven to be, as expected, a statistically significant factor that influences the length of breastfeeding. However, there was no statistical difference determined for the decision to breastfeed. In fact, contrary to expectations, considering that breastfeeding is the most economically advantageous form of diet for a child, mothers with higher family income were breastfeeding longer than those with lower income, excluding the group with the highest income (more than 2100 euro per month). Results similar to ours were obtained in the research from 2001, which has not demonstrated a significant difference in the proportion of lactating women in relation to family income. In her research, Hoyer explored the difference between mothers who have their own income and those that do not, and established a statistically significant difference in the length of breastfeeding, i.e. mothers who do not have their own income breastfed for a longer period of time, which can be understood as the absence of pressure on women to return to work (Hoyer, 1994).

Another important socio-demographic factor, the education level of the mother, proved to be statistically significant in both the decision to breastfeed and for the length of breastfeeding. According to our study, mothers, who at the time of birth of the first child have completed college and university education, opted more often for breastfeeding and breastfed longer than mothers who completed elementary and secondary education. The aforementioned study from 2003 demonstrated that mothers with the higher level of education breastfeed more often; for women with only primary education, it was only 31.3 % of lactating women, while among university-educated mothers, the percentage rises to

53.8 %. A study conducted on 773 mothers in the clinical hospital centre Split determined that mothers with higher education breastfed longer, as confirmed in a study conducted among Slovenian mothers (Zakarija-Grković, 2016). In contrast, Čatipović et al. mentioned that mothers with higher education gave up breastfeeding at the very beginning, so at the end of the child's first year, there was a greater proportion of mothers with lower education who breastfeed than those with higher education (Čatipović, 2002).

The importance of breastfeeding for premature infants is underlined, as there is a significant difference in the mortality rate of premature babies depending on breastfeeding (Frković, 2002). Čatipović et al. expressed concern about early weaning of children with the Apgar score below 7, lower birth weight, and premature children, and came to the conclusion that women who gave birth by Caesarean section were significantly more likely to give up breastfeeding in the early period when compared to those who have given birth vaginally (Čatipović, 2002). However, this number equalises in the later period. In this study, we observed the proportion of nursing mothers and the duration of breastfeeding in relation to the potential complications in pregnancy and preterm birth. We came to the result that, as expected, mothers who did not have complications in pregnancy breastfeed more often and, contrary to expectations, there was no significant difference in relation to preterm birth.

In this study, we investigated the relationship of acceptance of sociocultural attitudes about outward appearance and applying them to oneself with the rate and length of breastfeeding. In accordance with the hypothesis, we demonstrated a statistically significant association. A smaller percentage of mothers stated aesthetic appearance as a reason to stop breastfeeding or to not breastfeed at all (Hoyer, 1994). Mothers who accepted and applied the media and social criteria imposed on the importance of external appearance to a greater degree choose to breastfeed less often and breastfeed for a shorter period of time. However, according to the results of a research in Mexico, it seems that mothers who are nursing lose on average 4.1 kg more in a period of 3 months after delivery than those who do not breastfeed. The study stressed the importance of these results in the promotion of breastfeeding as the best option for both the child and the mother (Lopez-Olmedo, 2016).

CONCLUSION

The level of education and monthly income have a significant influence to both the decision to breastfeed and the duration of breastfeeding of the first child. Therefore, educational programmes should be intensified among young mothers and those with a lower level of education. Furthermore, the results of this research have demonstrated a negative influence that standards of modern society impose on physical appearance of women, which, as a result, affects their decision to breastfeed.

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PERCEPTION AND SATISFACTION WITH THE BODY IMAGE ACCORDING TO NUTRITIONAL STATUS AMONG ADOLESCENT BOYS FROM URBAN AND RURAL AREAS OF THE SARAJEVO CANTON

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ABSTRACT

Adolescence is a very sensitive period of life, characterized by intense growth and development, increased energy needs, and experimentation with independence. It is also the period in which body perception is developed, together with appearance dissatisfaction, which leads to risky behavior. It is considered that mostly girls pay attention to their appearance, but in this research, boys are chosen as respondents.
The aim was to evaluate the satisfaction of boys with their appearance based on the actualnutritional status and tendency to risky behavior that leads to eating disorders. Boys were divided into groups according to the place of residence (rural vs. urban).
We used anthropometric measurements (body weight and body height) for calculating BMI and a questionnaire designed for this study. The questionnaire consisted of questions regarding body satisfaction, being on a reduction diet, and the method used for body weight reduction.
Statistical significance is noticed in both groups (urban and rural) in relation to the attempt of reducing body weight (p<0.0005) and in relation to being on a weight reduction diet (rural: p=0.002; urban: p<0.0005). Nutritional status also affects urban boys' satisfaction with their appearance (p<0.0005).
Detailed results suggest that most of the boys in all weight categories are not satisfied with their appearance, most of the normalweight and underweight boys tried to reduce their weight and were on a weight reduction diet, which means that adolescent boys do not have a proper perception of body image according to the actual nutritional status.

Keywords: adolescents, body perception, nutritional status, weight reduction diet

INTRODUCTION

Adolescence is a very sensitive period characterized by intense growth and development. In that period, nutritional demands are very high and their unfulfillment can lead to stagnation of cognitive development and growth. Improper and inadequate nutrition can significantly affect the growth and development of children and adolescents and temporarily, or even permanently, endanger their health (Gibney, 2002). Adolescence is a

period of extreme sensitivity to social pressure from peers, often reinforced by the media, which is reflected in conformity in the behavior, attitudes, and patterns of feeding (Johnston and Haddad, 1996). The search for identity, the struggle for independence and acceptance, and concern about the looks are changes that have a big impact on lifestyle, eating habits, and nutrient intake in adolescents (Spear, 2002).

There are many studies of body image perception among girls showing that they are very conscious of their looks and body weight, and make a huge effort to influence its shape, often through very harsh restrictive diets. These attitudes and behaviors are now more widespread and commonly referred to as the *cult of thinness* (Rukavina, 2002). Diets are identified as a risk factor for eating disorders (McVey et al., 2002) and are associated with many health problems (Fisher et al., 1995). According to Perković et al. (2006), it is of particular concern that reduction dieting is appearing among adolescents with normal body weight.

There is a lack of data regarding boys and body satisfaction/dissatisfaction. Problems related to body image are evidently appearing among men and adolescent boys and need more professional/scientific attention.

The aim of this research was to evaluate boys' satisfaction with appearance based on the actual nutritional status and the tendency to risky behavior that leads to eating disorders.

SUBJECTS AND METHODS

Participants were 324 male adolescents (14-15 years old) living in rural (n=60) and urban (n=264) areas of Sarajevo Canton. The research was conducted in twelve primary schools located in nine municipalities of the Sarajevo Canton and the protocol was approved by the Ministry of Education, Sciences and Youth of the Sarajevo Canton. The adolescents were introduced to the research protocol and participated voluntarily.

The aim of the research was to evaluate boys' satisfaction with appearance based on the actual body mass index and tendency to risky behavior that leads to eating disorders.

We used anthropometric measurements (body weight and body height) for calculating BMI and a questionnaire designed for this study.

To calculate BMI-for-age percentiles, body weight (medical digital balance, OT 150 FWEB Gorenje) and height (portable stadiometer) were measured. BMI-for-age percentiles were defined in relation to the standards of the World Health Organization (de Onis et al., 2007). Information concerning body perception and dieting was collected by means of a questionnaire designed for this study. The questionnaire included questions regarding body satisfaction, being on a reduction diet, and the method used for reducing body weight.

Statistical analysis was performed using the statistical software package SPSS 19.0. (SPSS Inc, Chicago, Illinois, USA).Significant difference was considered at the level of $p<0.05$. For descriptive purposes, we used the arithmetic mean, frequencies, and percentages. For the assessment of normality, we used the Kolmogorov-Smirnov test and when the variables did not satisfy the conditions of normality, non-parametric Mann-Whitney U test was used. Dependence was tested by the Chi-square test and the bond strength by Spearman's rank correlation coefficient (ρ) and t-test for independent samples.

RESULTS AND DISCUSSION

Results of the research are summarized in three tables. Results presented in Table 1 show that the normal weight category, characterized as $5^{th} < 85^{th}$ percentile, is prevalent among both groups of participants (rural *vs* urban: 70.00%:58.30%). It is followed by the overweight (rural *vs* urban: 20.00%:24.20%) and the obesity (rural *vs* urban: 10.00%:14.40%) category. The underweight category is present only among urban boys (3.00%).

In rural areas, BMI does not affect satisfaction with body image (p=0.172, ρ=0.179). Still, more normal weight adolescents (79.17%) and the most obese adolescents (75.00%) were not satisfied with their appearance, while half of the overweight boys were not satisfied. In urban areas, BMI affects satisfaction with body image (p<0.0005, ρ=0.365). The great majority of normal weight adolescents (82.09%) were not satisfied with their body image. Overweight adolescents are equally dissatisfied and satisfied with their appearance, and 78.95% of obese adolescents are satisfied. Overall, adolescents do not have the correct perception of their body image.

Table 1. Correlation between adolescents' BMI and body satisfaction and being on a reduction diet

Place of Residence	BMI categories	Adolescent's BMI	Body satisfaction		p	ρ	Reduction diet		p	ρ
			No	Yes			No	Yes		
Rural	$5^{th} < 85^{th}$	42	38	10	0.172	0.179	4	42	0.002	-0.400
		70.00%	79.17%	20.83%			8.69%	91.31%		
	$85^{th} < 95^{th}$	12	4	4			3	4		
		20.00%	50.00%	50.00%			42.86%	57.14%		
	$\geq 95^{th}$	6	3	1			2	2		
		10.00%	75.00%	25.00%			50.00%	50.00%		
Urban	$< 5^{th}$	8	6	2	<0.0005	0.365	1	6	<0.0005	-0.299
		3.00%	75.00%	25.00%			14.28%	85.72%		
	$5^{th} < 85^{th}$	154	165	36			6	187		
		58.30%	82.09%	52.20%			3.11%	96.89%		
	$85^{th} < 95^{th}$	64	16	16			9	20		
		24.20%	50.00%	50.00%			31.03%	68.90%		
	$\geq 95^{th}$	38	4	15			4	14		
		14.40%	21.05%	78.95%			22.22%	77.78%		

Living in an urban or a rural location influences lifestyle, including changes in eating habits, which are more pronounced among adolescents in urban areas. They quickly accept innovations and are exposed to commercial messages and market (Esposito et al 2009). A study conducted in Croatia shows that consumption of fast food, soft drinks, and alcohol is more widespread and more associated with eating behaviors in urban than in rural areas (Colić-Barić et al., 2004).

The same group of boys (participating in this study) was asked about their eating habits and those answers were correlated with BMI in another study conducted by the first author, Taljić (2015). Results show statistical significance for more observed determinants of eating patterns for urban boys: the number of daily meals *(p*=0.039), regular consumption

of breakfast (p=0.009), the diversity of diet (p=0.049), and eating snacks (p=0.037). Boys who follow a proper eating pattern, they either do not consume snacks or consume them minimally, are within the normal weight category (72.30%). On the other hand, the majority of boys with an improper dietary pattern, who consume snacks more often, are also in the normal weight category (82.50%).Among adolescents who have diverse diet every day, the highest percentage is of those in the category of normal weight (80.80%), and a smaller percentage of those being overweight.A smaller percentage of obese and overweight and a higher percentage of normal weight adolescents regularly consume breakfast.A smaller percentage of obese and a higher percentage of normal weight adolescents have more than three meals a day and follow the form of proper nutrition.

Table 1 also shows that rural adolescents' BMI is correlated with being on a reduction diet (p=0.002, ρ=-0.400). A large percent of adolescents within the category of normal body weight was on a reduction diet (91.31%), as well as more than half of the overweight (57.14%) and half of the obese. Urban boys' BMI is correlated with the reduction diet (p<0.0005, ρ=-0.299). The majority of respondents in all categories were on a reduction diet. Data research conducted in Croatia showed that 50.00% of girls and 16.00% boys were on some kind of a reduction diet (Rukavina, 2002). As much as 49.00% of girls believe that their ideal body weight is less than the actual. It was found that as early as in elementary school, 8.00% of girls aged 11 were on a reduction diet, and that percentage increases to 29.00% at the age of 14 (Pokrajac-Bulian et al., 2002). According to research done by Hodžić and Smajić (2012) among adolescents (13-15 years old) in Sarajevo, reduction diets and BMI were dependent, statistically significant (p<0.0005). In that study, adolescents with normal weight were never on a diet and most of the obese (degree I) went on a diet more than once (32.50%). These data suggest that diet was held only by those who have a problem with obesity.

Results in Table 2 show that in rural areas, BMI affects weight reduction (p<0.0005, ρ=-0.524). Boys with normal weight (71.74%) have tried to reduce their weight, while most of the overweight (87.50%) and all obese boys did not try to reduce it. In urban areas, BMI affects weight reduction (p<0.0005, ρ=-0.421). Among them, even boys with normal weight (79.21%) have tried to reduce their weight.

Table 2. Correlation between adolescents' BMI and weight reduction

Place of Residence	BMI		Weight reduction		p	ρ
			No	Yes		
Rural	$5^{th} < 85^{th}$	%	13	33	<0.0005	-0.524
			28.26%	71.74%		
	$85^{th} < 95^{th}$	%	7	1		
			87.50%	12.50%		
	$\geq 95^{th}$	%	4	0		
			100.00%	0.00%		
Urban	$< 5^{th}$	%	0	8	<0.0005	-0.421
			0.00%	100.0%		
	$5^{th} < 85^{th}$	%	42	160		
			20.79%	79.21%		
	$85^{th} < 95^{th}$	%	20	11		
			64.52%	35.48%		
	$\geq 95^{th}$	%	14	5		
			73.68%	26.31%		

Some were exaggerating since all the respondents in the underweight category have tried to reduce their weight. Overweight (64.52%) and obese (73.68%) adolescents did not try to reduce their weight. It is obvious that adolescents do not have a proper picture of their body mass index.

According to the mentioned study by Hodžić and Smajić (2012) adolescents' opinion about their weight and BMI category were statistically dependent (p<0.0005): 50.00% of underweight and 22.22% of obese boys (degree II) think that their weight is just as it should be, while 10.80% normal weight boys think that they are overweight. Children were not aware of their underweight or overweight and obesity. The same unawareness is present among Croatian adolescents. Perković et al. (2006) found that according to the actual nutritional status, 21.20% of adolescents with normal body weight, 22.70% underweight and 30.10% overweight went on reduction diets.

The method of reducing body weight and rural boys' BMI are not correlated (p=0.967, ρ=-0.005). Most of the normal weight boys (48.00%) from rural areas reduced body weight by physical activity and then by skipping meals (32.00%), overweight (50.00%) by skipping meals, half of the obese by reducing the meal size, and the other half by physical activity.

The method of reducing body weight and urban boys' BMI are not correlated (p=0.601, ρ=-0.50). Normal weight (56.00%), overweight (40.91%), and obese (46.15%) boys were found to decrease their body weight by physical exercise. The second most common way is skipping meals, and among underweight, skipping meals, reducing the meal size and physical exercise are equally present.

Table 3. Correlation between BMI and the method of reducing weight

Place of Residence	BMI	The method						p	ρ
		Skipping meals	Reducing meal size	Fat free and snack free diet	Vomiting after meal	Usage of laxatives, shakes	Physical activity		
Rural	$5^{th} < 85^{th}$	8	3	1	1	0	12	0.967	-0.005
		32.00%	12.00%	4.00%	4.00%	0.00%	48.00%		
	$85^{th} < 95^{th}$	3	0	1	0	0	2		
		50.00%	0.00%	16.66%	0.00%	0.00%	33.33%		
	$\geq 95^{th}$	0	2	2	0	0	2		
		0.00%	50.00%	50.00%	0.00%	0.00%	50.00%		
Urban	$< 5^{th}$	1	1	0	0	0	1		
		33.33%	33.33%	0.00%	0.00%	0.00%	33.33%		
	$5^{th} < 85^{th}$	16	8	7	1	1	42		
		21.33%	10.67%	9.33%	1.33%	1.33%	56.00%	0.601	-0.50
	$85^{th} < 95^{th}$	6	6	0	0	1	9		
		27.27%	27.27%	0.00%	0.00%	4.54%	40.91%		
	$\geq 95^{th}$	1	4	2	0	0	6		
		7.69%	30.77%	15.38%	0.00%	0.00%	46.15%		

CONCLUSION

BMI in both groups (rural and urban) in relation to weight reduction and being on a reduction diet is statistically significant. Positive correlation was noticed between BMI values and body image satisfaction. In urban adolescents, BMI also influences the

satisfaction with their body image: boys with normal weight in both groups are not satisfied with their appearance; they tried to lose weight and were on reduction diets (BMI values are correlated negatively in cases of weight reduction or being on reduction diets). Other than diets, most of the participants used physical activity (which is a proper way of reducing weight), but also skipping meals and reducing the meal size.

Such data suggest that adolescent boys living in the Sarajevo Canton are also affected by modern society idealization of body and weight, which leads to risky behavior in this very sensitive period. This highlights the need for adequate nutrition education in primary and secondary schools, as well as for commitment to healthy lifestyles in children of both genders.

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PROCJENA KAKVOĆE PREHRANE MALE DJECE POMOĆU SKORA PREHRAMBENE KAKVOĆE

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SAŽETAK

Procjena kakvoće prehrane putem indeksa za procjenu kakvoće prehrane daje bolji pregled ukupnog obrasca prehrane, raznolikosti prehrane i usklađenosti s prehrambenim smjernicama nego procjena adekvatnosti unosa pojedinih hranjivih tvari. Premda indeksi za procjenu kakvoće prehrane imaju široku primjenu u odrasloj populaciji, kod djece je takav pristup manje zastupljen. Cilj ovog istraživanja bio je procijeniti kakvoću prehrane male djece pomoću indeksa razvijenog za tu specifičnu populacijsku skupinu-skora prehrambene kakvoće (engl. Diet Quality Score-DQS). U istraživanje je bilo uključeno 66 djevojčica i 65 dječaka u dobi od 1 do 3 godine. Dva neovisna dnevnika prehrane korištena su za izračun skora prehrambene kakvoće. Prosječan rezultat indeksa iznosio je 4,30±1,17 bodova, dok je maksimalna vrijednost koju je bilo moguće ostvariti iznosila 10 bodova (najbolja kakvoća prehrane). Iako nije utvrđena statistički značajna razlika između skora prehrambene kakvoće i dobi, s porastom dobi došlo je do opadanja rezultata indeksa. Utvrđena je statistički značajna povezanost između skora prehrambene kakvoće i unosa ugljikohidrata (p=0,002) te prehrambenih vlakana (p=0,004), kao i unosa tiamina (p=0,04) i riboflavina (p=0,02) te bakra (p=0,001) na razini cijelog uzorka ili pojedinačnih dobnih skupina. S obzirom na specifičnost skora prehrambene kakvoće za primjenu u populaciji male djece, u budućim istraživanjima na većem broju ispitanika bilo bi zanimljivo povezati ostvarene rezultate sa zdravstvenim statusom djece.

Ključne riječi: mala djeca, kakvoća prehrane, skor prehrambene kakvoće

UVOD

Procjena kakvoće prehrane u pedijatriji je od velikog interesa jer se prehrambene navike i ponašanja koja se razvijaju u djetinjstvu mogu pratiti tijekom vremena i tako predvidjeti s prehranom povezane bolesti u starijoj dobi (Craigie i sur., 2011). Primjerice, dojenje je povezano s nižom krivuljom rasta i poboljšanim kognitivnim razvojem, a unos natrija u prvih nekoliko mjeseci života je pozitivno povezan s krvnim tlakom u djetinjstvu i pubertetu (Smithers i sur., 2011). Štoviše, neadekvatna prehrana u djetinjstvu utječe na zdravlje djeteta i isto tako može utjecati na zdravlje u kasnijem

periodu života. Upravo zato je važno proučavati prehrambene obrasce u ranom djetinjstvu (Voortman i sur., 2015).
U posljednja dva desetljeća metode za procjenu kakvoće prehrane su evoluirale te su se pojavili brojni bodovni sustavi, odnosno indeksi. Široko je prihvaćeno da pojedinci ne konzumiraju izolirane hranjive tvari ili namirnice, već složene kombinacije hrane koja se sastoji od nekoliko hranjivih tvari i ne-nutrijenata (Kourlaba i Panagiotakos, 2008). Koncept indeksa obuhvaća procjenu kako kakvoće, tako i raznolikosti prehrane, omogućujući ispitivanje povezanosti cjelokupne prehrane i zdravstvenog stanja, a ne samo utjecaja hranjivih tvari. Kakvoća prehrane mjeri se bodovanjem prehrambenih obrazaca s obzirom na stupanj usklađivanja s nacionalnim prehrambenim smjernicama te s obzirom na zastupljenost različitih namirnica iz osnovnih skupina hrane (Wirth i Collins, 2009).
Upotreba indeksa kakvoće prehrane u pedijatrijskoj populaciji predstavlja područje istraživanja koje se jako brzo širi na međunarodnoj razini. Njihova primjena u razvijenim zemljama je usmjerena uglavnom na procjenu kakvoće prehrane, a tek nedavno u istraživanjima na bolestima povezanim s prehranom ili faktorima rizika kroničnih bolesti. Za razliku od razvijenih zemalja, razvoj i uporaba prehrambenih indeksa za dječju populaciju u zemljama u razvoju uglavnom su namijenjeni za ocjenu prehrambene adekvatnosti i rasta djeteta koji su glavni nutritivni problemi u tim državama (Lazarou i Newby, 2011). Međutim, pregledom literature, pokazalo se kako postoji mali broj razvijenih prehrambenih indeksa za djecu, no oni nisu namijenjeni za djecu predškolske dobi. Usmjereni su na prehrambenu raznolikost umjesto na kakvoću; razvijeni su za posebne zdravstvene ishode ili uključuju unos određenih nutrijenata za koje podatci najčešće nisu dostupni (Marshall i sur., 2014), stoga je cilj ovog istraživanja bio upravo procijeniti kakvoću prehrane djece od jedne do tri godine pomoću jednog od indeksa namijenjenog djeci predškolske dobi - skora prehrambene kakvoće.

MATERIJALI I METODE

Ispitanici

Tijekom istraživanja podatci su prikupljani u razdoblju od studenog 2015. godine do srpnja 2016. godine u pedijatrijskim ambulantama na području Grada Zagreba i Zagrebačke županije. U navedenom razdoblju dobiveni su potpuni podatci za 131 ispitanika. Svi roditelji su prethodno potpisali informirani pristanak za sudjelovanje u istraživanju u kojem su pojašnjena očekivanja i cilj istraživanja. Materijali korišteni prilikom prikupljanja podataka su uključivali upute za ispunjavanje obrazaca i vođenje dnevnika prehrane, opći upitnik, dnevnik prehrane za malu djecu, primjer dnevnika prehrane za malu djecu, knjigu fotografija s porcijama hrane i pića.

Dijetetičke metode

Kao dijetetička metoda korišten je dnevnik prehrane koji se sastoji od dva prazna obrasca za unos hrane i pića. Prilikom vođenja dnevnika, naglasak je bio na praćenju prehrambenog unosa za dva neuzastopna dana s minimalnim razmakom od sedam dana te za one dane kada se svi obroci jedu kod kuće, odnosno kada je dijete pod cjelodnevnim nadzorom roditelja. U dnevniku prehrane bio je naveden dio koji se odnosi na dojenje, na korištenje adaptirane

mliječne formule i na ostalu hranu i piće. Za svakog ispitanika, ovisno o trenutnim prehrambenim navikama, bilo je potrebno popuniti dio koji se na njega odnosi (npr. dojena djeca imala su ispunjen i dio vezan uz dojenje). Za procjenu energijskog i nutritivnog sastava namirnica korištene su hrvatske tablice o sastavu namirnica i pića (Kaić-Rak i Antonić, 1990). Za namirnice čiji energijski i nutritivni sastav nije bio dostupan, korištena je švicarska baza podataka (engl. Swiss food composition database) (FDHA, 2017). Isto tako, za neke gotove proizvode, poput grickalica i čokoladnih deserta, energijski i nutritivni sastav je ručno upisan s deklaracije proizvoda. Zbog teškoće određivanja volumena konzumiranog majčinog mlijeka, ono je izuzeto iz ukupnog energijskog i nutritivnog unosa ispitanika. Naposlijetku, za svakog ispitanika izračunat je energijski i nutritivni unos za pojedini dan te prosječan unos za oba dana.

Skor prehrambene kakvoće

Za procjenu kakvoće prehrane korišten je Skor prehrambene kakvoće, indeks koji je specifično razvijen za procjenu kakvoće sveukupne prehrane djece predškolske dobi (Voortman i sur., 2015). Za svakog ispitanika, odnosno za svaki prijavljeni dan, je izračunat kumulativni rezultat Skora prehrambene kakvoće ovisno o tome pripadaju li konzumirane namirnice nekoj od skupina namirnica indeksa (povrće (≥100 g/dan); voće (≥150 g/dan); kruh i žitarice (≥70 g/dan); riža, tjestenina, krumpir i mahunarke (≥70 g/dan); mlijeko i mliječni proizvodi (≥350 g/dan); meso, perad, jaja i zamjene za meso (≥35 g/dan); riba (≥15 g/dan); ulja i masti (≥25 g/dan); bomboni i grickalice (≤20 g/dan); i bezalkoholni napitci s dodanim šećerom (≤100 g/dan)). Maksimalna kumulativna vrijednost koju je bilo moguće ostvariti iznosila je 10 bodova. Dodijeljeni bodovi zapravo predstavljaju omjer unesene količine namirnica tijekom dana i preporučenog unosa za iste namirnice. Unosu koji je jednak ili iznad pridružene granične vrijednosti za određenu komponentu dodjeljuje se maksimalan broj bodova koji iznosi 1. Ovaj princip vrijedi za sve komponente indeksa izuzev skupine „Bomboni i grickalice" te „Bezalkoholni napitci s dodanim šećerom" kod kojih su dodijeljeni bodovi razlika maksimalnog broja bodova (1) te omjera unesene i preporučene količine namirnica.

Opći upitnik

Opći upitnik sastoji se od 26 pitanja. Prvi i najveći dio odnosi se na dijete, odnosno ispitanika. U tom dijelu obuhvaćeni su podatci o dojenju i dohrani te o uvođenju namirnica u prehranu i antropometrija djeteta. Pitanja o dojenju odnosila su se na to je li majka uopće dojila i je li dijete dojeno za vrijeme trajanja istraživanja. Pitanje o duljini dojenja podijeljeno je u četiri kategorije i to u trajanju kraćem od 6 mjeseci, između 6 i 12 mjeseci te 13 i 18 mjeseci te duljem od 18 mjeseci, dok je pitanje o isključivom dojenju podijeljeno u dvije kategorije, kraćem od 4 mjeseca i duljem od 4 mjeseca. Također, upitnik sadrži i pitanja o korištenju dodataka prehrani i postojanju alergija na hranu. Drugi dio upitnika namijenjen je roditeljima te uključuje pitanja o socio-ekonomskom statusu poput broja osoba u kućanstvu, ukupnog mjesečnog prihoda kućanstva te pitanja o majčinim prehrambenim navikama i antropometriji.

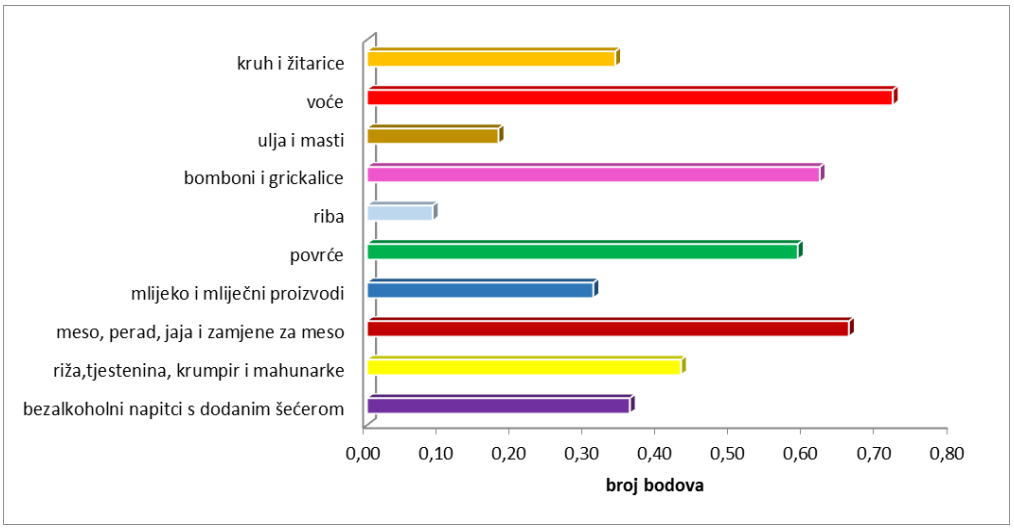
Statistička obrada

Kako bi se procijenio rezultat Skora prehrambene kakvoće u odnosu na unos hranjivih tvari, korišten je model linearne regresije gdje je unos hranjivih tvari predstavljao zavisnu

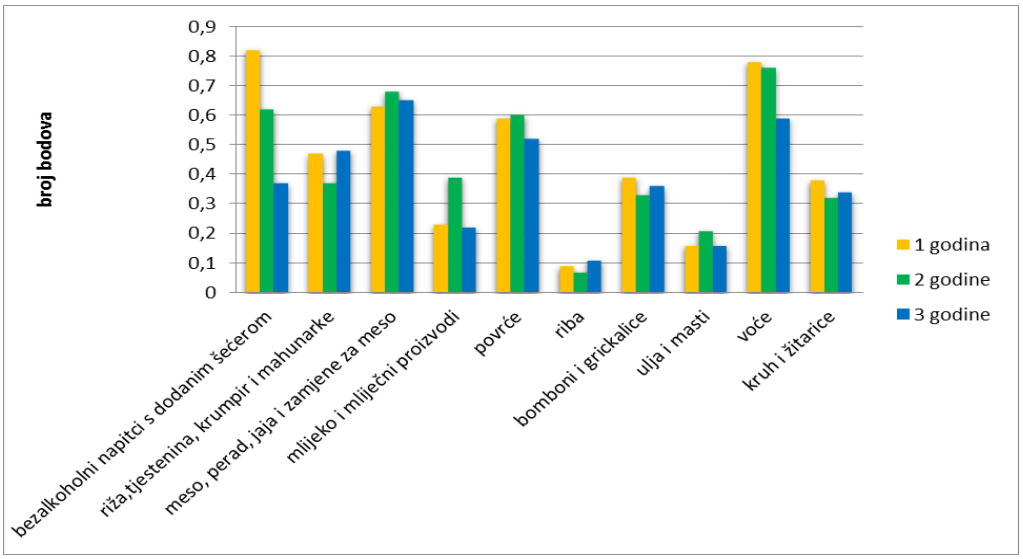
varijablu. Za procjenu rezultata Skora prehrambene kakvoće u ovisnosti o dobi i spolu djeteta, prehrambenim obilježjima te socio-demografskim čimbenicima korištena je jednofaktorska ANOVA. Obrada je provedena u Microsoft Office Excel programu (2010). Za svaku provedenu analizu, razina značajnosti (P-vrijednost) je iznosila 0,05.

REZULTATI I RASPRAVA

Među djecom koja su bila uključena u ovo istraživanje bilo je 66 djevojčica i 65 dječaka u dobi od jedne do tri godine. Rezultat prehrambenog indeksa za cijeli uzorak bio je u rasponu od 1,04 do 7,02 bodova sa srednjom vrijednošću (±SD) 4,30±1,17. Većina djece imala je relativno visoke rezultate za unos voća (0,72), mesa, jaja i zamjena za meso (0,66) te bombona i grickalica (0,61), ali relativno niske rezultate za unos ribe (0,09), ulja i masti (0,18) kao i za unos mlijeka i mliječnih proizvoda (0,31) (Slika 1.). Prema nizozemskom istraživanju (Voortman i sur., 2015) koje je provedeno s 3629 djece u dobi od 13 i 25 mjeseci, rezultat DQS bio je u rasponu od 0,6 do 8,8 sa srednjom vrijednošću od 4,1 ±1,3. Većina djece imala je relativno visoke rezultate za unos voća (0,80) te kruha i žitarica (0,81), a niske rezultate za unos mesa, jaja i zamjena za meso (0,23), ribe (0,15) te bombona i grickalica (0,18).
Za djecu u dobi od jedne godine koja su pokazala najbolji rezultat, rezultat Skora prehrambene kakvoće varirao je između 2,50 i 6,92 te je prosjek skupine iznosio 4,52 ±1,16 bodova. Točnije, pokazalo se kako djeca u ovoj dobnoj skupini češće konzumiraju voće (0,78) te meso i zamjene za meso (0,63), a najmanje namirnice iz skupine riba (0,09), ulja i masti (0,16) te bezalkoholne napitke s dodanim šećerom (0,82) (slika 2). Uspoređujući dobivene rezultate, nešto lošiji rezultat imala su djeca u dobi od dvije godine (4,34±1,14) dok su najlošiji rezultat ostvarila djeca u dobi od tri godine (3,99±1,21).



Slika 1. Rezultati skora prehrambene kakvoće prema skupinama namirnica
Fig. 1. Results of the Diet Quality Score according to the components of the index



Slika 2. Rezultati skora prehrambene kakvoće prema skupinama namirnica i dobi
Fig. 2. Results of the Diet Quality Score according to the components of the index and age

Statistički značajna razlika s obzirom na dob i spol u ovom istraživanju nije utvrđena, no dobiveni rezultati su pokazali kako s porastom dobi dolazi do opadanja rezultata indeksa te kako su dječaci ostvarili nešto viši rezultat u odnosu na djevojčice (Tablica 1).

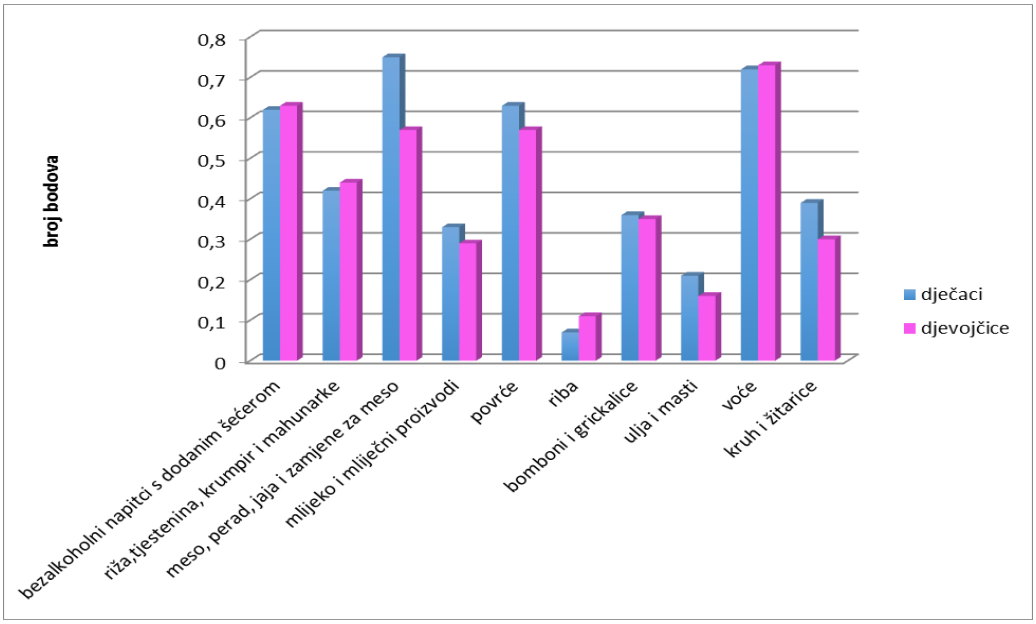
Tablica 1. Razlika u rezultatima skora prehrambene kakvoće s obzirom na spol i dob
Table 1. Difference between the results of the Diet Quality Score according to sex and age

Parametar	**Srednja vrijednost ± SD**	**F**	**P**
Spol			
Dječaci (n=65)	4,49±1,23	3,25	0,07
Djevojčice (n=66)	4,12±1,09		
Dob			
1 godina (n=37)	4,52±1,16	1,99	0,14
2 godine (n=61)	4,35±1,14		
3 godine (n=33)	3,97±1,21		

F: jednofaktorska ANOVA

Gledajući rezultate za pojedinačne komponente indeksa s obzirom na dob, isticao se rezultat jednogodišnjaka u odnosu na ostale dobne skupine za komponentu bezalkoholni napitci s dodanim šećerom ($p<0,001$); rezultat dvogodišnjaka za komponentu voće ($p=0,003$) te rezultat jednogodišnjaka i dvogodišnjaka za komponentu mlijeko i mliječni proizvodi ($p=0,002$). Za navedene komponente je utvrđena i statistički značajna razlika u odnosu na ostale komponente indeksa. Prijašnje studije pokazale su kako se kakvoća prehrane smanjuje što je dijete starije (Smithers i sur., 2011) što možemo povezati s provedenim istraživanjem gdje rezultat Skora prehrambene kakvoće opada s porastom dobi.

Ako se uzmu u obzir rezultati indeksa prema spolu, isti su varirali između 1,30 i 7,02 kod dječaka te između 1,04 i 6,92 kod djevojčica. Gledajući rezultate za pojedinačne komponente indeksa, dječaci su ostvarili viši rezultat za skupinu mesa, peradi, jaja i zamjena za meso te skupinu kruha i žitarica za razliku od djevojčica (slika 3). Iako prema Skoru prehrambene kakvoće nije utvrđena razlika među spolovima (tablica 1),s obzirom na komponente indeksa, statistički značajna razlika utvrđena je za unos mesa, peradi, jaja i zamjena za meso kod dječaka (p=0,001) za razliku od djevojčica kod kojih nije pronađena statistički značajna razlika ni za jednu od komponenti indeksa.
S obzirom na makronutrijente, utvrđena je statistički značajna povezanost između rezultata Skora prehrambene kakvoće i unosa ugljikohidrata (p=0,001) i prehrambenih vlakana (p=0,004) na razini cijelog uzorka. Prehrambeni indeks bio je pozitivno povezan s nekoliko vitamina i mineralnih tvari. Na razini cijelog uzorka to su tiamin (p=0,04) i riboflavin (p=0,02) te bakar (p=0,001). U istraživanju koje su proveli Voortman i sur. (2015), Skor prehrambene kakvoće bio je pozitivno povezan s unosom polisaharida (0,30) i prehrambenih vlakana (0,34) te obrnuto proporcionalno povezan s unosom monosaharida (-0,31) i disaharida (-0,26).
Skor koji je specifično osmišljen za ovu populacijsku skupinu primjenjivan je u samo još jednom istraživanju (Voortman i sur., 2015). Jedna od značajnih prednosti istraživanja je mali broj informacija o prehrani male djece u Hrvatskoj, a posebice ovim pristupom, međutim u istraživanje je uključen relativno mali uzorak te bi svakako dobivene rezultate valjalo potvrditi na većem, stratificiranom uzorku.



Slika 3. Rezultati skora prehrambene kakvoće prema skupinama namirnica i spolu
Fig.3. Results of the Diet Quality Score according to the components of the index and sex

ZAKLJUČAK

U istraživanju koje je provedeno s ciljem procjene kakvoće prehrane djece u dobi od jedne do tri godine pomoću Skora prehrambene kakvoće te utvrđivanja utjecaja različitih socioekonomskih i prehrambenih parametara na rezultate dobivene primjenom DQS može se zaključiti kako je u ispitivanom uzorku najviši rezultat prehrambenog indeksa iznosio 7,02, a najniži 1,04 sa srednjom vrijednošću (±SD) 4,30±1,17. Iako nije utvrđena statistički značajna povezanost između Skora prehrambene kakvoće i dobi, s porastom dobi došlo je do opadanja rezultata indeksa. Potrebno je uključiti više ispitanika te ispitanika iz različitih dijelova Hrvatske da bi rezultati bili reprezentativni za populaciju male djece u Hrvatskoj.S obzirom na specifičnost DQS za primjenu u populaciji male djece, u budućim istraživanjima bilo bi zanimljivo povezati ostvarene rezultate sa zdravstvenim statusom djece.

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***Topic: Nutrition /* Sekcija: Nutricionizam**

TODDLER DIET QUALITY ASSESSMENT USING A DIET QUALITY SCORE

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Assessing the quality of a diet using dietary indices gives a better overview of the overall dietary pattern, nutrition diversity, and compliance with the current guidelines, rather than assessing the adequacy of nutrient intake. Although dietary indices are widely used for adults, that approach is less represented for the paediatric population. The aim of this study was to determine the toddler diet quality using the index developed for this specific population group – the Diet Quality Score (DQS). The study was conducted with 66 girls and 65 boys age 1 to 3. Two independent diaries were used to calculate the DQS. The average score of the index was 4.30 ± 1.17 points, while the maximum possible result was 10 points (the best dietary quality). Although no statistically significant difference was found between the dietary index and age, with the increase in age, there was a decline in the results. A statistically significant association between the DQS and the intake of carbohydrates ($p=0.002$) and dietary fibre ($p=0.004$), as well as the intake of thiamine ($p=0.04$), riboflavin ($p=0.02$), and copper ($p=0.001$), was established for the whole group or individual age groups. Given the specifics of the DQS for use with toddlers in future research on a larger number of subjects, it would be interesting to link the achieved results with the child's health status on a larger number of subjects in future research.

Keywords: toddlers, diet quality, Diet Quality Score

VITAMIN D INTAKE AND STATUS IN OVERWEIGHT PEOPLE

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ABSTRACT

Vitamin D deficiency may contribute to the development of osteoporosis and other chronic diseases, such as diabetes and cardiovascular disease. Inadequate vitamin D status is highly prevalent worldwide and is associated with low dietary intake, insufficient sun exposure, aging, and excess weight. The aim of this study was to determine the vitamin D intake and status among overweight people.

The participants were adults of both genders with the mean age of 43.5 (n=36). A food frequency questionnaire (FFQ) and 24-hour recall were used to assess the vitamin D intake. Body composition was determined by using the bioelectrical impedance method. Vitamin D status was assessed by determining the concentration of the circulating 25-hydroxyvitamin D [25(OH)D].

The average daily vitamin D intake was 2.51 μg/day, according to the FFQ, and fish was the main source of vitamin D in the diet. The vitamin D intake of the majority of the participants (77.8%) was lower than 20% of the dietary reference intake. The average serum 25(OH)D concentration was 48.4 nmol/L. 88.9% of the participants had an inadequate vitamin D status (<75 nmol/L). A significant inverse association was determined between vitamin D intake and body fat mass.

The study has shown a high prevalence of inadequate vitamin D intake and status among overweight people.

Keywords: vitamin D intake, vitamin D status, overweight, body fat

INTRODUCTION

Vitamin D is a fat soluble vitamin, steroid hormone with numerous functions, most commonly known are calcium homeostasis, phosphorus homeostasis, and bone remodelling (Prentice et al., 2008). Aside from calcium homeostasis, the roles of vitamin D in bone health include differentiation and proliferation of bone marrow cells. Vitamin D has a role for the immunity of skin cells and prostate, ovary, and breast epithelial cells (DeLuca, 2004). People acquire vitamin D from their diet and by cutaneous synthesis after exposure to sunlight. Dietary sources contain vitamins in the

form of either vitamin D_3 (cholecalciferol) or vitamin D_2 (ergocalciferol). The richest natural sources of vitamin D are oily fish and egg yolks. The endogenous supply of vitamin D_3 depends on the exposure of the skin to UVB radiation at wavelengths of 290–315 nm (Prentice et al., 2008). However, the main source of vitamin D in the body is endogenous synthesis through occasional exposure to ultraviolet B rays from sunlight (Laktašić-Žerjavić et al., 2011).

The prevalence of vitamin D deficiency is high in different populations around the world. The lack of this vitamin is a global problem that affects all age groups; new-borns, children, adults, and the elderly. There are numerous factors that affect the level of vitamin D in the serum, including age, gender, body mass index, skin colour, air pollution and urbanization, clothing, sun exposure, and poor eating habits (Lips, 2007). People with excess body weight are at increased risk of vitamin D deficiency because the serum 25-hydroxyvitamin D [25(OH)D] concentration decreases with increased body fat (Palacios et al., 2011). There is also the possibility that reduced vitamin D concentrations contribute to the development of obesity and reduce the chance of weight loss (Earthman et al., 2012). Circulating concentration of the 25(OH)D is inversely related to body fat content (Arunabh et al., 2003).

The aim of this study was to determine the average daily vitamin D intake and vitamin D status among overweight people, and to investigate the possible association with anthropometric and biochemical parameters that are directly or indirectly related to vitamin D status.

SUBJECTS AND METHODS

Subjects

The study included 36 overweight subjects aged 20 to 65. The main selection criterion was the body mass index (BMI), which ranged from 25 to 29.9 kg/m^2. The subjects were recruited through the Association for Overweight Prevention, through acquaintances, by online invitations via social networks, and by the medical staff of the Clinical Hospital "Sveti Duh". 75% of the subjects were female and 25% were male.

Participation was voluntary, and all subjects signed an informed consent form. The Ethics Committee of the Institute for Medical Research and Occupational Health approved the study.

In this study, anthropometric, dietetic, and biochemical methods were used for the evaluation of the nutritional status. The data was collected between November of 2015 and March of 2016.

24-hour recall

Vitamin D intake was determined with 24-hour recall for the day before the blood samples were collected. The amount of food that the subjects consumed was described using household dishes and measures (e.g., cups, bowls, glasses, spoons). A book of food photographs was also used to assess whether they consumed a small, medium, or large portion size (Senta et al., 2004). The USDA National Nutrient Database (USDA, 2012), as well

as the Danish Food Composition Databank (Moller et al., 2005), and food labels were used to determine the amount of vitamin D in the food.
Among subjects using supplements, vitamin D intake was determined by the reported dosage of supplements consumed. The majority of subjects were using multivitamin supplements containing vitamin D. Most commonly used multivitamin supplements were Centravit (Dietpharm) and Elevit (Bayer), and one subject was taking a high dose of vitamin D (Vitamin D3 10 000 IU, Solgar).

Food frequency questionnaire

The Food Frequency Questionnaire (FFQ) was designed at the Faculty of Food Technology and Biotechnology, as part of the grant of the University of Zagreb titled "Body Weight as a Risk Factor for Inadequate Vitamin D Status: Preliminary Study". This questionnaire evaluated the intake of vitamin D over a period of past 2 months. The questionnaire contained 40 foods that contributed to the intake of vitamin D. Frequency of consumption and the usual portions were also offered: small, medium, and large. The subjects should choose the portion and the frequency of consumption of certain foods by choosing 9 offered frequencies (never or <1/month, 1/month, 2-3/month, 1/week, 2/week, 3-4/week, 5-6/week, 1/day, 2+/day). For the purpose of estimating the intake of vitamin D, it was determined that the small portion is equal to half of the medium portion and that the large portion is equal to one and a half of the medium portion (Lee and Nieman, 2003). To calculate the average daily vitamin D intake data on the consumption frequency of certain foods, the usual portion of food and the amount of vitamin D in 1 g of food were used.

Biochemical methods

Fasting venous blood samples were collected into tubes without anticoagulant for serum and centrifuged at 2000g for 5 minutes within 1 hour of collection. All samples were stored at −20°C until analysis. Blood samples were collected for measurements of serum 25(OH)D, serum intact parathyroid hormone (iPTH), serum creatinine, and serum calcium concentrations.
Serum 25(OH)D concentrations were measured by enzyme immunoassay (Immunodiagnostic Systems Limited, Boldon, UK). Serum calcium and creatinine concentrations were assessed by a standard laboratory method using the autoanalyzer Olympus AU400 (Olympus Life and Material Science Europa GmbH, Lismeehan, Ireland). Serum iPTH concentrations were determined using the Immulite Intact PTH (Diagnostic Products Corp, Los Angeles, CA, USA).
Biochemical parameters were measured at the Clinical Department of Chemistry, University Hospital Centre "Sestre milosrdnice" (Zagreb, Croatia).

Anthropometry

Anthropometric measurements included the measurement of body weight, body height, waist and hip circumference, and the determination of body composition. Body weight and height were measured without shoes and while wearing indoor clothes to the nearest 0.1 kg

and 0.1 cm, respectively. Body height was measured with a stadiometer SECA 437, and body weight was measured on an electronic digital scale (SECA, Hamburg, Germany). Body composition was estimated by the bioelectrical impedance method, using the Omron BF-500, and the percentage of body fat using the Omron body fat analyzer BF-300 (Omron Healthcare, Vernon Hills, Illinois, USA). The BMI was calculated using the standard formula (weight in kilograms/height in meters squared).

Statistical analyses

Statistical analyses were performed using the Microsoft Office Excel 2010 and Statistica software (version 10.0; StatSoft, Inc., Tulsa, Oklahoma, USA). The results are presented as the means ± SD. The normality of data distribution has been tested with the Shapiro-Wilk test. All observed variables were normally distributed except for the variables describing anthropometric characteristics (% of body fat, % of skeletal muscles, visceral fat level), biochemical parameters (25(OH)D), and vitamin D intake (24-h recall, FFQ, 24-h recall with supplements). The Grubbs test indicated a few outliers in the 25(OH)D concentration and in vitamin D intake, according to 24-h recall for subjects using supplements. The t-test was used to assess the differences between groups according to age. Associations of certain variables were tested using Spearman's correlation coefficients. The analyses were performed with a level of statistical significance set at 95% ($p<0.05$).

RESULTS AND DISCUSSION

The study included 36 overweight subjects (BMI from 25 to 29.9 kg/m^2). General characteristics of the sample are presented in Table 1. The subjects were divided into two groups according to their age (20-45; 46-65), and according to gender. Supplements containing vitamin D were used by 10 subjects, the majority of whom were female (n=9). The average body mass index of all subjects was 28.2 ± 1.3 kg/m^2. The BMI did not significantly differ according to gender ($p=0.138$). The average percentage of body fat determined by Omron BF-500 was 37.9 ± 6.9%. Female subjects had a significantly higher percentage of body fat ($p<0.001$), and a lower percentage of skeletal muscles ($p<0.001$) compared to male subjects. Male subjects had a significantly higher visceral fat level compared to female subjects ($p=0.002$). Variables such as body fat (%), skeletal muscles (%), and visceral fat level do not follow normal distribution ($p<0.05$).
Table 2 shows the average daily vitamin D intake. Dietary vitamin D intake was not significantly different between the two groups according to age determined by 24-hour recall and the food frequency questionnaire. Average daily vitamin D intake in accordance with the recommendation was estimated for 19.4% of the subjects according to 24-hour recall. All subjects with the adequate vitamin D intake were taking vitamin D supplements. The average daily vitamin D intake was 2.5 ± 2.1 μg/day, according to the FFQ, without supplementation. In this study, the average vitamin D intake was considerably lower than the dietary reference value (DRV) for vitamin D (15 μg/day) (EFSA, 2016). Vitamin D intake was lower than 20% of the dietary reference value for the majority of the subjects (77.8%).

Table 1. Anthropometric characteristics of the participants

Parameters	Total	Males	Females		
Sample (n)	36	9	27		
Age (years)					
20-45	20	9	11		
46-65	16	0	16		
Supplement users	10	1	9		
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	p	p
Body height (cm)	171.6 ± 10.0	184.1 ± 6.2	167.5 ± 7.1	<0.001*	0.690
Body weight (kg)	83.5 ± 10.5	97.7 ± 6.6	78.8 ± 6.5	<0.001*	0.087
BMI (kg/m^2)	28.2 ± 1.3	28.8 ± 0.9	28.1 ± 1.4	0.138	0.005
Waist circumference (cm)	94.7 ± 6.1	99.3 ± 8.2	93.1 ± 4.3	0.050	0.070
Hip circumference (cm)	111.5 ± 4.5	112.6 ± 5.5	111.1 ± 4.2	0.392	0.320
Body fat (%)	37.9 ± 6.9	28.4 ± 5.2	41.1 ± 3.7	<0.001*	0.001**
Visceral fat level	8.7 ± 1.9	10.3 ± 1.6	8.2 ± 1.7	0.002*	0.001**
Skeletal muscle (%)	27.5 ± 4.5	33.9 ± 3.4	25.4 ± 2.2	<0.001*	0.014**

*Significant difference at p<0.05 according to the t-test
**Null hypothesis is rejected, the variable from which the sample was extracted does not follow a normal distribution (p<0.05)

The foods contributing the most to vitamin D intake were fish (30.2%), milk (11.4%), eggs (10.6%), and fortified cereals (8.3%), according to the FFQ (Figure 1). Other foods that contributed to vitamin D intake were yogurt (3.1%), liver and meat (2.8%), cheese (2.0%), mushrooms (2.0%), and butter and margarine (1.6%). On average, supplements contributed with 23.8% to daily vitamin D intake. Fish was the main source of vitamin D in a diet, which was also reported by studies in France (ANSES, 2013). Fish, eggs, milk, and some fortified foods, such as cereals and margarine, had a small contribution to total vitamin D intake.

Table 2. Average daily vitamin D intake according to age, estimated by 24-hour recall and a food frequency questionnaire

Parameters	**24-hour recall**	**24-h recall + vitamin D supplements**	**FFQ(without supplements)**
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Sample (n=36)	4.0 ± 5.8	14.8 ± 41.8	2.5 ± 2.1
Age (years)			
20-45 (n=20)	5.1 ± 6.6	35.1 ± 110.4	2.7 ± 2.0
46-65 (n=16)	2.6 ± 4.3	5.1 ± 5.9	2.3 ± 2.3
p, t-test	0.191	0.241	0.593
p	<0.0001**	<0.0001**	<0.0001**

**Null hypothesis is rejected, the variable from which the sample was extracted does not follow a normal distribution (p<0.05)

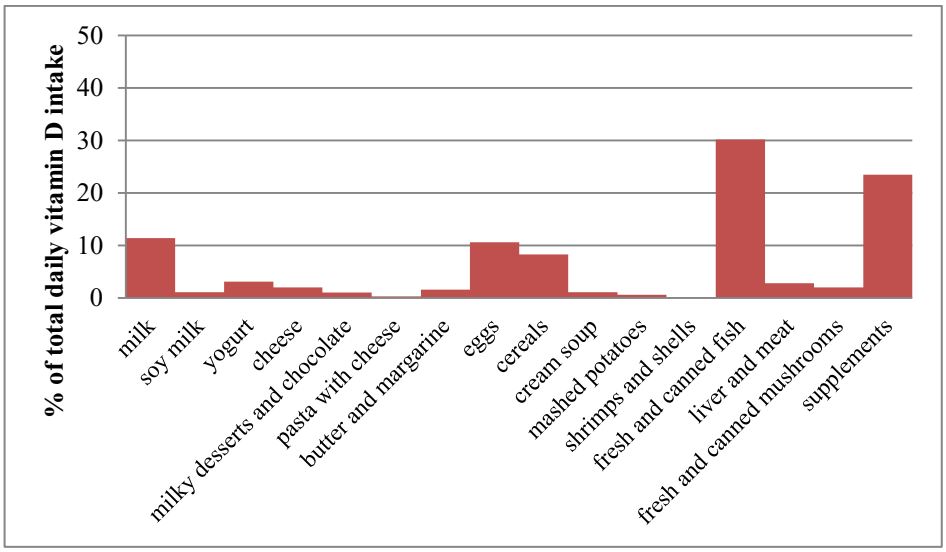

Fig. 1. Food groups as main sources of vitamin D in a daily diet

The concentration of average serum 25(OH)D and other biochemical parameters was presented in table 3. Subjects aged 46-65 had significantly lower creatinine concentrations (66.9 ± 5.2 µmol/L) than subjects aged 20-45 (77.2 ± 12.4 µmol/L) (p=0.002). Serum 25(OH)D concentrations did not differ significantly between the two groups according to age. The average serum 25(OH)D concentration was 48.4 nmol/L, which is lower than the desirable serum concentration (≥75 nmol/L). The average serum 25(OH)D concentration in subjects using supplements containing vitamin D was also lower than desirable.

Table 3. Biochemical parameters of the subjects (n=36) according to age (20-45, 46-65)

Parameters	Subjects using supplements (n=10)	Sample (n=36)	Age (years)		p	p
			20-45 (n=20)	46-65 (n=16)		
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD		
25(OH)D (mmol/L)	55.0 ± 31.6	48.4 ± 25.1	50.3 ± 19.4	45.9 ± 31.3	0.632	0.113
iPTH (pmol/L)	2.2 ± 0.4	2.8 ± 0.8	2.9 ± 0.8	2.7 ± 0.9	0.570	0.908
Creatinine (µmol/L)	68.5 ± 5.6	72.7 ± 0.8	77.2 ± 12.4	66.9 ± 5.2	0.002*	0.232
Calcium (mmol/L)	2.2 ± 0.1	2.3 ± 0.8	2.3 ± 0.1	2.4 ± 0.1	0.070	0.276

*Significant difference at p<0.05 according to the t-test

Concentrations of 25(OH)D between 25-49.9 nmol/L were present for 38.9% of the subjects, 36.1% had concentrations between 50-75 nmol/L, 13.9% had concentrations below 25 nmol/L, and 11.1% had above 75 nmol/L (Figure 2). In this study, 88.9% of the subjects were vitamin D insufficient, while 52.8% were vitamin D deficient. Some authors consider that adequate concentrations of vitamin D for calcium absorption and PTH stabilization are 75-100 nmol/L. Serum 25(OH)D concentrations below 75 nmol/L are considered as vitamin D insufficiency

(Laktašić-Žerjavić et al., 2011). Vitamin D status is influenced by several factors, including vitamin D intake, sun exposure, skin pigmentation, and obesity or excess weight (Forrest and Stuhldreher, 2011).

According to studies, serum 25(OH)D concentrations are lower as body weight increases (Earthman et al., 2012). No significant correlation was established between the BMI and 25(OH)D concentrations in this study (Figure 3). The increase in the BMI of 1 kg/m^2 results in a decrease in the concentration of 25(OH)D for 1 nmol/L and 1,25$(OH)_2$D for 0.9 pmol/L (Lagunova et al., 2011). However, an increased BMI does not necessarily mean increased body fat (Lagunova et al., 2009). Fat accumulation influences 25(OH)D levels. Vitamin D, as a fat soluble vitamin, accumulates in adipose tissue, and because of that, circulating 25(OH)D concentrations are lower (Earthman et al., 2012).

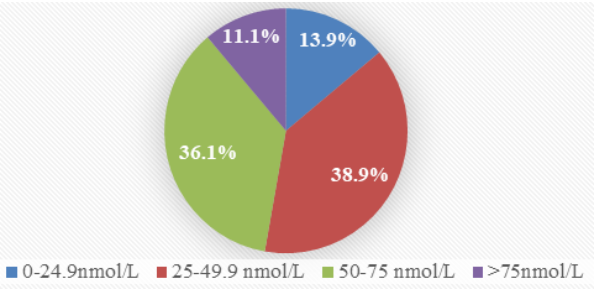

Fig. 2. Distribution of the serum 25(OH)D concentration in the total sample (n=36)

There is evidence that greater body fat content in obese or overweight subjects altered vitamin D metabolism in adipocytes (Pelczynska et al., 2016). It has been proposed that the production of 1,25$(OH)_2$D is enhanced in obese individuals and, thus, its higher concentrations exert negative feedback control on the hepatic synthesis of serum concentrations of 25(OH)D (Palacios et al., 2012). Significant correlation between serum 25(OH)D concentrations and the percentage of body fat was not determined in this study (Figure 4). In this study, a significant correlation was not determined between serum 25(OH)D and vitamin D intake according to 24-hour recall and the FFQ (Figure 5 and 6), which confirmed that vitamin D status depends on others factors as well, such as subcutaneous synthesis.

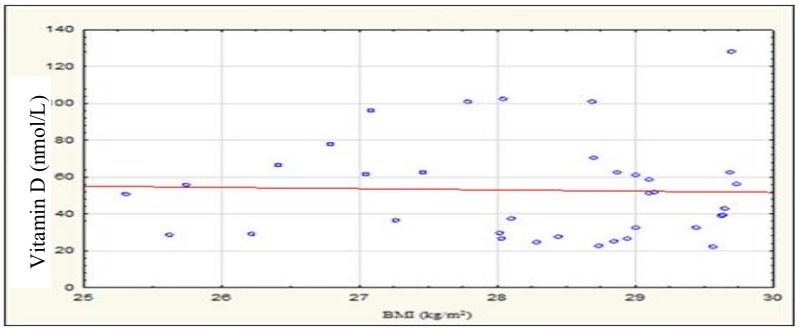

Fig.3. Correlation between serum 25(OH)D concentrations (nmol/L) and BMI (kg/m^2) (p=0.765)

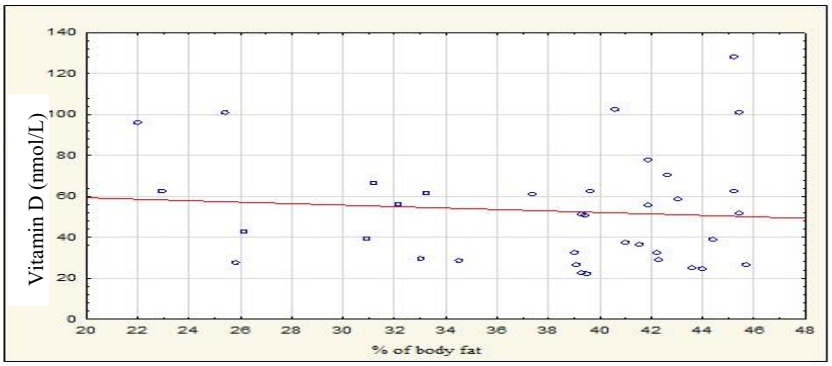

Fig. 4. Correlation between serum 25(OH)D concentrations (nmol/L) and the percentage of body fat (p=0.783)

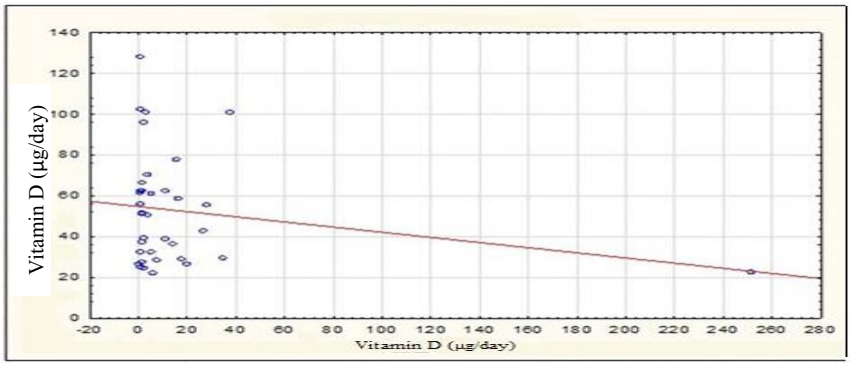

Fig. 5. Correlation between serum 25(OH)D concentrations (nmol/L) and vitamin D intake (µg/day) according to 24-h recall with supplements (p=0.328)

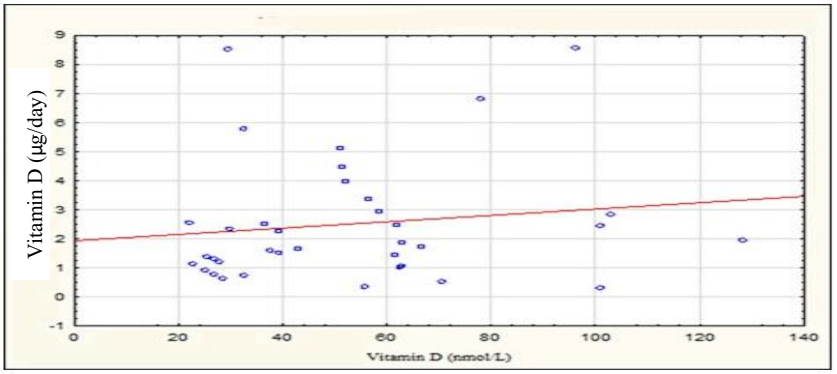

Fig. 6. Correlation between serum 25(OH)D concentrations (nmol/L) and vitamin D intake (µg/day) according to the FFQ (p=0.317)

CONCLUSIONS

This study has shown a high prevalence of inadequate vitamin D intake and status among overweight people. According to the food frequency questionnaire, none of the participants had an average daily vitamin D intake in accordance with the recommendation. Only the participants taking vitamin D supplements had an adequate daily vitamin D intake according to 24-h recall. Most of the participants (88.9%) are vitamin D insufficient. There was no significant association between the average daily intake of vitamin D and serum 25(OH)D concentrations. Serum 25(OH)D concentration did not significantly correlate with the percentage of body fat. Considering the prevalence of the inadequate vitamin D status in overweight people, it is recommended that the vitamin D intake should be increased (fish, milk and dairy products, eggs, meat, and mushrooms), and that there should be moderate exposure to the sun.

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ASSOCIATION OF FRUIT AND VEGETABLE INTAKE AND BONE MINERAL DENSITY IN ELDERLY WOMEN

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ABSTRACT

Different diseases and conditions are associated with inadequate diet, also including the decline in bone mineral density (BMD). A healthy and balanced diet includes the adequate consumption of fruit and vegetables, so the aim of this study was to determine if fruit and vegetable intakes are associated with bone mineral density in elderly women.
The subjects were 60 women of 65 to 90 years of age. 24-hour recall estimates the average daily fruit and vegetable intake and the intake of micronutrients important for bone health. Anthropometric parameters (body weight, body height, % of body fat) and biochemical parameters (serum folate, folate in erythrocytes, and serum vitamin D) were determined. BMD was measured at the femoral neck, femur, and lumbar spine by dual energy X-ray absorptiometry (DXA).
There was no significant correlation between fruit and vegetable intake and BMD in elderly women. A significant positive correlation was determined between vitamin D intake and BMD of the lumbar spine.
These results did not show that adequate fruit and vegetable intake contributes to the maintenance of BMD.

Keywords: bone mineral density, fruit, vegetables, elderly women

INTRODUCTION

Elderly women in the period after menopause have a predisposition to different diseases. One of these diseases is osteoporosis, which occurs due to a reduction of bone mineral density (BMD), which is frequent in advanced age because of the loss of oestrogen (Koršić, 2006). As osteoporosis is a significant health issue, it is very important to create healthy habits, which include a healthy diet in order to reduce an occurring problem as much as possible (Qiu et al., 2017).
As women in their lifetime pass through various hormonal changes and have special nutritional needs, diet is an essential component of their way of life (Mitmesser and Carr,

2004). Diets high in fruit and vegetables produce a more alkaline urine, because a high intake of fruits and vegetables accordingly lowers the acid load and reduces the urinary calcium excretion rates. High fibre intake could promote calcium absorption in the colon. Also, fibre components of fruits and vegetables might have this effect (New et al., 2000; Tucker et al., 2001).
Aside from the well-known foods that are good sources of minerals and vitamins that contribute to bone health, it is necessary to estimate the influence other foods that are present in the daily diet have on bones. Dietary recommendations state that it is necessary to consume five or more servings of fruit and vegetables per day, so the aim of this study was to determine if fruit and vegetable intakes are associated with BMD in elderly women.

SUBJECTS AND METHODS

Subjects

In this study, the subjects were 60 women of 65 to 90 years of age. Thirty-seven women were between 65 and 74 years of age, and 23 women were 75 years of age or older. The subjects were recruited for participation in the scientific project "Diet, Homocysteine, and Bone Health" (Ministry of Science and Education), and the subjects were the colleagues, friends or acquaintances of researchers, and the residents of nursing homes in the city of Zagreb. Twenty-eight women lived in nursing homes, while 32 women lived in their own households.
The subjects who were using medications known to interfere with bone metabolism (e.g., hormone replacement therapy, bisphosphonates) or had diabetes, liver disease, or renal disease, were not included in the study.
Participation was voluntary, and all subjects signed an informed consent form. The Ethical Committee of the Institute for Medical Research and Occupational Health approved the study.

Dietary intake

The dietary assessment method 24-hour dietary recall was used to determine an average intake of micronutrients, and an average fruit and vegetable intake. All kinds of fresh fruit and dried fruit, but no fruit juices, were included in the fruit group. Potatoes and legumes were not included in the vegetable group.
The food and drink consumed was evaluated for three non-consecutive days (two working days and one day during the weekend). In order to determine the quantity of consumed food, household dishes and measures were used (e.g., cups, bowls, glasses, spoons), as well as food photographs of small, medium, and large portion sizes.
The amount of micronutrients important for bone health was calculated using national food composition tables (Kaić-Rak and Antonić, 1990). For foods the composition of which was not available in the national tables, the data from the Danish Food Composition Databank (Moller et al., 2005), the US Department of Agriculture National Nutrient Database (USDA, 2012), and from food labels was used.

Anthropometry

Body weight and height were measured without shoes and while wearing indoor clothes to the nearest 0.1 kg and 0.1 cm, respectively. Height was measured on a portable stadiometer and body weight was measured on a portable scale (Model 220; SECA, Hamburg, Germany). They were used to calculate the body mass index (BMI; kg/m^2). Waist and hip circumference were measured by obtaining tape measurements around the waist and hips. Percentage of body fat was estimated by the bioelectrical impedance method using the Omron body fat analyser BF-300 (Omron Healthcare, Vernon Hills, Illinois, USA).

Blood analyses

In this study, folate concentrations in serum and red blood cells, and 25-hydroxyvitamin D concentrations in serum were determined. Fasting venous blood samples were collected into tubes without anticoagulant for serum and centrifuged at 2000g for 5 minutes, within 1 hour of collection. All samples were stored at −20°C until analysis.
Folate concentrations were measured using the Abbott AxSYM system (Abbott Laboratories, Abbott Park, Illinois, USA), according to the manufacturer's instructions. AxSYM measures folate using the ion capture technology. The status biomarker 25-hydroxyvitamin D (25(OH)D) was used for the evaluation of vitamin D. 25(OH)D concentrations were measured using an enzyme immunoassay, and the commercial kit was used according to the manufacturer's instructions.
Folate and 25-hydroxyvitamin D concentrations were measured at the Clinical Department of Chemistry, University Hospital Centre "Sestre milosrdnice" (Zagreb, Croatia).
Reference values for folate in serum were 16 to 35 nmol/L, and in erythrocytes they were 572 to 1843 nmol/L. The reference values for 25(OH)D were 47.7 to 144 nmol/L.

BMD measurements

The method of dual-energy X-ray absorptiometry (DXA), which is regarded as the "gold standard" for the estimation of BMD, was used to determine bone mineral density (g/cm^2). A GE Lunar Prodigy Bone Densitometer, Madison, WI, was used in this study.
BMD was measured at the femoral neck, femur, and lumbar spine. Bone densitometry was carried out at the Institute for Medical Research and Occupational Health, Zagreb. Osteoporosis and osteopenia were determined according to the WHO criteria (1994).

Statistical analyses

Statistical analyses were performed using Microsoft Office Excel 2010 and Statistica software (version 10.0; StatSoft, Inc, Tulsa, Oklahoma, USA). The results are presented as the means ± SD. The Shapiro-Wilk test was used to test the normality of data distribution. The data was normally distributed. The t-test was used to assess the differences between groups according to fruit and vegetable intake. Associations of certain variables were tested using partial Spearman's correlation coefficients. The level of statistical significance was determined at $p<0.05$.

RESULTS AND DISCUSSION

In this study, the subjects were 60 women, mean age 72.9 ± 6.9. Average anthropometric parameters, biochemical parameters, and BMD of the subjects are presented in Table 1. The average body weight was 73.6 ± 15.0 kg, and the average body height was 160.8 ± 8.6 cm. The body mass index was calculated as a ratio of body weight in kilograms and body height in square meters. The average BMI was 28.4 ± 5.2 kg/m^2, which indicates excess body weight.
The average serum folate, red blood cell folate, and serum vitamin D concentrations were 20.9 nmol/L, 845.5 nmol/L, and 54.1 nmol/L, respectively. The average values of all biochemical parameters were within the reference interval. Concentrations of 25(OH)D lower than 50 nmol/L are considered as a deficiency of vitamin D. A severe form of vitamin D deficiency (25(OH)D < 20-25 nmol/L) during longer periods will cause a serious disorder of bone metabolism, i.e. it may cause rickets or osteomalacia, depending on age (Holick, 2007; Holick, 2009; Lips, 2004). In a study with 194 postmenopausal women aged 50 or over from Croatia the mean serum concentration of 25(OH)D was 49.1 ± 17.1 nmol/L (Laktašić-Žerjavić et al., 2013), which is similar to the average concentration in this study.
According to the average T-score, osteopenia was determined at the lumbar spine and femoral neck (Table 1). In a study by Prynne et al. (2006), the average values of lumbar spine BMD and femoral neck BMD were similar to the results of this study. One of the study groups in that study consisted of elderly women, 67 subjects of 60 to 83 years of age. The average value of the lumbar spine BMD was 1.062 g/cm², while the femoral neck BMD was 0.843 cm/g² (Prynne et al., 2006). In the study which included 2 000 women aged 65 and older, carried out by Liu et al., the average value of the lumbar spine BMD was 0.753 g/cm^2 (Liu et al., 2015), which is less than in this study. The average value of the femoral neck BMD in a study by Liu et al. was 0.584 g/cm², which is less than in this study, where the average value of the BMD of the femoral neck was 0.787 g/cm² (Table 1).

Table 1. Average age, anthropometric parameters, biochemical parameters, and BMD (n=60)

Parameters	**Mean ± SD**
Age (years)	72.9 ± 6.9
Body height (cm)	160.8 ± 8.6
Body weight (kg)	73.6 ± 15.0
BMI (kg/m^2)	28.4 ± 5.2
Waist circumference (cm)	94.8 ± 12.5
Hip circumference (cm)	110.3 ± 11.6
Body fat (%)	42.2 ± 4.4
Serum folate (nmol/L)	20.9 ± 7.7
Folate in erythrocytes (nmol/L)	845.5 ± 323.2
Serum vitamin D (nmol/L)	54.1 ± 26.0
BMD lumbar spine (g/cm²)	1.015 ± 0.209
T-score lumbar spine	-1.1 ± 1.6
BMD femoral neck (g/cm²)	0.787 ± 0.153
T-score femoral neck	-1.5 ± 1.0
BMD femur (g/cm²)	0.867 ± 0.151
T-score femur	-1.0 ± 1.2

BMI = body mass index; BMD = bone mineral density

Table 2. Average daily micronutrient intake important for bone health

Parameters	Mean ± SD	% DRI
Calcium (mg)	614.1 ± 256.9	51.2 ± 21.4
Magnesium (mg)	215.3 ± 93.2	67.3 ± 29.1
Phosphorus (mg)	886.8 ± 262.3	126.7 ± 37.5
Potassium (mg)	2257.9 ± 795.6	48.0 ± 16.9
Sodium (mg)	2768.7 ± 858.1	223.7 ± 71.1
Folate (µg)	210.4 ± 78.7	52.6 ± 19.7
Vitamin D (µg)	1.5 ± 1.2	8.8 ± 6.8
Vitamin C (mg)	97.9 ± 72.6	130.6 ± 96.8

DRI = dietary reference intake

Table 2 shows the average daily dietary intake of micronutrients important for bone health. Average daily intake of calcium, magnesium, potassium, folate, and vitamin D was not in accordance with the dietary reference intake (DRI), while average daily intake of phosphorus, sodium, and vitamin C was higher than recommended (Table 2). The average daily intake of micronutrients important for bone health was higher in a study by New et al. than in this study. In that study, the average daily vitamin D intake was 3.41 μg, calcium intake was 1101 mg, magnesium intake was 326 mg, potassium intake 3404 mg, and phosphorus intake was 1536 mg for women older than 65 (New et al., 2000). Ilich et al. also studied the intake of micronutrients by older women, in order to analyse the impact of the diet on bones (Ilich et al., 2003). The average intake of above mentioned micronutrients for 136 subjects, whose mean age was 68, was higher in comparison to the results of this study.

The average daily intake of fruit and vegetables is presented in Table 3. Numerous studies suggest that a high fruit and vegetable intake has been associated with increased bone mineral density, decreased bone loss, and reduced bone turnover (Benetou et al., 2016).

Table 3. Average daily fruit and vegetable intake

Parameters	Mean ± SD	Min	Max
Fruit (g)	235.9 ± 187.9	43.3	954.0
Vegetables (g)	119.9 ± 82.0	0.0	388.8
Fruit and vegetables (g)	355.9 ± 227.3	82.5	1139.0

According to the minimum values, some of the subjects did not consume vegetables at all during the three days when the 24-hour recalls were carried out. The average daily fruit intake was 235.9 g and vegetable intake was 119.9 g (Table 3). The average daily fruit and vegetable intake was lower than recommended for more than 400 g per day (Antonić Degač et al., 2002).

Liu et al. studied daily intake of fruits and vegetables for 2000 men and 2000 women aged 65 and older. The average daily fruit intake was 247.0 g and the average daily vegetable intake was 238.1 g for older women (Liu et al., 2015). The average daily fruit intake was similar to the results of this study, and the average daily vegetable intake was higher than in this study. In that study, the total vegetable intake included

the intake of legumes, and in this study, legumes were not included in the estimated vegetable intake.

A significant difference was not determined for BMD at all measured sites between the two groups of subjects, according to fruit and vegetable intake (≤400 g and >400 g per day) (Table 4). The results of the similar study by Zalloua et al. (2007) do not entirely correspond to these results. They also examined the association between fruit and vegetable intake and BMD at the hip and total body in a Chinese population of both genders, aged 25 to 64. The subjects were divided into two groups based on their weekly consumption of fruit and vegetables. The first group consisted of subjects who consumed ≤250 g, and the second group of those who consumed >250 g of fruit per week. The subjects who had higher fruit intake had significantly higher total body BMD for both genders ($p<0.05$). According to vegetable intake, subjects were divided into two groups, with the consumption of >1500 g and ≤1500 g of vegetables per week. Higher vegetable intake did not seem to have a detectable statistical impact on BMD (Zalloua et al., 2007).

The group of subjects who had an average daily fruit and vegetable intake >400 g had a significantly higher average daily intake of magnesium, potassium, vitamin C, and folate ($p<0.05$), than the group of subjects who had an average daily fruit and vegetable intake ≤400 g (Table 5).

Table 4. Average bone mineral density according to fruit and vegetable intake (mean ± SD)

Parameters	Fruit and vegetable intake		p
	≤400 g (n=39)	>400 g (n=21)	
BMD lumbar spine (g/cm^2)	1.021 ± 0.237	1.003 ± 0.149	0.764
T-score lumbar spine	-1.1 ± 1.8	-1.3 ± 1.2	0.616
BMD femoral neck (g/cm^2)	0.784 ± 0.164	0.794 ± 0.133	0.799
T-score femoral neck	-1.5 ± 1.0	-1.5 ± 1.0	0.899
BMD femur (g/cm^2)	0.868 ± 0.169	0.866 ± 0.114	0.969
T-score femur	-1.0 ± 1.3	-1.0 ± 0.9	0.841

BMD = bone mineral density

Table 5. Micronutrient intake according to fruit and vegetable intake (mean ± SD)

Parameters	Fruit and vegetable intake		p
	≤400 g (n=39)	>400 g (n=21)	
Calcium (mg)	597.9 ± 264.2	644.2 ± 246.3	0.510
Magnesium (mg)	188.6 ± 43.8	265.0 ± 134.1	0.001
Potassium (mg)	1972.8 ± 540.3	2787.4 ± 927.1	<0.001
Sodium (mg)	2795.8 ± 814.6	2718.3 ± 952.6	0.741
Vitamin C (mg)	68.8 ± 43.7	152.0 ± 84.7	<0.001
Folate (μg)	185.0 ± 65.9	257.5 ± 80.0	<0.001

No significant correlation was determined between bone mineral density and the average daily intake of micronutrients (Table 6). A significant positive correlation was determined between vitamin D intake and the BMD of lumbar spine ($r=0.342$; $p=0.007$) (Table 6).

This confirms the importance of vitamin D for bone health, due to its role in improving intestinal calcium absorption and bone mineral matrix (Gennari, 2001).
Tucker et al. investigated the association between dietary components contributing to an alkaline environment (dietary potassium, magnesium, and fruit and vegetables) and BMD for elderly men and women. They observed that a higher potassium intake was significantly associated with higher BMD at all four measured sites for men (femoral neck, trochanter, Ward' s area, and radius) and at three sites for women (trochanter, Ward' s area, and radius).
Magnesium intake was associated with greater BMD at one hip site for both men and women and in the forearm for men. Fruit and vegetable intake was associated with BMD at 3 sites for men (femoral neck, Ward' s area, and radius) and 2 sites for women (trochanter and radius) (Tucker et al., 1999).
Some studies confirmed the positive influence of fruit and vegetables on bone mineral density (New et al., 2000; Prynne et al., 2006). They stated that a greater fruit and vegetable intake may have positive effects on bone mineral status, especially for younger persons (adolescents) and for elderly women. Those effects are visible on the spine and femoral neck (Prynne et al., 2006).

Table 6. Spearman's correlation coefficients between bone mineral density and micronutrients intake

Parameters	**Calcium (mg)**	**Magnesium (mg)**	**Phosphorus (mg)**	**Potassium (mg)**	**Sodium (mg)**	**Vitamin C (mg)**	**Folate (µg)**	**Vitamin D (µg)**
BMD lumbar spine (g/cm^2)	0.043 (0.744)	0.016 (0.906)	0.213 (0.102)	0.110 (0.403)	-0.032 (0.808)	0.019 (0.883)	-0.020 (0.880)	0.342 (0.007)
T-score lumbar spine	0.032 (0.808)	0.024 (0.858)	0.228 (0.080)	0.114 (0.385)	-0.034 (0.797)	0.004 (0.977)	-0.007 (0.954)	0.323 (0.012)
BMD femoral neck (g/cm^2)	0.049 (0.709)	-0.016 (0.905)	0.140 (0.287)	-0.038 (0.772)	-0.114 (0.384)	0.042 (0.751)	-0.097 (0.463)	0.161 (0.220)
T-score femoral neck	0.072 (0.584)	0.009 (0.947)	0.184 (0.160)	-0.054 (0.683)	-0.046 (0.729)	0.002 (0.989)	-0.112 (0.396)	0.172 (0.190)
BMD femur (g/cm^2)	-0.004 (0.975)	-0.070 (0.597)	0.094 (0.477)	-0.135 (0.303)	-0.056 (0.670)	0.013 (0.919)	-0.128 (0.329)	0.201 (0.123)
T-score femur	0.007 (0.957)	-0.038 (0.774)	0.124 (0.344)	-0.126 (0.337)	-0.013 (0.921)	0.006 (0.962)	-0.127 (0.332)	0.199 (0.128)

p-values are in the brackets

Table 7. Spearman's correlation coefficients between bone mineral density and fruit and vegetable intake

Parameters	Fruit	Vegetables	Fruit and vegetables
BMD lumbar spine (g/cm^2)	-0.01	0.06	0.04
T-score lumbar spine	0.01	0.06	0.05
BMD femoral neck (g/cm^2)	-0.14	0.12	-0.04
T-score femoral neck	-0.17	0.12	-0.06
BMD femur (g/cm^2)	-0.17	0.05	-0.08
T-score femur	-0.18	0.03	-0.09

But in this study, it was not confirmed that fruit and vegetable intake have a positive influence on BMD (Table 7). In a study with Swedish men and women aged 45 to 83, Byberg et al. determined that a fruit and vegetable intake below the recommended 5 servings/day is associated with a higher risk of hip fracture. Fruit and vegetable intake above this recommendation does not seem to further lower the risk of hip fracture (Byberg et al., 2015). Greater intake of fruit and vegetables was independently associated with a higher BMD and a lower presence of osteoporosis in middle-aged and elderly Chinese subjects. Fruit tended to have a higher contribution to favourable association than vegetables (Qiu et al., 2017). Greater fruit intake was independently associated with better bone mineral status among men and women aged 65 and older (Liu et al., 2015).
The limitation of the study is the small number of subjects, so the results of this study should be interpreted with caution, because the small number of subjects reduced the statistical power of our data. It would be better if a higher number of 24-h recalls were collected, in order to more accurately estimate the fruit and vegetable intake.
This is the only study that investigated the possible impact of fruit and vegetable intake on bone mineral density in a Croatian population.

CONCLUSIONS

In this study, no significant difference was determined in BMD of lumbar spine, femur, and femoral neck between the two groups of subjects, according to an average daily intake of fruit and vegetables (>400 g and ≤400 g). A significant positive correlation was determined between an average daily vitamin D intake and BMD of the lumbar spine. No significant correlation was determined between an average daily fruit and vegetable intake and BMD at all measured sites for elderly women. The results of this study did not show that a diet high in fruit and vegetables contributes to the maintenance of BMD.

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Topic: Dietetics and diet therapy

Sekcija: Dijetetika i dijetoterapija

THE IMPORTANCE OF NUTRITION EDUCATION FOR DIABETICS – TYPE 1 VERSUS TYPE 2 DIABETICS

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ABSTRACT

Nutrition education is an integral part of the diabetic therapy that aims at achieving good glycaemic control to prevent complications. This is very important for type 1 diabetics (DMT1), where nutrition and insulin therapy must be matched. The aims of the study were: the evaluation of differences in nutrition education between DMT1 and type 2 diabetics (DMT2), the influence of education on glycaemic control and diet quality, and the self-assessment of health-related quality of life. A descriptive study was conducted on patients with DMT1 (n=101) and DMT2 (n=90) from Croatia using a study-specific questionnaire. Diet quality did not differ significantly between DMT1 and DMT2 patients. However, 18.0% of DMT1 and 20.0% of DMT2 patients do not possess adequate knowledge of the diabetic diet, nor stick to relating nutritional guidelines. Poor glycaemic control had 48.5% of DMT1 and 73.5% of DMT2 patients ($p<0.001$). Psychophysical health is better among the DMT2 patients ($p<0.001$), while the DMT1 patients have a better social life ($p=0.015$) and the overall quality of life ($p<0.001$). The results show that diabetics have poor nutrition knowledge, clearly showing the need for professional, continuous nutrition education. Education would help diabetics in improving their quality of life and glycaemia control, which decreases disease complications.

Keywords: diabetes type 1, diabetes type 2, diet quality, glycaemia control, quality of life

INTRODUCTION

Diabetes mellitus (DM) is a life-long metabolic disorder of carbohydrates, proteins and fats which leads to state of hyperglycemia. The global prevalence of DM in 2015 was 8.8%, and the positive trend continues (IDF, 2015). Uncontrolled hyperglycaemia is an underlying cause of a wide range of micro- and macrovascular complications. Today, more than two-thirds of deaths in diabetics are linked to cardiovascular diseases (Low Wang et al., 2016).
DM type 1 (DMT1) is characterized by an autoimmune destruction of β cells of the pancreas resulting in insulitis, inflammatory infiltration of the islets of Langerhans (In't Veld, 2011). Consequently, insulinopenia develops, i.e. major reduction

(around 80%) or complete absence of insulin synthesized in the pancreas. Symptoms are hyperglycemia, polyuria, polydipsia, sudden loss of weight, dehydration with consequent electrolyte disturbance, and ketoacidosis. Disease has acute start and develops within a couple of days or weeks. It affects predominantly young people till the age of 30, but the highest incidence is between the ages of 10 and 14, with no gender differences in frequency (Poljičanin et al., 2015; Stipančić et al., 2012).
Conversely, DMT2 is a polygenetic group of disorders, characterized by hyperglycemia due to disturbance in insulin secretion and/or its effect on target cells in the body, which results in a decreased number of glucose transporters (i.e. peripheral resistance) (Tripathi and Srivastava, 2006). The disease develops gradually, mainly affects older adults (from 45 years of age and older) (IDF, 2015) and it usually takes 9-12 years from the onset of the disease to its diagnosis (Harris et al., 1992). At diagnosis, about 50% of beta cell function is lost and symptoms include hyperglycemia, polyuria, polydipsia, dehydration with consequent electrolyte disturbance, fatigue, blurred vision, a high rate of infections and hyperosmolar hyperglycemic non-ketotic syndrome (Vijan, 2015). Unlike DMT1, obesity represents a major risk factor in DMT2; at diagnosis, up to 88% of DMT2 patients are overweight or obese (Wilding, 2014).The main diagnostic and treatment success criteria is glycated hemoglobin, HbA1c. This is a fraction of hemoglobin on which glucose in the bloodstream naturally attaches regardless of insulin and reflects average blood glucose levels over duration of 2-3 months (IDF, 2015).
The treatment puts the main focus on self-management and starts with education about the disease itself, oral medications and/or insulin, principles of the diabetic diet and the importance of regular physical activity (ADA, 2015). The only and major difference in the treatment protocol between DMT1 and DMT2 is in the type of the medicament therapy due to differences in disease etiology, progression and later prognosis (ADA, 2015; Vrca Botica et al. 2012; Kokić et al., 2011). Basic treatment principles are the adjustment of the dietary and lifestyle habits aiming to achieve and maintain good glycaemia control , consequently preventing and delaying micro- and macrovascular complications (ESC/EASD, 2013) in order to maintain good quality of life and prolong life expectancy (ADA, 2015). In order to achieve these goals, continuous, professional education is considered crucial (ADA, 2015; IDF, 2015).
The aim of the study was to evaluate the differences in nutrition education between DMT1 and DMT2 patients, and to determine the influence of education on glycaemic control, diet quality, and the self-assessment of health-related quality of life.

SUBJECTS AND METHODS

A descriptive study was conducted on patients with DMT1 (n=101) and DMT2 (n=90) from Croatia using a study-specific questionnaire. The questionaire was anonymous, and participants were asked to complete it only once. DMT1 patients were recruited via an online form (using open source Google Forms) and parents provided the information for the underaged children. DMT2 patients were recruited in pharmacies across the Osijek-Baranja region.

Questionnaire

The questionnaire consisted of three parts and it was developed on the basis of the previous research experience and guidelines for the treatment of DMT1 and DMT2 (IDF, 2012; 2017; ADA, 2015).
The first part of the questionnaire included questions about general and socio-economic characteristics, e.g. age, gender, body mass, body weight, education, monthly income per person, etc. The participants' self-reported current body mass and height were then used for the calculation of the body mass index (BMI). Calculated BMI was used to categorize participants according to their state of nourishment (WHO, 2006).
The second part of the questionnaire included questions about diagnosis, course of the disease and the treatment. Glycaemic control was considered good if glycated haemoglobin (HbA1c) was ≤6.5% or fasting blood glucose was ≤7.0 mmol/L (IDF, 2012; 2017; ADA, 2015).
Three questions in a form of a visual-analogue scale examined participants' subjective assessment on how much diabetes affects their social life (SocL), psychophysical health (PsH) and quality of life (QoL). The participants were asked to place a vertical line on the scale which was measured with a ruler, counted and transfered into a score. The score ranged from 0 to 100, and higher scores correlated with better aspects of life.
The third part of the questionnaire assessed the quality of the diet through a set of questions that encompassed all aspects of the diabetic diet and scored according to the current recommendations for diabetics (IDF, 2012; 2017; ADA, 2015). Questions were set as a multiple choice questions and only one answer could be given for each question. Each answer was assigned from 1 (the least preferred habit) to 5 points (the most preferred habit). The points were summed and gave the overall score, ranging from 25 to 125. Higher overall score correlated with a better diet and lifestyle habits.

Statistical analysis

Statistical analysis was performed by software Statistica (v. 13.3, StarSoft Inc., USA), with the level of significance p=0.05. Normality of the data distribution was tested by the nonparametric Kolmogorov-Smirnov test for the comparison of medians and arithmetic mean, and histograms plotting. Categorical data are presented as absolute and relative frequencies, while for numerical data median and interquartile range is used using descriptive statistical methods. For the comparison of categorical data within and between groups Fischer's exact test was used. The differences between two independent groups were tested with Mann-Whitney U Test.

RESULTS AND DISCUSSION

The study encompassed 101 DMT1 patients, mean age 25.0 ± 8.9 years (6 to 50 years, 22.8% males, 77.2% females) and 90 DMT2 patients, average age 68.0 ± 9.4 years (46 to 87 years, 46.0% males and 54.0% females).
As previously emphasized, the ultimate goal of nutrition education is to teach a diabetic how to independently regulate disease in order to manage everyday life activities without the risk of developing acute or chronic complications (Kokić et al., 2011). All

complications significantly alter the quality of life of diabetics and reduce life expectancy (DCCT, 1993; Nathan, 2014; Pacilli et al., 2016).
Although there was no difference in the quality of the diet or the level of education between DMT1 and DMT2 patients (Table 1), previous studies show that education on nutrition is necessary to improve control of glycaemia in both DM types (Baretić et al, 2017; Badruddin et al., 2002). However, 18.0% of DMT1 and 20.0% of DMT2 patients do not possesss adequate knowledge of the diabetic diet, nor stick to relating nutritional guidelines. Poor glycaemic control was found in 48.5% of DMT1 and 73.5% of DMT2 patients ($p<0.001$, Table 1). The same findings have been reported by the other authors (Badruddin et al., 2002; Gerstl et al., 2008).

Table 1. Glycaemia control, diet quality and the level of nutrition education between DMT1 and DMT2 patients

Characteristics of diabetics		**Diabetes mellitus type**		**p**
		Type 1	**Type 2**	
Diet quality (points)[+]		71.3 (66.3 – 76.3)	73.6 (68.0 – 77.6)	0.057[*]
Nutrition education[++]	Well educated	82 (82.0)	72 (80.0)	0.433[**]
	Not educated	18 (18.0)	18 (20.0)	
Glycaemia control[++]	Good[1]	52 (51.5)	18 (26.5)	*0.001*[**]
	Bad[1]	49 (48.5)	50 (73.5)	

[+]median (25 % - 75%); [++]n (%); [1]According to the recommended levels for HbA1c level, i.e. fasting blood glucose; *Mann-Whitney U test; **Fischer's exact test; statistical significance at $p<0.05$

The results of this research show the need for education on nutrition by a nutritionist/dietitian (for uneducated diabetics), but also for re-education of DMT1 and DMT2 diabetics due to our findings of their poor glycaemia control (Table 1). This becomes even more important if we know that 82% of DMT1 and 80% of DMT2 diabetics were educated, but almost half of DMT1 and tree quarters of DMT2 diabetics do not achieve recommended HbA1c levels (Table 1). Basic principles of education on nutrition for diabetes include setting up a regular meal pattern with consistent caloric and carbohydrate intake, while the number of meals itself depends on oral and/or insulin therapy (3-5 meals per day) (ADA, 2008.; Kokić *et al.,* 2011). Overall energy intake should be adjusted to a goal regarding patients weight reduction or control (Wheeler, 2012.). Even though the ideal diet for diabetic patients remains to be determined on an individual level, certain general guidelines should be followed no matter what regime is in place (Gingras et al., 2015; Delahanty et al., 2009; ADA, 2015).
DMT2 patients have better psychophysical health than DMT1 patients ($p<0.001$), while DMT1 patients have better social life ($p=0.015$) and the overall quality of life ($p<0.001$) in comparison to DMT2 patients (Figure 1). DMT2 patients have better PsH because they were diagnosed with DM at an older age when they were capable of comprehending the challenges of the diseases. Also, as the diseases progresses, the quality of life and social aspects of life inevitably worsenes. On the other hand, for young children diagnosed with DMT1 the most of the burden is taken by their parents/care-takers by the time they reach adolescence. As long as parents have some control, the disease will not affect the quality of life in DMT1 diabetics.

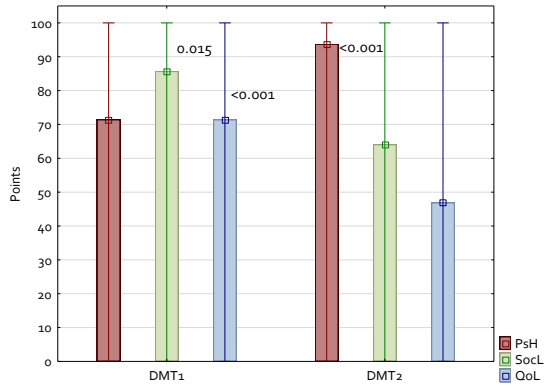

Fig. 1. The self-assessment on the level of influence diabetes has on three dimensions of life between DMT1 and DMT2 patients (Mann-Whitney U Test; statistical significance at $p<0.05$)

The quality of life in diabetics is most significantly affected by awareness of the complications and risk-factors of diabetes (Kalda et al., 2008; da Costa and Vieira, 2015).
In general, adolescents are more resistant to accepting the disease than younger children because they no longer depend on their parents or guardians for care and are responsible for their own health. Psychosocial issues also influence the behaviour of adolescents, reflecting their attitudes towards diabetes. Hormonal alterations, immaturity, difficulties in acquiring autonomous control, and a low rate of disease acceptance may hinder the daily control of blood glucose levels (da Costa and Vieira, 2015). For DMT2 patients, patient's age, duration of the disease, and patient's BMI significantly correlate with the quality of life (Kalda et al., 2008). Additionaly, obese DMT2 patients, especially females, experience anxiety or depression in comparison to their counterparts of a normal weight (Svenningsson et al., 2012). Also, diabetics with developed cardiovascular complications (i.e. hyperlipidemia and heart diseases) had significantly worse social functioning, physical functioning and mental health (Kazemi-Galougahi et al., 2012).

CONCLUSIONS

The results show that diabetics have poor nutrition knowledge, clearly showing the need for professional, continuous nutrition education. Education would help diabetics in improving their quality of life and control of glycaemia, consequently decreasing the burden of the disease (fewer complications).

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Topic: Dietetics and diet therapy / **Sekcija: Dijetetika i dijetoterapija**

KARDIOVASKULARNI RIZIK U DJECE I ADOLESCENATA S RESTRIKTIVNIM TIPOM ANOREKSIJE NERVOZE

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SAŽETAK

Anoreksija nervoza (AN) jedan je od poremećaja jedenja koji danas spada među najčešće kronične bolesti adolescenata s visokom stopom smrtnosti, a najčešće se veže za ženski spol i adolescentsku dob. Retrospektivna studija obuhvatila je 183 djevojčice i adolescentice (od 2005. do 2015. godine) Centra za poremećaje u jedenju djece i adolescenata Kliničkog bolničkog centra Sestre milosrdnice Zagreb. Prosječna dob pacijentica bila je 14,8 ± 2,5 godina (7 do 23 godine) s dijagnozom AN u trajanju od prosječno 12,8 ± 13,9 mjeseci (0,7 do 108 mjeseci). Prosječni indeks tjelesne mase (BMI) je bio 15,8 ± 1,9 kg/m^2 s postotkom idealne tjelesne mase (IBW) 77,7 ± 9,9% i prosječnim gubitkom na masi 21,7 ± 9,9%. Normalan menstrualni ciklus je bio prisutan kod 20,6%, a sekundarnu amenoreju je imalo 74,4% AN pacijentica. Povećan kardiovaskularni (CVD) rizik utvrđen je kod trećine AN pacijentica (35,5%) i gotovo polovice restriktivnog tipa AN (47,4%). Povećan kardiovaskularni rizik kod AN pacijentica povezan je s lošijim antropološkim parametrima (p=0,018) i amenorejom (p<0,001). Rezultati potvrđuju povezanost povećanog CVD rizika i endotipa AN te potvrđuju važnost tjelesnog oporavka kod AN pacijenata.

Ključne riječi: anoreksija nervoza, djeca i adolescenti, restriktivni tip, kardiovaskularni rizik

UVOD

Poremećaji jedenja u današnje vrijeme postaju sve učestalija pojava među svim dobnim skupinama i oba spola. Defniraju se kao trajan poremećaj odnosa prema jelu i slici o izgledu vlastitog tijela koji za posljedicu ima poremećen unos hrane sa znatnim oštećenjem fizičkog zdravlja i psihosocijalnog funkcioniranja (Sambol i Cikaš, 2015). Najčešći predstavnici poremećaja su anoreksija nervoza, bulimija nervoza, emocionalni poremećaji izbjegavanja hrane, sindrom sveobuhvatnog odbijanja hrane i pića i tzv. *pica* (Vidović, 2009).
Anoreksija nervoza (AN) danas spada među najčešće kronične bolesti kod adolescenata, najčešće ženskog spola, s visokom stopom smrtnosti u odnosu na druge bolesti u toj dobi (Žaja, 2014; Keski-Rahkonen i Mustelin, 2016).

AN primarno podrazumijeva psihijatrijski poremećaj kod kojeg je gubitku apetita prethodilo dugotrajno gladovanje s ciljem redukcije tjelesne mase praćeno strahom od prirasta na masi i iskrivljenim doživljajem vlastitog tijela (Lesar i Žaja, 2014). Etiologija AN uključuje biološke (genetske), psihološke i socijalne čimbenike, od kojih svaki znatno utječe na razvoj bolesti, ali nijedan od njih pojedinačno nije nužan niti dovoljan za punu ekspresiju bolesti (Žaja, 2014). Neki od faktora koji utječu na nastanak bolesti su poremećaj ličnosti, emocionalni poremećaji, pritisak obitelji i okoline, ali i opsjednutosti mislima o mršavosti (Vidović, 2009).
Danas se AN dijagnosticira prema Dijagnostičkom i statističkom priručniku mentalnih poremećaja (DSM), verziji 5, i primarno se pojavljuje u dva oblika - restriktivna (AN-RT) i bulimično-purgativna (AN-BP) s razlikom u odnosu prema hrani. Za restriktivni tip karakteristično je strogo ograničavanje i smanjenje količine unosa hrane i kalorija, kao i izlaganje pretjeranom vježbanju, koje je prisutno u približno 40% slučajeva (Lesar i Žaja, 2014).
Posljedice AN su brojne i pogađaju sve tjelesne sustave (Žaja, 2014). Akutne medicinske komplikacije AN su dobro opisane, no spoznaje o kroničnim komplikacijama koje utječu na koštano i kardiovaskularno zdravlje manje su poznate.

ISPITANICI I METODE

Cilj rada bio je ispitati kardiovaskularni rizik u djece i adolescenata s restriktivnim tipom AN promatrano kroz lipidni status.
Provedena je retrospektivna studija koja je obuhvatila 183 djece i adolescenata liječenih u Centru za poremećaje u jedenju kod djece i adolescenata Kliničkog bolničkog centra Sestre milosrdnice Zagreb u razdoblju od 2005. do 2015. godine. Dijagnoza poremećaja u jedenju postavljena je prema važećim kriterijima za određeno vremensko razdoblje, odnosno kriteriju DSM-IV i DSM-V. Istraživanjem je obuhvaćena cijela skupina uz izdvajanje restriktivnog endotipa AN. Svi ispitanici su bili ženskog spola, a karakteristike ispitanica prikazane su u tablici 1. Oboljele su u Centar obično bile upućene iz školskih zdravstvenih službi (prema suspektnoj procjeni na izostanak napredovanja u rastu i razvoju, a prema dijagramu rasta i tjelesne mase za dob i spol) ili nakon što je obitelj izravno stupila u kontakt s bolnicom. Iz daljnje obrade u sklopu Centra isključene su pacijentice kod kojih su dokazane druge somatske bolesti kao što su: dijabetes tipa 1, celijakija, Crohnova bolest, hipotireoza, reumatoidni artritis i alergije na hranu.
Istraživanje je odobreno od strane Etičkog povjerenstva Kliničkog bolničkog centra Sestre milosrdnice Zagreb.

Antropometrijska procjena

Antropometrijski podaci su dobiveni fizičkim pregledom i mjerenjima tjelesne mase (TM) koja je mjerena medicinskom vagom s utezima (Seca, UK) i tjelesne visine (TV) koja je mjerena stadiometrom (Seca, UK) bez obuće. Idealna tjelesna masa (IBW) je određena iz percentilnih krivulja za tjelesnu masu po dobi za djevojčice (Kuczmarski i sur., 2000), a udio idealne tjelesne mase je računat prema jednadžbi (1):

% IBW = (TM (kg) / IBW (kg)) x 100 (1)

Kriterij TM za dijagnozu AN kod odraslih osoba je BMI ispod 17,5 kg/m^2. To odgovara rezultatu standardne devijacije BMI-a (SDS) ispod 2,00 (Lindgren, 1995) koji je korišten kao kriterij tjelesne mase za adolescente (Hebebrand, 2004).
Pratila se godina pojave bolesti i trajanje bolesti u mjesecima, kao i TM prije bolesti te se gubitak kilograma od početka bolesti (% gubitka) računao prema jednadžbi (2):

% gubitka = [(TM prije anoreksije - TM) / TM prije anoreksije] x 100 (2)

Gubitak mase izračunat je kao razlika između maksimalne zabilježene mase i procjenjene mase. Vrijeme trajanje gubitka mase izračunato je kao razlika u vremenu između maksimalne zabilježene mase iz medicinskih procjena, dok je stopa gubitka mase izračunata kao gubitak mase podijeljen s trajanjem. Stoga se prilikom procjene gubitka tjelesne mase, trajanje i brzina gubitka tjelesne mase nije uzeo u obzir tijek mijenjanja mase između dva promatranja. Konačna stopa gubitka mase izračunata je od zadnje zabilježene mase prije procjene.

Biokemijske analize

Sve biokemijske analize provedene su na Kliničkom zavodu za kemiju Kliničkog bolničkog centra Sestre milosrdnice Zagreb u sklopu rutinske kliničke obrade pacijenata.
U uzorcima krvi prikupljenim na inicijalnom pregledu po prijemu u Centar određeni su: serumski elektroliti (natrij, kalij, kloridi, kalcij, fosfor, magnezij), pH krvi, ukupni proteini, glukoza, feritin, transferin, trigliceridi, ukupni kolesterol, lipoprotein niske gustoće (HDL), lipoprotein visoke gustoće (LDL), ureja, kreatinin, tireotropin stimulirajući hormon (TSH), trijodtironin (T3), slobodni T3, tetrajodtironin (T4), slobodni T4, folikul stimulirajući hormon (FSH), luteinizirajući hormon (LH) i estradiol (E2).
Lipidni profil je određen prema metodama propisanim od strane Međunarodne federacije za kliničku kemiju i laboratorijsku medicinu (eng. *The International Federation of Clinical Chemistry and Laboratory Medicine*, IFCC).
S obzirom na to da je kardiovaskularni rizik procijenjen prema lipidnom statusu, navedene su specifične metode samo za te parametre.
Ukupni kolesterol određen je standardnom kolorimetrijskom metodom s kolesterol oksidazom (CHOD-PAP metoda, uz kolesterol oksidazu (CHOD) i peroksidazu (PAP)), HDL kolesterol određen je direktnom fotometrijskom metodom uz primjenu kolesterol esteraze, dok su trigliceridi određeni kolorimetrijskom enzimskom metodom (GPO-PAP metoda, u prisustvu glicerol fosfat oksidaze (GPO) i peroksidaze (PAP)). Za analizu kolesterola i HDL kolesterola do siječnja 2005. godine korištena je Advia 1650 (Bayer), a nakon toga Architect (Abbott). LDL kolesterol je izračunat kao razlika ukupnog kolesterola i HDL kolesterola.

Kao indikator kardiovaskularnog rizika korišten je ukupni kolesterol kojemu je referentni raspon za oba spola do 18 godina <4,7 mmol/L, a za odrasle osobe <5,0 mmol/L.

Statistička analiza

Statistička analiza napravljena je programskim sustavom Statistica (inačica 12.0, StatSoft Inc., USA), uz odabranu razinu značajnosti od p=0,050.
Normalnost razdiobe podataka utvrđena je primjenom Kolmogorov-Smirnov testa. Za usporedbu kategoričkih podataka unutar i među skupinama korišten je Hi-kvadrat test te je primijenjen T-test za zavisna odnosno nezavisna mjerenja. Za izračun korelacija numeričkih podataka korišten je Pearsonov test korelacije. Svi prikupljeni kategorički podaci predstavljeni su apsolutnim i relativnim frekvencijama, dok su numerički podaci opisani aritmetičkom sredinom i standardnom devijacijom, uz navođenje minimalnih i maksimalnih vrijednosti.

REZULTATI I RASPRAVA

Prosječna dob pacijentica bila je 14,8 ± 2,5 godina, no minimalna dob je bila 7, a maksimalna dob 21 godina. Dob kod pojave bolesti bila je 14,1 ± 2,5 godina, a bolest je prosječno trajala 12,8 ± 13,9 mjeseci (od 0,7 do 108 mjeseci) (Tablica 1). Ovi su podaci u skladu s istraživanjima koja govore kako je najveći broj AN pacijentica u dobi od 14 godina, no kako se javlja i sve ranije pa obolijevanje u dobi od 7 godina nije rijetkost. Do 40% svih dijagnosticiranih slučajeva AN nalazi se u dobnoj skupini od 15 do 19 godina. Istraživanja pokazuju da je najveća prevalencija anoreksije zabilježena u dobnoj skupini od 10 do 19 godina te je 2000. godine iznosila 34,6 slučajeva na 100 000 stanovnika (Jagielska i Kacperska, 2017; Sambol i Cikač, 2015; Vidović i sur., 2008; Lazarević i sur., 2013). AN se sve češće javlja u dječjoj dobi što podrazumijeva poremećaj nastao prije 14. godine, a istraživanja devedesetih godina prošlog stoljeća bilježe nagli porast broja djece koja su dovedena na liječenje upravo u toj dobi (Žaja, 2014).

Tablica 1. Demografska obilježja ispitanica vezana uz pojavu AN
Table 1. Demographic characteristics of children and adolescents with AN

Karakteristike	N	Srednja vrijednost	SD	Minimum	Maksimum
Dob (godine)	183	14,8	2,5	7	21
Dob kod pojave bolesti (godine)	183	14,1	2,4	7	21
Trajanje bolesti (mjeseci)	183	12,8	13,9	0,7	108

SD – standardna devijacija

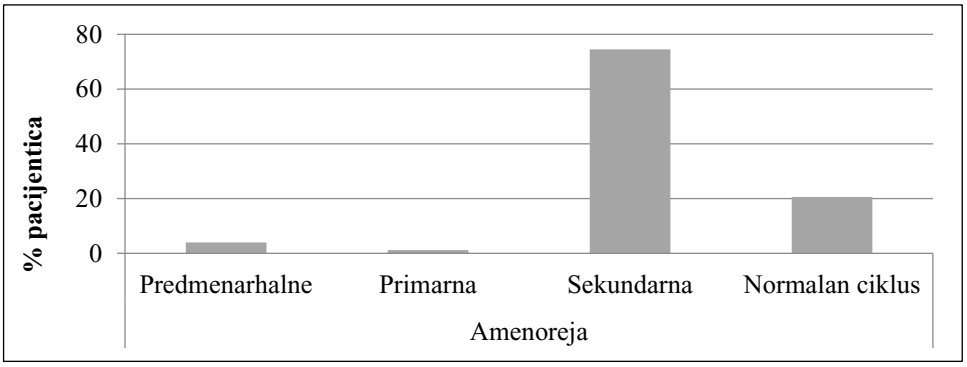
S obzirom na antropomtrijske parametre (Tablica 2), vidljivo je kako je prosječni indeks tjelesne mase (BMI) bio 15,8 ± 1,9 kg/m^2 te da je postotak idealne mase (IBW) bio prosječno 77,7 ± 9,9% uz prosječni gubitak tjelesne mase od 21,7 ± 9,9%.

Tablica 2. Antropometrijski parametri ispitanica s dijagnozom AN
Table 2. Anthropometric parameters children and adolescents with AN

Antropometrijski parametri	n	Srednja vrijednost	SD	Minimum	Maximum
Indeks tjelesne mase (kg/m^2)	183	15,8	1,9	11,3	20,0
Idealna tjelesna masa (%)	183	77,7	9,9	44,4	99,8
Gubitak tjelesne mase (%)	177	21,7	9,9	0,0	58,7

SD – standardna devijacija

Među ispitanicama, premenarhalnih je bilo 3,9%, s primarnom amenorejom 1,1%, sekundarnom amenorejom 74,4% dok je normalan menstrualni ciklus imalo 20,6% ispitanica (Slika 1). Amenoreja u AN razvija se kao posljedica sekundarne supresije hormonske veze hipotalamus-hipofiza-jajnik i energetskog deficita posredovanog leptinom (Golden i Carlson, 2008; Golden i Jacobson, 2016). Razine LH u serumu, FSH i E2 su niske, uz simptome odgođenog puberteta prate i primarnu i sekundarnu amenoreju. Uspostavljanje menstrualnog ciklusa je iznimno važno i za zdravlje kostiju. Pacijenti s AN koji su povećali TM, ali nisu povratili menstrualni ciklus imaju nižu mineralnu gustoću kostiju od onih koji su dobili na TM i povratili menstrualni ciklus (Misra i sur., 2006, Balenović i sur., 2008). Iako još nije poznat učinak povratka menstrualnog ciklusa na rizik od kardiovaskularnog zdravlja, ponovnim uspostavljanjem menstrualnog ciklusa obnavlja se razina estrogena, stoga se oporavak TM povezuje s nastavkom spontanih ciklusa i prepoznat je kao važna objektivna mjera vraćanja biološkog zdravlja (Žaja, 2014; Swenne, 2016).
Prema lipidnom profilu pacijentica s AN utvrđeno je kako povišene vrijednosti ukupnog kolesterola ima 35,5% (61/172) AN pacijentica i čak 47,4% pacijentica s restriktivnim tipom AN (27/152).



Slika 1. Udio ispitanica prema menstrualnom ciklusu (N=183)
Fig. 1. The percentage of children and adolescents with AN according to their menstrual cycle (N=183)

Svi parametri lipidnog profila se mogu koristiti kao indikatori kardiovaskularnog rizika, no HDL kolesterol jednak je LDL kolesterolu (Frontini i sur., 2008), a koristi se i ukupni kolesterol (ESC/EAS, 2011, Stone i sur., 2014). Koncentracija HDL kolesterola kod

adolescenata je indikator kardiovaskularnih bolesti u odrasloj dobi i predstavlja veći rizik od vrijednosti ukupnog kolesterola (Stone i sur., 2014) i ukoliko je <1,2 mmol/L postavlja se dijagnoza dislipidemije (Dai i sur., 2014). Pacijentice s AN su imale prosječnu vrijednost HDL kolesterola 1,7 ± 0,4 mmol/L (rezultati nisu prikazani).

Mnoga istraživanja upućuju na to da oboljeli od AN imaju povišene koncentracije kolesterola i LDL kolesterola unatoč karakteristikama da visok udio kolesterola uobičajeno imaju pacijenati s povećanom tjelesnom masom i aterogenom prehranom (Mordasini i sur., 1978; Feillet i sur., 2000; Weinbrenner i sur., 2004; Ohwada i sur., 2006; Misra i sur., 2006; Matzkin i sur., 2007; Swenne, 2016). Podaci dostupni u literaturi vezano za pedijatrijsku populaciju su oskudni jer se velika većina istraživanja odnosi na odraslu populaciju. Generalno, povećan udio lipida povećava rizik razvoja kardiovaskularnih bolesti što dodatno otežava uvjete oporavka i liječenja pacijenata koji boluju od AN, a dodatno povećava i rizik za smrtnost ovih pacijenata (Swenne, 2016; Sniderman i sur., 2010).

Ranija istraživanja Mordasini i sur. (1978) i Weinbrenner i sur. (2004) su utvrdila kako visoke vrijednosti kolesterola negativno koreliraju s tjelesnom masom i indeksom tjelesne mase (BMI). Ovim istraživanjem je to i potvrđeno: utvrđena je statistički značajna negativna povezanost između ukupnog serumskog kolesterola i BMI kod AN pacijentica (r=-0,341). Rezultati upućuju na jasnu korelaciju serumskog kolesterola s endotipom AN, potvrđujući važnu ulogu izgladnjivanja u povećanju kardiovaskularnog rizika kod AN pacijenata. 1965. godine Klinefelter je prvi put opisao hiperkolesterolemiju u AN koja je vrlo neočekivana s obzirom na restriktivnu prehranu koju provode oboljeli (Klinefelter, 1965). Etiologija hiperkolesterolemije u poremećajima jedenja ostaje za sada enigma. Mogući mehanizmi uključuju povećanu sintezu kolesterola, odgađanje metabolizma kolesterola, kao i povećanu mobilizaciju lipida iz adipoznih tkiva kako bi se zadovoljili zahtjevi za energijom. Većina studija potvrđuje kako se kod AN hiperkolesterolemija liječi s oporavkom TM. Endogeni estrogen ima važne metaboličke učinke na metabolizam lipida te utječe na enzime kolesterola, sinteze i degradacije lipoproteina (Barton, 2013).

Daljnjom analizom utvrđena je statistički značajno niži IBW (75,23 ± 9,45% u usporedbi s 78,98 ± 9,79%, p=0,018) kod ispitanica koje su imale povećani kardiovaskularni rizik u odnosu prema onima s nižim rizikom.

Među pacijenticama s restrikitvnim tipom AN u skupini onih koje imaju povećan kardiovaskularni rizik, utvrđen je statistički značajno manji broj pacijentica s normalnim menstrualnim ciklusom u usporedbi s onima u skupini s niskim kardiovaskularnim rizikom (13 u usporedbi s 21 među onima niskog kardiovaskularnog rizika, p<0,001). U skladu s drugim istraživanjima, rezultati ove studije naglašavaju važnost očuvanja ili oporavka menstruacije kao jednog od glavnih ciljeva liječenja AN kako bi se izbjegle dugoročne posljedice amenoreje i dislipidemije (Swenne, 2016; Skafar i sur., 1997).

ZAKLJUČAK

Dobiveni rezultati jasno potvrđuju povezanost povećanog kardiovaskularnog rizika i endotipa AN te potvrđuju važnost tjelesnog oporavka oboljele djece i adolescenata s AN, kako bi se izbjegle dugoročne posljedice, ponajprije u pogledu kardiovaskularnog zdravlja.

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Topic: Dietetics and diet therapy / **Sekcija: Dijetetika i dijetoterapija**

CARDIOVASCULAR RISK IN CHILDREN AND ADOLESCENTS WITH RESTRICTIVE TYPE OF ANOREXIA NERVOSA

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Anorexia nervosa (AN), one of the eating disorders that today represents one of the most common chronic disease in adolescents with high mortality rate, is mainly related to female gender and adolescent age. The research was conducted in collaboration with the Centre for eating disorders in children and adolescents, Sestre milosrdnice University Hospital Center Zagreb. Observational retrospective study included 183 girls and adolescents from the Centre's registry (2005 to 2015 period). The average age of patients was 14.8 ± 2.5 years (7 to 21 years), with AN diagnosed for an average of 12.8 ± 13.9 months (0.7 to 108 months). Their average BMI was 15.8 ± 1.9 kg/m2 with IBW of 77.7 ± 9.9% and average weight loss of 21.7 ± 9.9%. Normal menstrual cycle was found in 20.6% patients and secondary amenorrhea in 74.4% of AN patients. The increased cardiovascular (CVD) risk was found in one third of AN patients (35.5%) and almost half in restrictive type AN patients (47.4%). The increased CVD risk correlates with worse anthropometric parameters ($p=0.018$) and amenorrhea ($p<0.001$). The results confirm correlation between the increased CVD risk and AN endotype and importance of nutritional recovery and normalization of the menstrual cycle as the main goal of AN treatment.

Keywords: anorexia nervosa, children and adolescents, restrictive type, cardiovascular risk

Topic: Functional food and food supplemnents

Sekcija: Funkcionalna hrana i dodaci prehrani

Topic: Functional food and food supplemnents / **Sekcija: Funkcionalna hrana i dodaci prehrani**

TREATMENT OF PAIN AND INFLAMMATION AMONG ATHLETES USING HERBAL THERAPY AND NUTRITION

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review article/pregledni rad

ABSTRACT

During training and competition, athletes can be injured, or feel fatigue and pain in the locomotor system, as well as psychological tension. The use of conventional drugs is controlled by applying tests for doping control, apart from some herbal substances which are authorized for use. The aim of our research was to determine the effectiveness of herbal extracts, and their safety for use by athletes. Materials and methods: We used data acquired by searching two databases: https://www.ncbi.nlm.nih.gov/pubmed and https://nccih.nih.gov. Results and discussion: 15 substances were analysed in terms of evident research, efficiency, and safety in use. Conclusion: most of the substances have already proven effective in reducing pain and inflammation (such as turmeric, ginger, polyphenols, bromelain, gavez, golden root) if applied locally and taken orally, but there is evidence that some aren't safe (arnica willow, devil's claw, lord lineage).

Keywords: athletes, pain, herbal therapy

INTRODUCTION

During training and competition, athletes can be injured, or feel fatigue and pain in the locomotor system, as well as psychological tension. The treatment of these symptoms means that the athlete will achieve greater success and better results. Conventional medical treatments with drugs lead to doping controls, but they also have side effects affecting other organs and tissues, which can be damaged, and thus reduce the psychophysical ability of the athlete.
Non-pharmacological treatment of pain and inflammation includes physical therapy and alternative medicine (Bartels et al., 2006).
Herbal therapy is a part of AM, and it is used in the form of pills, grease, local baths, and compresses. It is not used daily by athletes, but it is used in Russia, China and other western countries by eminent athletes (Popova Ramova and Angelovska, 2016).
The aim of our study is to show to what extent some herbal extracts are safe, effective, and scientifically proved.

MATERIALS AND METHODS

We found out about this and got the idea by researching one review published on the website of the National Center for Integrative Health in the USA, in 2016, which confirms the efficacy and safety of multiple products that can be used in the treatment of athletes.

We did the second research in the medical data base Pubmed, using keywords: sports injuries, herbal therapy. The data is grouped by evidence of data, effectiveness, and safety. There were 14 herbal extracts that were analysed.

RESULTS AND DISCUSSION

1. **Turmeric** has a long history for the treatment of inflammation by Ajuverdic medicine (Peddada et al., 2015; Madhu et al., 2013).

Recorded data	Effectiveness	Safety
Few studies (2013-15)	Proved labour effect on muscles and OA pain	Safe if consumed by adults, can stimulate vomiting and nausea.

2. Bromelain is a mix of enzymes found in pineapple. It is used in diet supplements, usually in the treatment of swelling in the nose and inflammation, arthritis, cancer, bad digestion, and muscle pain, but the exact mechanism is unknown (Brien et al., 2004; De la Barrera-Núñez et al., 2014).

Recorded data	Effectiveness	Safety
Only a few studies	Potential effect in the treatment of OA, but further research is required in the future to provide valid conclusions	Gastric problems, increased heard pulse, allergic reactions

3. Willow has been used for years for the treatment of pain, headaches and inflammation of burses and tendons. White willow bark contains salicylates (Vlachojannis et al., 2009; Shara and Stohs, 2015).

Recorded data	Effectiveness	Safety
Only a few studies	Positive effect on muscle pain	Side effects by patients allergic to salicylates

4. Omega 3 fatty acids - There are a few proofs for omega 3 fatty acids that can be used in the treatment of RA (Haghiac et al., 2015).

Recorded data	Effectiveness	Safety
Very little proof	Decreases morning stiffness, and the need for anti-inflammatory drugs	Prolonged bleeding time

5. Devil's claw - originated in Africa, it is used as a supplement in the treatment of arthritis, gout, muscle pain, and the inflammation of tendons and ligaments (Chantre et al., 2000).

Recorded data	Effectiveness	Safety
Few studies with an evident quality of research	Decrease of pain, take orally, by OA and locally for grease	Decreases glucose, and improves blood pressure

6. Ginger – is used in Asia for the treatment of gastric pain and vomiting, but also for the treatment of arthritis, joints, and muscles (Black et al., 2010).

Recorded data	Effectiveness	Safety
Few studies (2008-10)	Decreases muscle pain, and pain by OA	Vomiting like dry intake

7. Lord vine- originated in China, Japan, and Korea. It is used in TCM for inflammation and hyper activity of the immune system (Vlachojannis et al., 2008).

Recorded data	Effectiveness	Safety
No evident studies	No consistent proof on OA	The root extract is toxic, hair loss, men's impotence, decreases bone mass

8. *Symphytum officinale* - Comfrey has been used in folk medicine, as root extract for the treatment of painful conditions of joints and muscles (Kucera et al., 2000; da Silva et al., 2015).

Recorded data	Effectiveness	Safety
Proven decrease of pain, inflammation, and swelling	Effective for back pain, myositis and joint pain, and sprains	Sage like grease or tincture for local application

9. Arnica - has a long history of traditional use after accidents by people and athletes, especially for muscle pain and painful conditions (da Silva et al., 2015; Venkatramani et al., 2013).

Recorded data	Effectiveness	Safety
Local treatment of tendons	For local application with a 5% grease or gel	Safe for local application and as a homeopathic drug, oral consummation causes optical nerve damage

10. Menthol was extracted from *Mentha piperita* and has a long history of use in TM as an anti-inflammatory substance for gastric problems (Di Lorenzo et al., 2013).

Recorded data	Effectiveness	Safety
Gels and greases for local use with an icy effect	Muscle stiffness, back pain, muscle spasms, sprains, haematomas	Safe, except in high concentrations,

11. Capsaicin is extracted from chili peppers. It is used as a dietetic supplement and as local grease (Maroon et al., 2006).

Recorded data	Effectiveness	Safety
Secretolytic Antioxidans	Pain treatment	Cannot touch mucosa

12. Shen Zhen is an herbal substance that is taken orally by many athletes from the East, to increase alertness or recover faster after injury (Youl Kang et al., 2002).

Recorded data	Effectiveness	Safety
Not enough studies	Effects similar to anabolic decrease of the cholesterol level and glucose, increases concentration	Cannot be used with anti-depressants, calcium blockers, and high pulse rate.

13. G**olden root** or Arctic root is used in TM in several regions of the world, increases mental and physical abilities (Ahmed et al., 2015).

Recorded data	Effectiveness	Safety
Evident decrease of inflammation	Labour proved	Safe

14. Polyphenols

Polyphenols from apples **-** recommended for athletes. Experiments done on animals have shown that if fed with apple polyphenols, they gain increased muscle contraction of the gastrocnemius, and increased levels of antioxidant proteins that protect the muscle from damage.
Polyphenols from grapes **-** resveratrol is from the group of phenol stilbenoids, that exist in the skin and seeds of grapes, but also in other plants, such as blueberries, blackberries, cranberries, and peanuts. There is data regarding its effect on human health, such as anti-cancer, protects the heart, blood vessels, and muscles. This effect is probably due to the reduction of fatty acid and lipid accumulation in the skeletal muscle. Causes greater sensitivity to insulin receptors.
Cherry juice - it's proved effective in reducing pain and improving the function of muscles. Consumption of cherries or their products prevents muscle damage under extreme load. It should be consumed at least three days before extreme muscle loading (Connolly et al., 2006).

CONCLUSIONS

Curcumin has an anti-inflammatory effect but there is insufficient data, it is generally safe. Bromelain has an anti-inflammatory effect but it is not yet proven, it can have possible unwanted effects. The extract of willow bark has an anti-inflammatory effect, because it contains salicylic acid, it has side effects for people allergic to it. Omega 3 fatty acids are used by people with inflamed joints, with uncertain evidence, and you should be cautious if you are receiving anticoagulant therapy. Devil's claw has anti-inflammatory effects, it is safe for local use, but oral use can decrease blood sugar. Ginger has an anti-emetic effect, but also an anti-inflammatory effect on joints and muscles. Lord vine has an anti-inflammatory effect but many side effects. Comfrey has the best local pain relief effect, especially for children after their third year. Arnica - used in sports injuries with anti-inflammatory effects, safe for topical use, but oral use can damage the optic nerve. Menthol, applied locally or as a tea, has anti-inflammatory effects. Capsaicin is a part of the creams for topical use with a heating effect, it has anti-inflammatory effects and can cause irritation of the skin and mucosa. Zhen Shen is anabolic which enhances sport performance and its side effects are insomnia and tachycardia. Golden root reduces fatigue and inflammation; it is used more by athletes because it is safe.

Polyphenols from apples, cherries, and grapes have a positive effect on muscle contraction and durability.

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Topic: Functional food and food supplemnents / **Sekcija: Funkcionalna hrana i dodaci prehrani**

FIBRE INTAKE AS A TOOL FOR MANIPULATING GUT MICROBIOTA IN AN OBESE INDIVIDUAL

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ABSTRACT

Microbiota plays an increasingly important role for the wellbeing of the host. It has been correlated with a plethora of conditions, raging form gastrointestinal to immunological and metabolic diseases. It is believed microbiota plays an important role in the onset, development, and treatment of obesity, with the exact mechanisms still unclear. Diet is one of the major factors affecting the microbiota - especially the intake of dietary fibres (prebiotics), which is believed to positively affect the microbiota composition. We had tested the concept of modulating the microbiota composition using prebiotics in a physically active obese male. Dietary intake and fibre intake were assessed before the intervention, using a food frequency questionnaire. The intervention consisted of daily supplementation with the commercial prebiotic Nutriose® until the maximum daily intake of fibre was achieved. No major change of dietary style, calorie intake, and lifestyle was recommended. The intervention was carried out for 30 days, and body height, weight, and composition were measured, and stool samples were taken at the beginning, after 15 days, and after 30 days of intervention. Anthropometric parameters showed a 5% decrease of body weight with a significant reduction of body fat (12.2%) and a decrease in visceral fat from 15 to 13. Microbiota composition showed a significant increase of beneficial bacterial genus *Bifidobacterium*, which increased tenfold during the intervention.

Keywords: microbiota, obesity, prebiotics, dietary supplements

INTRODUCTION

Excess weight and obesity are one of most the prevalent preventable medical conditions worldwide, with obesity affecting more than 13% and excess weight almost 40% of adults globally (WHO Factsheet, 2017). The prevalence of those conditions depending on sex varies greatly between countries, with women more affected overall (Kanter and Caballero, 2012). In Croatia, 20.37 % of the adult population is obese, with more women affected, the same trend as seen globally (Ivanković et al., 2012). Both conditions are characterized by the excess of body adipose tissue, that may have negative effects on health, especially contributing to cardiovascular and metabolic diseases. (Caballero, 2007). The most frequently used index for the diagnosis of obesity, despite its limitations, is the Body Mass

Index (BMI). Defined as the ratio between body weight, expressed in kilograms, and body height, expressed in square meters, it is applied to the entire population, regardless of sex, age, or geography. With simple numeric values and defined body categories, it offers a valuable assessment tool for mortality and morbidity risks, especially when adopted by specific populations (Weng et al., 2006).

The mechanisms by which people become overweight and obese are still only partially understood. The main contribution is believed to be the imbalance between energy intake and expenditure (Hill and Melanson, 1999). This has been exacerbated in the modern society, with the abundance of energy rich foods and the decrease of physical activity, due to prevailing sedentary forms of work and greater urbanization. Recently, other factors have also been recognized as important in the onset and development of obesity, such as genetics and the environment (Graham et al., 2015).

One of the factors gaining importance in the last decade, in the onset and development of obesity, is the gastrointestinal microbiota. Microbiota, defined as microorganisms living on and in us, has been observed to differ between murine models of obese and normal weight individuals (Ley et al., 2005), although results of human studies still remain controversial (Ley et al., 2005; Duncan et al., 2008). The observed difference is in the ratio of dominant bacterial phyla Firmicutes and Bacteroidetes, with higher Firmicutes abundance being correlated with obesity. With weight loss on either fat-restricted or carbohydrate-restricted low-calorie diet, the proportion of Bacteroidetes increases (Turnbaugh et al., 2006; Ley et al., 2006). It was also observed that faeces of obese mice had significantly less energy remaining compared to lean mice, suggesting that the bacteria from the phyla Firmicutes have better energy harvesting capabilities, which might provide additional energy to the host and thus result in obesity (Turnbaugh et al., 2006). To establish a causal link between microbiota and obesity, microbiota from obese and lean individuals was transplanted to germ-free or antibiotic-treated mice, resulting in the transfer of the lean or obese phenotype (Ridaura et al., 2013; Ellekilde et al., 2014). These studies have highlighted the possibility of using microbiota as a tool to fight excess weight and obesity by manipulating its composition. Besides using faecal transplant to introduce changes in the microbiota, there are other methods available, most prominent being the use of probiotics and prebiotics. Several strains of probiotic bacteria have been tested as a tool for weight loss. Although the results still remain inconclusive, a greater effect on reducing body weight was shown when multiple species of probiotic bacteria were consumed (Zhang et al., 2015). Also discovered was the difference in the attained effect for specific probiotic strains on different population groups, e.g. *Lactobacillus rhamnosus* CGMCC1.3724 was effective in women and not in men (Sanchez et al., 2014). It can be concluded from recent research that the best results will be obtained when probiotic supplementation is optimized for specific populations, or even individuals. Prebiotic supplementation is a subtler approach for manipulating gut microbiota. Prebiotics are indigestible polysaccharides which are believed to boost the growth of beneficial gastrointestinal microbes, namely the genera *Lactobacillus* and *Bifidobacteria* (Roberfroid et al., 2010; Gibson and Fuller, 2000). Supplementation with prebiotics has been shown to lessen adiposity in obese mice (Neyrinck et al., 2011), obese adults (Parnell and Reimer, 2009), and in overweight and obese children (Nicolucci et al., 2017). It was shown to change the gene expression pattern in white adipose tissue of obese mice, leading to an increase in lipolysis, a decrease in adipogenesis, and an increased metabolic response to hormones such as leptin - all

contributing to lower adiposity (Everard et al., 2011; Dewulf et al., 2011; Barengolts, 2016). A dietary increase in prebiotics can result in increased bloating and bowel movements, due to greater fermentation and the production of short chain fatty acids (Marteau and Seksik, 2004**).** In general, the populations of developed western countries have a dietary fibre intake lower than recommended, with the estimated fibre intake of American adult population average of 15.7-17.0 g (Grooms et al., 2013), which is also reflected on the microbiota composition (Wu et al., 2011). In this study, we had tested the concept of personalizing the quantity of prebiotic supplementation, in an effort to modulate the microbiota composition and assessing its effect on body weight and adiposity of a physically active obese male subject. Using a food frequency questionnaire, the subject's daily dietary fibre intake was estimated and was supplemented with prebiotics, up to the maximum recommended dose of 38 g of fibre per day (Institute of Medicine, 2005). As a prebiotic digestion-resistant dextrin with improved gastrointestinal tolerance - NUTRIOSE® was used (Pasman et al., 2006).

MATERIALS AND METHODS

Volunteers were recruited using a web form. Fourteen volunteers in total signed up to participate in the study investigating the dynamics of gastrointestinal microbiota in *in vitro* conditions (http://biodinamik.pbf.hr). Among the registered volunteers, 6 were female and 8 were male, 5 had normal BMI, 6 were overweight, and 3 were obese. The inclusion criteria for participation in the study were: BMI greater than 30 and no antibiotic treatment in the last 3 months. Two volunteers satisfied the inclusion criteria but only one of the volunteers was willing to participate in the study for the period of one month. The subject was an obese 34-year-old male with the BMI of 33 kg/m^2. He was a former professional athlete, still physically active at least one hour per day. He was apparently healthy and did not receive any antibiotic treatments in the last 3 months, did not take any dietary supplements, and was a non-smoker. He filled out the food frequency questionnaire, with assistance from trained personnel, to assess his daily fibre intake. The food frequency questionnaire was adopted and modified from The Personalized Nutrition Project (Zeevi et al., 2015), and the daily fibre intake was assessed using the computer program PRODI (version 6.3, Nutri-Science GmbH, Freiburg, Germany). As a reference for maximum allowed daily intake of fibre, the Dietary Reference Intakes (Institute of Medicine, 2005) were used. Basic anthropometric measurements were conducted at three points in time - before prebiotic supplementation, and after two and four weeks of supplementation. The total supplementation period was four weeks. Body height (cm) was measured using the portable stadiometer (Seca, Type 217, Vogel & Halke Gmbh & Co., Germany), and body weight (kg) using a digital flat scale for mobile use (Seca, Type 877, Vogel & Halke Gmbh & Co., Germany). Height was measured with the precision of 0.1 cm, and body weight with the precision of 0.1 kg. During the measurement, the subject was barefoot with minimum clothing. The measurements were performed according to standard instructions (Lee and Nieman, 2012). The body mass index (BMI) was calculated from the obtained measures and expressed in the unit of measurement kg/m^2. Total body fat, visceral fat, and the amount of muscles were measured using the OMRON BF-500 body composition monitor that uses bioelectrical impedance, along

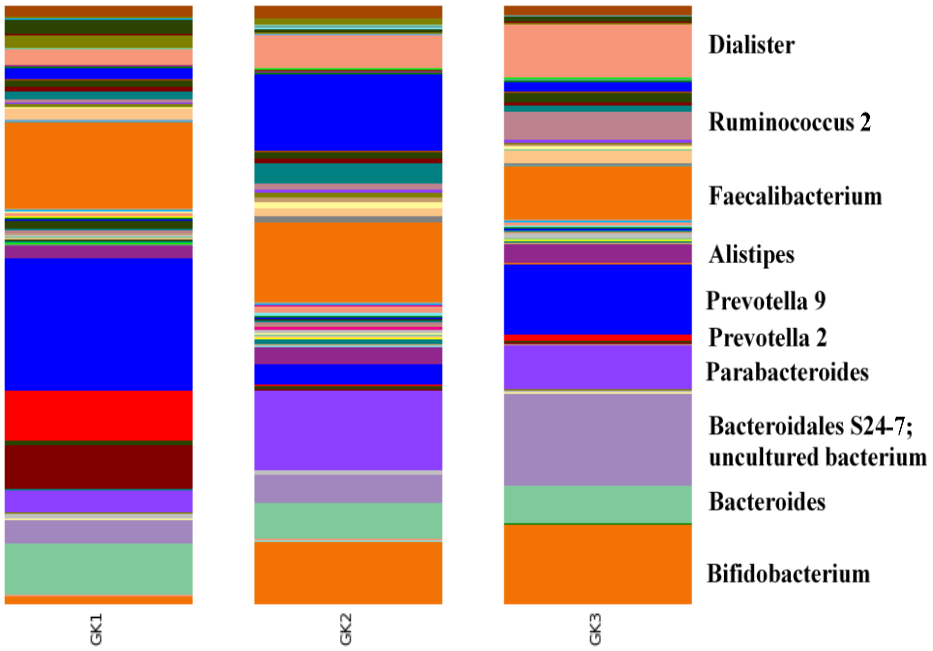
with height, weight, age, and gender information to generate results. All measurements were done in the nutrition lab by trained personnel.

To determine the gastrointestinal microbiota composition, faecal samples were collected by the subject at three points in time, at the same time when the anthropometric measurements were conducted - before starting, after 2 weeks, and after 4 weeks of prebiotic supplementation. Faecal samples were immediately frozen at -20 °C and were kept frozen until the extraction of DNA. The automatic DNA extraction system Promega Maxwell® 16, with the Maxwell® 16 DNA Tissue Purification Kit was used. The DNA extraction procedure was carried out according to the manufacturers' instructions. The concentration of isolated DNA was measured using the Shimadzu BioSpec-nano system and 20 µL of extracted DNA was sent to the sequencing centre. Variable regions 3 and 4 of the gene coding for 16S rRNA were amplified using PCR, and respective fragments were sequenced using the Illumina MiSeq platform paired-end protocol. The sequencing results were processed using the QIIME platform (Caporaso et al., 2010). The paired-end raw *fastq* files were first joined and quality filtered, and then demultiplexed using respective barcodes for each sample. Operational Taxonomic Units (OTUs) were calculated at 97% sequence identity by clustering using the *usearch* program, as implemented in QIIME. The taxonomic assignments of OTUs were based on Silva release 123 (Quast et al., 2013).

RESULTS AND DISCUSSION

The anthropometric measurements of the subject showed that his height was 178 cm and he weighed 104.4 kg at the time of the first measurement - corresponding to the BMI of 33 kg/m^2. Based on the answers from the Food Frequency Questionnaire (FFQ), his daily fibre intake was estimated at 26 g/day. The estimated intake is slightly higher than that of an average adult American (Grooms et al., 2013) but it is still lower than the maximum recommended daily intake of 38 g of dietary fibre (Institute of Medicine, 2005). Higher than average consumption of fibre is also visible in the microbiota composition (Fig. 1), with *Prevotella* species representing 30.5% of the present bacterial genera. The higher abundance of *Prevotella* is linked to higher dietary consumption of carbohydrates, especially fibre (Wu et al., 2011). Based on the estimated fibre intake from the FFQ and the recommended maximum daily intake, the subject was given a 15 g/day supplementation of Nutriose® distributed in two daily doses, which is equivalent to 13 grams of dietary fibre. Based on the answers from the FFQ, the subject was advised to increase the number of meals per day from the current 2 to the recommended 5 meals per day. No other dietary or lifestyle interventions were suggested. The subject had kept a 30-day dietary log, which showed that his average calorie intake was 2600 ± 300 kcal daily. During the four weeks of prebiotic supplementation, the subject had lost 5.2 kg or 5% of his starting weight and his BMI has decreased from 33 kg/m^2 to 31.3 kg/m^2 (Table 1).

The reduction of body weight resulted in the reduction of body fat percentage from 31.1% to 27.3% and the visceral fat rating from 15 to 13. The weight loss of the subject is more drastic than reported in the previous study using Nutriose® supplementation - which was 1.5 kg for the body weight and 0.5 kg/m^2 for the BMI in the cohort of overweight Chinese adults during 12 weeks of supplementation (Guerin-Deremaux et al., 2011).



GK1 - faecal sample before supplementation; GK2 - faecal sample after 2 weeks of 15 g/day Nutriose® supplementation; GK3 - faecal sample after 4 weeks of 15 g/day Nutriose® supplementation

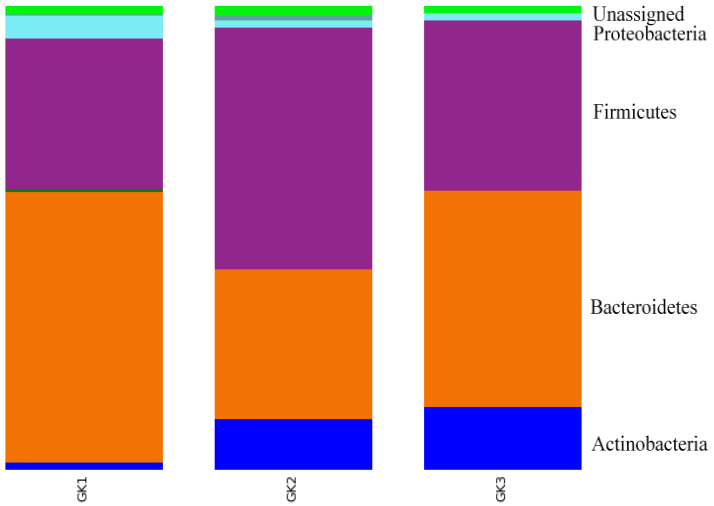
Fig. 1. Taxonomic distribution of gastrointestinal microbiota at the genus level

Table 1. Anthropometric details and body composition of the subject

Date	**31/01/2017**	**17/02/2017**	**08/03/2017**
Body height (cm)	178	178	178
Body weight (kg)	104.4	100.1	99.2
BMI (kg/m^2)	33	31.6	31.3
Body muscle **%**	32.8	33	34.9
Body fat **%**	31.1	30.6	27.3
Visceral fat rating	15	14	13

The reason for that might be the active lifestyle of our test subject, with at least one hour of reported physical activity daily.The study by Guerin-Deremaux (2011) does not report activity levels of the test subjects, but claims that physical activity levels probably had little impact on the body composition outcomes. It must also be pointed out that weight loss in the test group was only one-sixth of the amount expected by Guerin-Deremaux (2011), based on the reduction in energy intake due to the increased satiety effect of Nutriose®. The subject in the study did not report improved satiety during the supplementation period as was previously reported, but has confirmed finding that Nutriose® does not cause any gastrointestinal discomfort (Guerin-Deremaux et al., 2011). As already mentioned, the decreased abundance of the bacterial phyla *Bacteroidetes* is associated with obesity, and its abundance increases with weight loss (Ley et al., 2006). As can be seen in Fig. 2., the test

subject had high abundance of phyla *Bacteroidetes,* making up 58.5% of all bacterial phyla detected in the first faecal sample, dropping to 32.3% in the second sample, and increasing to 46.5% in the final sample. This proportion of *Bacteroidetes* is usually associated with lean individuals and the observed variance in *Bacteroidetes* abundance can be attributed to normal variation of gastrointestinal microbiota. The proportion of *Firmicutes* varies from 32.5% in the first sample, to 52% in the second, and to 37% in the third sample. The increase of the *Firmicutes* in the second sample is mostly due to the increase of the bacterial family *Erysipelotrichia*, typical for diets high in fat, and can be attributed to the temporary change in the diet richness (Greiner and Bäckhed, 2011). Prebiotics affect gastrointestinal microbiota in a way that increases the abundance of beneficial bacteria - mostly from the genera *Bifidobacterium* and *Lactobacillus* (Roberfroid et al., 2010; Gibson and Fuller, 2000; Druart et al., 2014). Genus *Lactobacillus* was not identified in any of the samples. Genus *Bifidobacterium* was present in the test subject in low abundance in the first sample (1.3%), and is steadily increasing during supplementation with the prebiotic to 13.5% in the third sample.



GK1 - faecal sample before supplementation; GK2 - faecal sample after 2 weeks of 15 g/day Nutriose® supplementation; GK3 - faecal sample after 4 weeks of 15 g/day Nutriose® supplementation

Fig. 2. Taxonomic distribution of gastrointestinal microbiota at the phylum level

The increase in Bifidobacteria is usually seen during various prebiotic supplementations (Brüssow, 2013) and is in accordance with results obtained in this study. The Clostridium cluster XIVa and *Roseburia* genus were detected in the test subject as less than 1% of the present microbiota. Those microorganisms were reported by Hobden (2013) to be significantly increased during Nutriose® administration to the continuous culture human colonic model system (Hobden et al., 2013). The reasons for the discrepancy is most likely the difference in the starting microbiota, as the Clostridium cluster was detected in the test subject at only 0.1% and *Roseburia* genus at 0.5% abundance. In the trial on healthy volunteers, the supplementation of Nutriose® stimulated the proliferation of *Bacteroides* and inhibited *Clostridium perfringens* (Lefranc-Millot et al., 2012). In the test subject, the

genus *Bacteroides* has decreased during supplementation from 8.6% in the first sample to 6.3% in the last sample, but the phylum *Bacteroidetes* remained in abundance correlated with lean individuals (Ley et al., 2006).

CONCLUSIONS

This research, which was intended as a proof of concept research to establish the benefits of personalization of fibre supplementation to obese individuals, showed promising results. The test subject reduced his body mass, the percentage of body fat, and visceral fat during the 4-week supplementation with 15 g/day of the prebiotic Nutriose®. The test subject did not follow any special dietary or lifestyle guidelines during the supplementation, which might prove beneficial for long-term compliance to such a regimen. Although the sample size is too small, the results obtained showed promising perspective and a study needs to be carried out on a larger group, taking into account the daily physical activity regime of the subjects.

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Topic: Functional food and food supplemnents / **Sekcija: Funkcionalna hrana i dodaci prehrani**

PRIRODNI DODATCI PREHRANI KAO NOSITELJI NUTRITIVNE KVALITETE, LJEKOVITOG POTENCIJALA I ODRŽIVOSTI PROIZVODA

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review article/pregledni rad

SAŽETAK

Od davnina su ljudi hranu dobivali direktno iz prirode te je tek krajem 19-tog i početkom 20-tog stoljeća došlo do ekspanzijskog razvoja moderne industrije koja je razvojem novih tehnologija omogućila i razvoj velikog broja prehrambenih i farmaceutskih proizvoda. Ovaj razvoj utjecao je i na razvoj lijekova čime se potisnula filozofija „hrane kao lijeka“ gotovo jedno cijelo stoljeće. Zbog pojave mnogih bolesti tijekom 20-tog stoljeća, moderna industrija svoj razvoj ponovo počinje temeljiti na obogaćivanju hrane prirodnim dodatcima prehrani. Prepoznatljiv je trend proizvodnje "funkcionalne hrane", odnosno hrane koja posjeduje povoljno djelovanje na zdravlje uz pripadajuća nutritivna svojstva. Uočeno je da je dodavanjem začinskih i ljekovitih biljaka moguće poboljšati okus i miris hrane. Velik broj istraživanja dokazao je da i navedene skupine biljaka sadrže spojeve s izraženim baktericidnim i fungicidnim djelovanjem te, kao prirodni dodatci prehrani, sprječavanju kvarenje hrane i održivost namirnica. Pored toga, izuzetno su snažni antioksidanti. Uporaba prirodnih dodataka prehrani danas je izražena kroz sve tehnologije proizvodnje, a najviše u tehnologiji mesa i mesnih prerađevina, mliječnih i pekarskih proizvoda te kroz uporabu u jestivim ambalažnim filmovima i punilima u prehrambenoj i farmaceutskoj industriji. Dodavanjem začinskih i ljekovitih biljaka u različite vrste proizvoda možemo značajno unaprijediti nutritivnu kvalitetu te ljekoviti potencijal i održivost proizvoda.

Ključne riječi: prirodni dodaci prehrani, funkcionalna hrana, biljke, nutritivna kvaliteta

UVOD

Kroz cijelu ljudsku povijest provlači se spoznaja o povezanosti prehrane i bolesti. Upravo ta povezanost rezultirala je stvaranjem novog koncepta funkcionalne hrane i dodataka prehrani u nastojanju da poboljšaju postojeće zdravstveno stanje ili smanje rizik od razvoja različitih bolesti. Aditivi, dodatci hrani, su tvari koje se obično ne konzumiraju kao sama hrana, a dodaju se hrani namjerno kroz tehnološke postupke kao što je, npr. konzerviranje hrane. Upravo biljni pripravci, eterična ulja i izolirane komponente biljaka (npr. pigmenti) mogu služiti kao prikladna zamjena sintetskim aditivima (Saarela, 2011). Funkcionalni

proizvodi, nutraceutici i dodaci prehrani su pojmovi koji kod potrošača ali i znanstvene zajednice još uvijek stvaraju izazov u pogledu razlikovanja.
Koncept funkcionalne hrane predstavljen je prvi put u Japanu sredinom osamdesetih godina XX. stoljeća u cilju vladinih nastojanja da se poboljša zdravstveno stanje ili smanji rizik od bolesti koje su uzrokovale velike troškove zdravstva (Serafini i sur., 2012; Granato i sur., 2017; Čalić i sur., 2011). Izraz „funkcionalna hrana“ je prije svega marketinški termin i nema službenu definiciju u
legislativi Europske unije (Serafini i sur., 2012). Neslužbena definicija glasi: „Funkcionalna hrana nije tableta, kapsula ili bilo koji drugi oblik dijetetskog suplementa, mora sačuvati prirodnost hrane i koristiti se kao dio uobičajene“ prehrane (Serafini i sur., 2012). Također, treba imati znanstveni dokaz o pozitivnom djelovanju na jednu ili više tjelesnih funkcija čovjeka, osim adekvatnog unosa nutrijenta, na način da poboljšava stanje organizma i/ili smanjuje rizik od obolijevanja (Serafini i sur., 2012). Funkcionalni proizvod trebao bi imati direktan utjecaj na fiziološke sustave, kao što su: imunološki, živčani, endokrinološki, probavni ili cirkulacijski sustav (Aronson, 2017). Postoji više različitih pojašnjenja pojma funkcionalne hrane. IFIC (International Food Information Council) objašnjava funkcionalnu hranu kao onu hranu koja pruža veću dobrobit za zdravlje nego osnovna prehrana, dok je FUFOSE (The European Commission Concerted Action on Functional Food Science in Europe) u par smjernica dala jedinstvene značajke funkcionalne hrane, a to su:

- Uobičajena i svakodnevna prehrana koja se konzumira kao dio uobičajene prehrane.
- Prirodnog je sastava bez sintetskih dodataka s komponentama koje su već prirodno prisutne u toj hrani, ali je njihova koncentracija povećana.
- Ima pozitivan utjecaj na fiziološke funkcije organizma.
- Poboljšava zdravstveni status organizma i/ili smanjuje rizik od obolijevanja.
- Ima znanstvene dokaze za svoju djelotvornost (Čalić i sur., 2011).

Funkcionalna hrana može se podijeliti u više kategorija:

1. Hrana u nemodificiranom i neprerađenom obliku koja sadrži biološki aktivne tvari s pozitivnim djelovanjem na organizam (npr. voće, povrće, začinsko bilje).
2. Obogaćeni proizvodi kod kojih je količina jednog nutrijenta uvećana ili je pak dodan novi nutrijent koji se uobičajeno ne nalazi u toj namirnici (mlijeko obogaćeno vitaminom D, sokovi s dodanim kalcijem).
3. Izmijenjeni proizvodi kod kojih je jedan od sastojaka zamijenjen s drugim nutrijentom koji ima pozitivan učinak (proizvodi sa smanjenim udjelom masti u kojima je mast zamijenjena vlaknima).
4. Poboljšani proizvodi u kojima je jedna ili više komponenti hrane prirodno obogaćena kroz specifične načine uzgoja biljaka i životinja (voće i povrće s povećanim udjelom vitamina) (Čalić i sur., 2011).

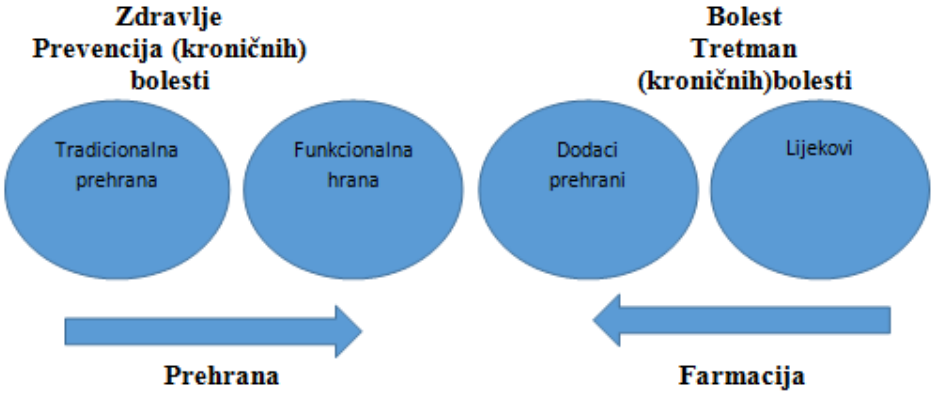
Osim pojma funkcionalne hrane 1990-ih godina, pojavio se i pojam nutraceutik (Saarela, 2011) koji do danas također nema službenu definiciju. Za mnoge autore i znanstvenike ovi pojmovi su međusobno zamjenjivi te često dolazi do njihovog izjednačavanja u literaturi. Prema Saarela (2011), nutraceutik je svaka tvar koja je hrana ili dio hrane i pruža medicinske ili zdravstvene pogodnosti, uključujući prevenciju i liječenje bolesti. Može biti prisutan u obliku tablete, praha ili u drugim oblicima kao pročišćeni i koncentriran, pri

čemu mu je dokazana djelotvornost protiv kroničnih bolesti (Keservani i sur., 2010; Saarela, 2011; Aronson, 2017). Također, Europska asocijacija nutraceutika određuje da nutraceutici ne smiju biti tvari sintetičkog podrijetla ili kemijski spojevi formulirani za određene indikacije, ali bi trebali sadržavati hranjive sastojke (djelomično u koncentriranom obliku), odnosno biološki aktivne tvari (Aronson, 2017; Augustin i Sanguansri, 2012). Nutraceutici i biološki aktivne tvari mogu uključivati vitamine, minerale, aminokiseline i peptide, prebiotike, probiotike, ali i začine i druge biljne vrste (Augustin i Sanguansri, 2012). Primjeri nutraceutika koji imaju fiziološku ulogu, a koji se koriste u prehrani, navedeni su u Tablici 1.

Tablica 1. Primjeri bioaktivnih spojeva koji djeluju kao nutraceutici i primjer potencijalnog učinka na zdravlje (Augustin i Sanguansri, 2012)
Table 1. Examples of bioactive compounds acting as nutraceuticals and an example of the potential health effects (Augustin and Sanguansri, 2012)

Bioaktivni spoj	**Primjer**	**Potencijalni učinak na zdravlje**
Prebiotici	Inulin, oligosaharidi	Održavanje zdravlja crijeva i reguliranje crijevne mikroflore
Probiotici	*Lactobacili, Bifidobacterium*	
Fitokemikalije	Beta-karoten, likopen, flavonoidi, proantocijanidini, polifenoli, alicin	Poboljšanje zdravlja crijeva, imunomodulacija
ω-3 masne kiseline	Dokozaheksaenska kiselina (DHA) i Eikozapentaenska kiselina (EPA)	Smanjenje rizika od razvoja kardiovaskularnih bolesti , karcinoma, dijabetesa, i degenerativnih bolesti
Bioaktivni peptidi	Izolirani peptidi mlijeka	Poboljšanje zdravlja kardiovaskularnog sustava
Karotenoidi	Beta-karoten, likopen, lutein, zeaksantin, astaksantin	Smanjenje rizika od razvoja očnih bolesti i određenih vrsta karcinoma
Biljke i začini	Eterična ulja, različiti biljni pripravci	Širok spektar dobrobiti na zdravlje

U širem smislu, nutraceutici obuhvaćaju i funkcionalnu hranu i obogaćene proizvode i dodatke prehrani. Funkcionalni i obogaćeni proizvodi se konzumiraju u okviru uobičajene prehrane, dok se dodaci prehrani uzimaju oralno u obliku tableta, kapsula i tinktura u maloj količini kao dodatak uobičajenoj prehrani (Gulati i sur., 2014; Augustin i Sanguansri, 2012). Ljekovite biljke predstavljaju veliku skupinu nutraceutika koje zbog kompleksnosti spojeva u svom sastavu također nisu jasno definirane Zakonom o hrani u Europskoj uniji. Određenim dijelom su definirane Zakonom o lijekovima kao „cjeloviti, razdijeljeni i usitnjeni dijelovi biljaka, algi, gljiva, lišajeva i njihovi proizvodi dobiveni ekstrakcijom, destilacijom, frakcioniranjem, pročišćavanjem, koncentriranjem ili fermentacijom (Gulati i sur., 2014).
Budući da biljke mogu biti lijek, dodatak prehrani ili hrana, biljni nutraceutici predstavljaju izazov u pogledu kvalitete, sigurnosti i samog definiranja u Europskoj uniji jer ne postoji jasna granica koja određuje što je hrana, a što lijek (Gulati i sur., 2014).



Slika 1. Shematski prikaz poveznice prehrane i farmacije (Eussen i sur., 2011).
Fig. 1. A schematic of the connection between nutrition and pharmaceuticals (Eussen et al., 2011).

U kontekstu Europske unije, značajna je baza Europske agencije za sigurnost hrane (European Food Safeety Authority) koja sadrži autorizirane i neautorizirane zdravstvene tvrdnje, kao i Europska agencija za lijekove (European Medicines Agency) pri kojoj djeluje odbor za biljne medicinske proizvode (Comittee on Herbal Medicinal Products) koji je odgovoran za sastavljanje i procjenjivanje znanstvenih podataka o biljnim, biološki aktivnim komponentama, njihovoj pripremi i kombiniranju.
Bez obzira definiramo li biljne proizvode kao hranu, nutraceutik, funkcionalni proizvod, dodatak prehrani ili pak receptni lijek, činjenica je da predstavljaju izvor ljekovitih, biološki aktivnih tvari koje imaju ulogu održavanja i/ili poboljšavanja zdravlja, prevencije i liječenja bolesti.

PRIRODNI DODATCI PREHRANI U ULOZI FUNKCIONALNE HRANE

Tradicionalno su lijekovi korišteni za liječenje bolesti ili ublažavanje simptoma bolesti dok je prehrana, s druge strane, ponajprije bila usmjerena na sprečavanje bolesti osiguravanjem optimalne ravnoteže makro- i mikronutrijenata (Eussen i sur., 2011).
Brojna istraživanja provedena su kako bi se potvrdila činjenica da različite biljke i biljni ekstrakti pokazuju funkcionalna svojstva kada se konzumiraju kao dio uobičajene prehrane. Biljke predstavljaju iznimno važnu skupinu u prehrani, ne samo zbog osnovnih nutrijenata (ugljikohidrati, proteini, masne kiseline, vitamini i minerali) već i zbog biološki aktivnih tvari koje su proizvod njihovog sekundarnog metabolizma. Najvažnije čine alkaloidi, karotenoidi, glukozinolati, fitosteroli, polifenoli, saponini, sulfidi i sulfoksidi (Thomas i sur., 2016). Različit broj biljnih vrsta, uključujući zeleni nadzemni dio biljke, plodove, listove i koru drveća, koriste se širom svijeta kao izvor biološki aktivnih tvari kako bi prevenirale ili pomogle u liječenju različitih bolesti ili zdravstvenih komplikacija (Granato, 2017). Svjetska zdravstvena organizacija (WHO) procjenjuje da 80% svjetske populacije ovisi o tradicionalnoj medicini kao primarnoj zdravstvenoj zaštiti pretežno kroz uporabu biljnih ekstrakta i njihovih bioaktivnih tvari

(Da Silva i sur., 2016). Budući da u Europskoj uniji ne postoji jasna granica između dodatka prehrani i biljnog lijeka, većina ljudi biljne proizvode uzima za (samo)liječenje, bilo kao samostalnu, ili pomoćnu terapiju. Tada doza biljke ili biljnog ekstrakta čini razliku između dodatka prehrani i lijeka. Međutim, većina istraživanja se odnosi samo na jedno ili nekoliko djelovanja biljke ili biljnog ekstrakta *in vivo* i/ili *in vitro*, bez provođenja kliničkih istraživanja koja bi potkrijepila njihovu navodnu primjenu (Granato, 2017). Biljke s tradicionalnom primjenom su one koje imaju određena medicinska svojstva, a koristi ih određena skupina ljudi na jednoj ili više lokacija te se smatra da su sigurne za upotrebu (Granato, 2017). U objavi Europske komisije 2004. godine, navedeno je da postoji pojednostavljeni postupak registracije proizvoda „s tradicionalnom uporabom" u pogledu učinkovitosti tradicionalnih biljnih lijekova. Za ove lijekove nisu potrebni dodatni podatci iz pretkliničkih i kliničkih ispitivanja, sve dok je njihova učinkovitost "vjerodostojna na temelju dugotrajne uporabe i iskustva" (Eussen i sur., 2011).

Za pojedine biljke, poput kantariona (gospina trava) (*Hypericum perforatum*), postoje brojna klinička istraživanja dok za pojedine vrste imamo samo njihovu tradicionalnu primjenu u brojnim zemljama svijeta. Tu nadasve značajnu ulogu čini ESCOP (*The European Scientific Cooperative on Phytotherapy*) koji daje pregled terapijskog djelovanja najznačajnijih biljnih medicinski proizvoda ili pripravaka te minimalne djelotvorne doze, na osnovi znanstvenih dokaza vodećih stručnjaka diljem Europe.

Agencija za hranu i lijekove Sjedinjenih Američkih Država je prepoznala više od 150 biljaka koje daju eterična ulja, smole i destilate, a koje su sigurne za konzumaciju bez ograničenja unosa. Takve biljke nose oznaku GRAS (*Generally Recognized As Safe*) kao opće prepoznate i sigurne. U tu skupinu ubrajaju se origano (*Origanum vulgare* L.), ružmarin (*Rosmarinus officinalis* L.), kadulja (*Salvia officinalis* L.), timijan (*Thymus vulgaris* L.), bosiljak (*Ocimum basilicum L.*), lovor (*Laurus nobilis L.*), metvica (*Mentha piperita*), peršin (*Petroselinum crispum* Mill.), estragon (*Artemisia dracunculus* L.), korijander (*Coriandrum sativum L.*), maslačak (*Taraxacum officinale* Web. Ex Wigg.), lavanda (*Lavandula officinalis* Wigg.), vrijesak (*Sarureia hortensis* L.) (Costa i sur., 2015).

Dobivanje i ekstrakcija novih prirodnih spojeva s biološkom aktivnošću koji bi se inkorporirali u funkcionalnu hranu je postalo najviše istraživano područje u prehrambenoj tehnologiji i znanosti o prehrani (Da Silva i sur., 2016).

LJEKOVITI POTENCIJAL DODATAKA PREHRANI

Razvojem moderne industrije povećala se i potražnja za više stabilnih, funkcionalnih i jednostavnijih dodataka prehrani koje imaju fleksibilnost za dodavanje u različite prehrambene proizvode. Pored toga, današnji razvoj tehnologije neprestano teži za što naprednijim metodama koje će doprinijeti što boljoj usporedbi odnosa prednosti i rizika lijekova, funkcionalne hrane i dodataka prehrani te procijeni dodane vrijednosti funkcionalne hrane ili dodataka prehrani terapiji lijekovima (Augustin i Sanguansri, 2012).

Prehrambene proizvode karakteriziraju unutarnja i vanjska svojstva kvalitete. Unutarnja svojstva kvalitete hrane odnose se na funkcionalne i prehrambene prednosti, kao što su koncentracija i sastav biljnih spojeva. To su ona svojstva koja izravno proizlaze iz biljaka. S druge strane, vanjske značajke kvalitete hrane nisu izravno

povezane s funkcionalnim i prehrambenim prednostima, nego su više sredstvo dodavanja vrijednosti osnovnom proizvodu putem ambalaže, robne marke, cijene, zemlje podrijetla ili metode proizvodnje kao što su organska proizvodnja i sl. (Wiesner i sur., 2017).

Pored osnovnih nutritivnih funkcija, funkcionalna hrana utječe na poboljšanje zdravlja kroz obogaćivanje formulacija prirodnih dodataka prehrani (Henry, 2010; Howlett, 2008) te se značajna ekspanzija ponude i potražnje na tržištu dogodila kada su potrošači postali svjesni da uporaba ovih dodataka značajno doprinosi promicanju zdravlja (Augustin i Sanguansri, 2012). Prirodni dodaci prehrani, čija intenzivna svojstva direktno proizlaze iz ljekovitih biljaka, mogu biti spojevi, skupine spojeva ili eterična ulja (Carocho i sur., 2014). U novije vrijeme zanimanje prehrambene industrije za prirodnim tvarima koje je moguće izravno dodati u proizvode ili za sinergiju s drugim spojevima rapidno raste. Pored prehrambene industrije povećano je zanimanje integracije ovih komponenti i u inovativna rješenja pakiranja u modificiranoj atmosferi (MAP) te u farmaceutskoj, parfemskoj i kozmetičkoj industriji koje prepoznaju njihove prednosti (Carocho i sur., 2014).

Ljekovite biljke su izvori prirodnih sastojaka te uporaba začina i ljekovitih biljaka seže u daleku povijest gdje su već tada upotrebljavane u ljekovite svrhe. One i danas doprinose promicanju zdravlja ljudi te se, iako se konzumiraju u malim količinama, biološki utjecaj njihova unosa i antioksidacijska svojstava ne mogu zanemariti (Costa i sur., 2015). One su jedan od najboljih izvora prirodnih spojeva za koje je pokazano da imaju antioksidacijski učinak (Carlsen i sur., 2010; Hinneberg i sur., 2006). Pored toga, sadrže i proteine, vlakna, hlapljive sastojke (eterično ulje), vitamine (A, C i B kompleks), minerale (kalcij, fosfor, natrij, kalij i željezo) te fitokemikalije. Fitokemikalije su biološki aktivne tvari prisutne u malim količinama koje djeluju kao antioksidanti, baktericidi ili antivirusni lijekovi (Muchuweti i sur., 2007; Naczk i sur., 2004).

Općenito, ljekovite biljke su kompleksne matrice fenolnih spojeva (Costa i sur., 2015). Ovi spojevi su prisutni u biljkama, ali njihova distribucija ovisi o dijelu biljke / tkiva (Robards, 2003). Fenolni spojevi su jedna od glavnih skupina koje doprinose svojstvima ljekovitih biljaka, uključujući prevenciju raka, kardiovaskularnih i neurodegenerativnih bolesti. Mnogi čimbenici mogu utjecati na njihov sadržaj poput vrste biljke i njezine kemijske strukture, odabrane metode ekstrakcije te uvjeta skladištenja. Prilikom ekstrakcije biološki aktivnih tvari i proizvodnje biljnih ekstrakata danas se i dalje najčešće primjenjuju klasične metode ekstrakcije (KME). KME su obično dugotrajne i visoka temperatura ekstrakcije može dovesti do degradacije termolabilnih fitokemikalija što utječe na kvalitetu i bioaktivno djelovanje konačnog proizvoda. Pored toga, u KME vrlo se često kao sredstva za ekstrakciju primjenjuju toksična organska otapala te primjena ovih otapala ima negativan utjecaj na sigurnost konačnog proizvoda. U procesima ekstrakcije biljnog materijala zahtjeve suvremene proizvodnje gotovo u potpunosti ispunjava ekstrakcija s CO_2 u superkritičnom stanju ($ESCO_2$) (Jokić i sur., 2014).

Pored ekstrakcije spojeva s antibakterijskom, antivirusnom ili antifungicidnom aktivnošću, posebna pozornost posvećuje se ekstrakciji antioksidacijskih spojeva zbog njihove važne uloge u očuvanju hrane i promicanju zdravlja. Antioksidanti su najčešće proučavani spojevi s funkcionalnim svojstvima te igraju važnu ulogu u prehrambenoj

tehnologiji zbog njihove sposobnosti prevencije lipidne peroksidacije. Obično, proizvodnja hrane, procesi obrade i skladištenje, mogu generirati važne gubitke antioksidanata koji ograničavaju zaštitu od oksidacije lipida. Određivanje fenola u ljekovitim biljkama proučavano je kroz mnoga istraživanja, no tumačenje dobivenih rezultata je otežano s obzirom na to da nema standardiziranog postupka za pripremu uzoraka i analizu fenolnih spojeva u tim matricama (Costa i sur., 2015). Epidemiološke studije pokazale su da je unos prirodnih fenolnih antioksidanata u korelaciji s smanjenom incidencijom bolesti kao što su koronarna srčana bolest, dobna degeneracija oka i karcinom (McCullough i sur., 2012).

Aktivnost polifenola uključuju antioksidacijsku, antimutagensku, antikancerogenu, antialergijsku, protuupalnu, antivirusnu, antiulceralnu, antidiaritmijsku, antihepatoksičnu, antiproliferativnu i dr. (Carocho i sur., 2014; Muchuweti i sur., 2007).

Međutim, polifenoli su važni za ljudsku prehranu i iz drugih razloga. U prehrani zapada, klorogenska kiselina zbog konzumacije kave je glavni izvor polifenola. Nadalje, flavonoli kvercetin i kamferol su najviše konzumirani flavonoidi i prisutni su u visokim koncentracijama, primjerice, u luku ili povrću porodice *Brassicaceae*. Flavoni, kao što su apigenin i luteolin, prisutni su na primjer u biljnim listovima ili salati. Antocijanini djeluju kao pokazatelj zrelosti plodova, npr. pri visokim koncentracijama u aroniji. Kondenzirani tanini koji uzrokuju astringenska svojstva povezana s katehinom vina ili čaja (flavan-3-ol) dobro su proučeni zbog njihovih antikancerogenih učinaka. Osim toga, gorak okus grejpa uzrokuje naringenin, flavanon koji je poznato da ometa apsorpciju i djelovanje određenih farmaceutskih lijekova, na primjer statina. Budući da izoflavonoidi imaju aktivnost estrogena, izoflavonoidi iz sojinih proizvoda privukli su veliku pažnju kao dodaci prehrani zbog pozitivnog učinka tijekom menopauze kod žena (Wiesner i sur., 2017).

Općenito, polifenoli mogu zaštititi od bolesti s etiologijom i patofiziologijom povezanim s reaktivnim kisikovim vrstama (Armatu i sur., 2010). Pronađeno je i da fenolni spojevi inhibiraju virusnu replikaciju (HIV), imunodeficijenciju, human simplex virus (HSV) i glukozilne transferaze *Streptococcus mutans* povezane s karijesom zuba (Proestos i sur., 2005). Fenolni spojevi djeluju kao redukcijska sredstva, donatori vodika i razgrađivači kisika (Proestos i sur., 2006) te su proučavani kroz brojne pregledne radove.

Ljekovite biljke mogu doprinijeti očuvanju zdravlja svojim antioksidacijskim svojstvima, ali i mogućnošću smanjenja unosa dodane soli u hranu (Costa i sur., 2015; Embuscado, 2015). Naime, aromatične biljke zbog svoje ljekovitosti ne samo da doprinose okusu i mirisu proizvoda nego mogu utjecati na smanjenje štetnih nusproizvoda tijekom pripreme hrane. Smith i sur. (2008) u svom radu navode kako primjena marinada koje sadrže aromatične biljke i začine bogate antioksidantima mogu značajno smanjiti stvaranje heterocikličkih amina prilikom termičke obrade mesa na grilu (204 °C).

Nove vrste proizvoda koje se obično koriste kao dodaci prehrani, u ulozi jestivih ambalažnih filmova na tabletama i pilulama, mogu pružiti važne zdravstvene prednosti. Jedna od posebnih i značajnih primjena jestive ambalaže je da se može upotrijebiti za kapsuliranje spojeva aroma, antioksidanata, antimikrobnih sredstava te pigmenata (Debeaufort i sur., 1998). Upotrijebljena za kapsuliranje, mora biti „food grade“ (s visokim stupnjem sigurnosti hrane). Jedan od problema koji može nastati u radu s prirodnim sastojcima u usporedbi s prerađenim sastojcima hrane je normalna varijacija u sastavu i kvaliteti koja postoji u izvornom obliku iz kojeg su izolirani. Broj procesa i procesnih uvjeta za njihovu izolaciju također utječe na njihova svojstva

(Augustin i Sanguansri, 2012). U preglednoj Tablici 2. radova autora Costa i sur. (2015) prikazana su ljekovita svojstva određenih biljaka generalno prepoznatih kao sigurnih.

Tablica 2. Ljekovita svojstva određenih biljaka (Costa i sur., 2015)
Table 2. Medicinal properties of certain plants (Costa et al., 2015)

Ime biljke	Botaničko ime vrste biljke	Porodica	Ljekovita svojstva biljke
Bosiljak	*Ocimum basilicum* L.	Lamiaceae	Liječenje glavobolje, kašlja, bradavica, parazita, zatvora, bronhitisa, laringitisa, angine, gastrointestinalnih tegoba i poremećaja, bolesti bubrega
Lovor	*Laurus nobilis* L.	Lauraceae	Listovi se koriste za liječenje visokog šećera u krvi, migrena, glavobolje, bakterijskih i gljivičnih infekcija te kod čira želuca. Posjeduju protuupalna i antioksidacijska svojstva. Eterično ulje se koristi kod reumatizma i dermatitisa.
Korijander	*Coriandrum sativum* L.	Umbelliferae (Apiaceae)	Liječenje anoreksije, kod povraćanja, dispepsije, nadutosti i dijareje
Maslačak	*Taraxacum officinale* Web. Ex Wigg.	Cichoriaceae	Kolerotik, diuretik, antireumatik, laksativ, protuupalno sredstvo, stimulans za apetit
Lavanda	*Lavandula officinalis* Chaix.	Lamiaceae	Liječenje dispepsije, lagani sedativ, diuretik, spazmolitik.
Mažuran, slatki	*Origanum majorana* L. syn. *Majorana hortensis* Moench)	Lamiaceae	Pomaže kod gastrointestinalnih poremećaja, grčeva, depresije, vrtoglavice, glavobolja, migrene, grčevitog kašlja, djeluje kao diuretik
Origano	*Origanum vulgare* L.	Lamiaceae	Pomaže kod oboljenja dijafragme te djeluje kao karminativ, antispazmolitik, antiseptik, tonik
Peršin	*Petroselinum crispum* (Mill.)	Apiaceae	Abortivno sredstvo, oralni hipoglikemijski lijek, antianemik
Ružmarin	*Rosmarinus officinalis* L.	Lamiaceae	Infuzija služi za oralnu primjenu kod tegoba s probavom, djeluje kao diuretik te kod različitih bolesti kardiovaskularnog sustava
Kadulja	*Salvia officinalis L.*	Lamiaceae	Koristi se kao biljni čaj za iscjeljivanje rana, ublažavanje želučanih tegoba, kod oboljenja jetre i reumatskih bolova. Također se koristi kod upale usne šupljine i grla.
Vrijesak	*Satureia hortensis L.*	Lamiaceae	Koristi se za liječenje bolova u mišićima, zaraznih bolesti, oboljenja želuca (kao tonik i karminativ) te intestinalnih poremećaja kao što su grčevi, mučnina, dijareja te ostale probavne smetnje. Ima antispazmolitično, antidiaroidno, antioksidacijsko, sedativno i antimikrobno djelovanje. Također, djeluje i kao ekspektorant i afrodizijak.
Metvica	*Mentha spicata* L.	Lamiaceae	Pomaže kod dispneje, nadutosti, dispepsije, djeluje kao sedativ i želučani tonik, repelent.
Taragon	*Artemisia dracunculus* L.	Compositeae (Asteraceae)	Koristi se za liječenje glavobolja, vrtoglavica i epilepsije.
Majčina dušica	*Thymus vulgaris* L. and *Thymus zygis* var. gracilis Boiss.	Lamiaceae	Antiseptik, karminativ, ima antimikrobna i antioksidativna svojstva. Koristi se kao fumigant (sredstvo za dimljenje) i za ispiranje u usta.

UTJECAJ PRIRODNIH DODATAKA NA ODRŽIVOST PREHRAMBENIH PROIZVODA

Biljni ekstrakti, integrirani u hrani, imaju različite nutritivne i ljekovite uloge. Brojne studije su dokazale da izravno dodavanje biljnih eteričnih ulja i ekstrakata u prehrambene proizvode pokazuje antimikrobno ili antioksidacijsko djelovanje. Ovisno o vrsti spojeva koji se dodaju, mogu utjecati na održivost proizvoda (Theivendran i sur., 2006). Također, naneseni u obliku različitih jestivih filmova, mogu služiti kao nosači antimikrobnih spojeva kako bi se održale visoke koncentracije konzervansa u hrani. Osim toga, imaju široku primjenu i mogu se naći kao poboljšivači viskoznosti, stabilizatori, dezintegranti, solubilizatori, emulgatori, sredstva za suspendiranje, sredstva za geliranje i bioadhezivi u premazima za film, mikrokuglice, nanočestice itd. (Avachat i sur., 2011).

Jestivi ambalažni filmovi

Jestivi ambalažni filmovi više se smatraju dodatcima prehrani nego sastojcima jer ne daju značajnu nutritivnu vrijednost obloženoj hrani. Utjecaj jestivih filmova i premaza na okus tijekom konzumiranja jestivog pakiranog prehrambenog proizvoda bi trebao biti što je moguće manji (Debeaufort i sur., 1998), stoga je izravno dodavanje eteričnih ulja u prehrambene proizvode ograničeno. Eterična ulja predstavljaju intenzivnu aromu koja može predstavljati problem kada intenzitet arome prelazi prihvatljivi prag potrošaču (Hyldgaard i sur., 2012). Eterična ulja i ekstrakti biljaka, kao takvi, ugrađeni u polimerne matrice, pored antioksidacijske uloge, utječu i na odgađanje oksidacije lipida i denaturacije proteina (Da Silva i sur., 2016). Dodatkom odabranih organskih kiselina ili eteričnog ulja u jestive filmove i premaze, moguće je utjecati na kontrolu rasta bakterija. Poznato je i da fenolni spojevi kao što su karvakrol, timol i eugenol pokazuju veliki antimikrobni učinak i uglavnom su prisutni u eteričnim uljima kao glavne komponente. Eterična ulja mogu biti nanesena na jestivi film ili inkapsulirana u jestive i biorazgradive polimere ili vrećice. Moguće je i inkapsulirati eterična ulja u nanoemulziju čime se sprječava interakcija eteričnih ulja s hranjivim matricama. Ugrađivanje antibakterijskih, antifungalnih i antioksidativnih aktivnih komponenti može dovesti do promjena u fizičko-kemijskim svojstvima proizvoda (Hyldgaard i sur., 2012).

Mikrokapsuliranje eteričnog ulja

Mikrokapsuliranje je tehnika očuvanja kakvoće osjetljivih komponenti aromatičnih biljnih ulja i ekstrakata koja se može koristiti u prehrambenoj industriji (Badee i sur., 2012). Badee i sur. (2012) utvrdili su da je kapsuliranjem paprene metvice s gumom arabikom u usporedbi s drugim materijalima omogućilo zadržavanje najviše okusa. Ahn i sur. (2008) utvrdili su da ekstrakti od ružmarina i drugi prirodni biljni ekstrakti kao što su klice brokule mogu inhibirati oksidaciju lipida mikrokapsuliranjem visoko oleinskog ulja suncokreta, čime se može doprinijeti poboljšanju kvalitete mikrokapsuliranih uljnih proizvoda u prehrambenoj industriji. Mikrokapsuliranje lipidnih produkata koristi se u proizvodnji praškastog ulja i masti (Ahn i sur., 2008). Almeida i sur. (2013) razvili su proces koji potencijalno može služiti za proizvodnju sastojaka s različitim vrstama škroba

pomoću superkritične impregnacije otapala izbjegavajući degradaciju eteričnih ulja i dobivajući visoku antioksidacijsku aktivnost produkta.

Primjena eteričnih ulja u mesnoj industriji

Jestivi ambalažni filmovi proizvedeni od mliječnih proteina, obogaćeni eteričnim uljem origana (*Origano vulgare* L.), pokazali su antioksidacijsku i antimikrobnu aktivnost u dodiru s mesom (Oussalah i sur., 2004).
Istraživanju provedena u radu Oussalah i sur., 2004. pokazala su da je imobilizacijom organskih kiselina na jestivim premazima baziranim na kalcijevom alginatnom gelu ili proteinima sirutke bilo moguće kontrolirati *Listeria monocytogenes* na tkivu govedine. Njihova istraživanja pokazala su da je ugradnjom eteričnih ulja u mliječne proteinske jestive filmove nanesene na mišićno meso moguće smanjiti aktivnost mikroba i povećati antioksidacijsku aktivnost tijekom 7 dana skladištenja. Njihovi rezultati podržali su i hipotezu da inhibicija *E. coli* O157: H7 i rast *Pseudomonas spp.* ovisi o prirodi fenolnih spojeva i njihovoj koncentraciji u eteričnim uljima Filmovi bazirani na dodatku eteričnog ulja origana pokazali su najučinkovitiju antimikrobnu aktivnost na inhibicija *E. coli* O157: H7 (Oussalah i sur., 2004). Uporaba jestivih filmova koji sadrže eterična ulja kao metoda očuvanja mesa je obećavajuća te omogućuje i progresivno oslobađanje fenolnih spojeva tijekom skladištenja. Aktivnosti biljnih ekstrakata ružmarina, sjemenki grožđa, zelenog čaja i gingo bilobe pokazali su utjecaj na očuvanje hrane (Theivendran i sur.,2006). Ovi rezultati su u suglasnosti s istraživanjima autora Rababah i sur. (2004) čija su istraživanja pokazala uspješnu primjenu sjemenki grožđa i ekstrakta zelenog čaja kao konzervansa u mesnim sustavima.
Eterično ulje ružmarina (*Rosmarinus officinalis* L.) dodano u kobasice mljevenog mesa pokazalo je smanjenje razine rezidualnog nitrita, smanjenu razinu oksidacije lipida i migraciju flavonoida kao što su hesperidin i narirutin (Viuda-Martos i sur., 2010). Istraživanja provedena na ekstraktu ružmarina (*Rosmarinus officinalis* L.) dodanom zamrznutim i svježim svinjskim kobasicama u različitim koncentracijama, uspoređena su s rezultatima dodavanja BHA (butil hidroksi anisol) i BHT (butil hidroksi toluen) te je uočeno da su ekstrakti s eteričnim uljem ružmarina jednako dobri antioksidanti kao BHA i BHT i u nekim slučajevima pružaju još bolje rezultate (Sebranek i sur., 2005). Eterično ulje ružmarina dodano u mesne okruglice pokazalo je učinkovitost u smanjenju oksidacije lipida i laganom smanjenju broja bakterija mliječne kiseline u mesu (Fernandez-Lopez i sur., 2005).

Primjena eteričnih ulja u industriji ribe

Dodavanje eteričnog ulja origana na oradu (*Spaurus aurata*) u različitim koncentracijama, u kombinaciji s MAP, pokazalo je učinak konzervansa jer se smanjila oksidacija lipida te je riba bila senzorno prihvatljivija duži period, u usporedbi s MAP pakiranjem bez dodanog eteričnog ulja (Goulas i Kontominas, 2007). Ekstrakti eteričnog ulja ružmarina (*Rosmarinus officinalis* L.) i origana (*Origanum vulgare* L.) korišteni su za obogaćivanje jestivih filmova želatine i pokazali su da smanjuju mikrobni rast i oksidaciju lipida kod dimljenih riba (Gomez-Estaca i sur., 2007).

Primjena eteričnih ulja u proizvodnji sira

Dodatak eteričnih ulja ulja lovora (*Pimenta racemosa*), klinčića (*Syzygium aromaticum*), cimeta (*Cinnamomum verum*) i timijana (*Thymus vulgaris* L.) u mekane sireve pokazao se kao učinkovit način inhibicije bakterija *Listeria monocytogenes* i *Salmonella enteritidis*. To je pokazalo da eterična ulja mogu biti djelotvorna antimikrobna sredstva za mliječne proizvode (Smith-Palmer i sur., 2001).

Primjena eteričnih ulja u pekarskoj industriji

Istraživanja provedena u radu autora Otoni i sur., (2014) pokazala su da emulzijski filmovi obogaćeni eteričnim uljima origana (*Origanum vulgare*) i klinčića (*Syzygium aromaticum*) pokazuju antimikrobno djelovanje protiv kvarenja pekarskih proizvoda. Oba eterična ulja djelovala su kao plastifikatori metil-celuloznih filmova te osigurali antimikrobno djelovanje protiv kvasaca i plijesni u rezanom kruhu. Jestivi filmovi obogaćeni eteričnim uljima dokazala su bolju učinkovitost utjecaja na trajnost pekarskih proizvoda u usporedbi s komercijalnim antifungalnim sredstvima koja se trenutno koriste u pekarskoj industriji. Proveli su i ispitivanja utjecaja veličine kapljice na poboljšanje antimikrobnih svojstava. Njihova istraživanja su pokazala da smanjenje veličine kapljica povećava antimikrobno ponašanje eteričnih ulja zbog činjenice da manja veličina čestite ima veći mogućnost prodiranja u unutrašnjost proizvoda.

Nedostatci uporabe eteričnih ulja u industriji

Za neka eterična ulja, kao što su eukaliptus, klinčić, kadulja i dr., mnoga istraživanja su potvrdila toksična i nadražujuća svojstva. Unatoč tome, većina tih ulja dostupna su za kupnju, kao samo eterična ulja ili inkorporirana kao dio farmaceutskih ili kozmetičkih proizvoda, što ukazuje da toksična svojstva ne zabranjuju njihovu upotrebu. Ipak, istraživanja o toksičnim ili nadražujućim svojstvima, posebno kada se razmatraju svi novi proizvodi za ljudsku upotrebu, bilo medicinski ili na neki drugi način, aktivno se provode (Hammer i sur.,1999).
Istraživanje koje su proveli Hammer i sur., (1999), potvrđuje da mnoga eterična ulja i biljni ekstrakti posjeduju *in vitro* antibakterijsko i antifungalno djelovanje. Međutim, ako se biljna ulja i ekstrakti koriste za očuvanje hrane ili u ljekovite svrhe, morat će se riješiti pitanja sigurnosti i toksičnosti (Hammer i sur.,1999).
Također, dodatkom eteričnih ulja u hranu moguće je utjecati i na narušavanje senzornih svojstava. Jedno od istraživanja koja su proveli Bagamboula i sur., (2004) upravo dokazuje da primjena određenih eteričnih ulja ometa senzorska svojstva kod salate pri čemu smeđi i dobiva snažan miris.
Kvaliteta eteričnih ulja glavni je čimbenik koji definira cijenu proizvoda te će eterična ulja bogatija bioaktivnim komponentama i izoliranim spojevima biti značajnije skuplja i samim tim poskupljivati proizvodni proces.

ZAKLJUČAK

Bez obzira definiramo li biljne proizvode kao hranu, funkcionalnu hranu, nutraceutike, dodatke prehrani ili lijekove na recept, činjenica je da oni predstavljaju izvor ljekovitih (bioloških i

farmakološki) aktivnih tvari koji imaju ulogu održavanja i / ili poboljšanja zdravlja, sprečavanju i liječenju bolesti. Do uspostave jasnih definicija ovih pojmova, sigurno je da imaju jedan zajednički cilj, a to je povećanje kvalitete života. U vremenu kada se sve više okrećemo tvarima koje nam priroda pruža, upotreba biljaka s ljekovitim djelovanjem nalazi široku primjenu u različitim granama industrije koje su usko povezane sa zdravstvenim stanjem pojedinca. Trenutno, jedno od najvažnijih istraživačkih područja znanosti i tehnologije hrane jest izolacija i karakterizacija novih prirodnih sastojaka s biološkom aktivnošću koja se može dodatno ugraditi u funkcionalnu hranu, pridonoseći dobrobiti potrošača. Upravo su eterična ulja vjerodostojni primjeri najraširenije uporabe prirodnih antimikrobnih produkata, potencijalne zamjene sintetskih konzervansa. Njihovom integracijom u proizvode zadovoljeni su zahtjevi potrošača za prirodnim i sigurnim proizvodima na tržištu. Upravo je mnogim dostupnim stručnim i znanstvenim istraživanjima potvrđeno djelovanje prirodnih dodataka, što je također
doprinijelo povećanju konzumacije ovih proizvoda. Predmet daljnjih istraživanja trebali bi biti korisni i štetni učinci kombiniranja funkcionalne hrane, prehrambenih dodataka i lijekova, zatim poticanje samoliječenja kroz uporabu funkcionalne hrane i dodataka prehrani te dugoročna sigurnost proizvoda.

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NATURAL FOOD SUPPLEMENTS AS CARRIERS OF NUTRITION QUALITY, HEALTHFUL POTENTIAL AND THE SUSTAINABILITY OF THE PRODUCT

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ABSTRACT

For centuries, people have received food directly from nature, and by the late 19th and early 20th century there was an expansion of the modern industry, which enabled the development of a large number of food and pharmaceutical products through the development of new technologies. This development has also influenced the development of drugs, which has suppressed the philosophy of "food as medicine" for almost a whole century. Due to the appearance of many illnesses during the 20th century, the modern industry is beginning to develop enriching food with natural nutritional supplements again. A well-known trend is the production of "functional food" or food that has a beneficial effect on health and the associated nutritional properties. It was noted that by adding spicy and medicinal plants it is possible to improve the taste and smell of food. A large number of studies have also shown that these groups of plants contain compounds with expressed bactericidal and fungicidal activity, as well as natural nutritional supplements to prevent food degradation and food sustainability. In addition, they are extremely powerful antioxidants. The use of natural nutritional supplements is now expressed in all production technologies, mostly in the meat and meat products technology, dairy and bakery products, and through the use of edible packaging films and fillers in the food and pharmaceutical industry. By adding species and medicinal plants to different types of products, it is possible to significantly improve the nutritional quality and the healing potential and sustainability of the product.

Keywords: natural food supplements, functional food, plants, nutrition quality

***Topic: Functional food and food supplemnents /* Sekcija: Funkcionalna hrana i dodaci prehrani**

Lepidium meyenii (BRASSICACEAE) AND *Moringa oleifera* (MORINGACEAE) AS SUPERFOOD: GLUCOSINOLATES AND OXIDATIVE STABILITY

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ABSTRACT

Glucosinolates are secondary plant metabolites found in 16 families of the Brassicales order, including plants from the Brassicaceae and Moringaceae families. Volatile compounds formed by thermal, enzymatic, or chemical degradation of glucosinolates are known for their biological activity, especially the isothiocyanates. Two plants, considered as superfood for their high phytonutrient contents, were investigated: maca (*Lepidium meyenii*) and moringa (*Moringa oleifera*). Glucosinolates, as compounds characteristic for these two plants, were determined by an indirect method, using their degradation products. The volatile isolates collected by various methods of hydrodistillation and extraction, were analysed using gas chromatography coupled with mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FTIR). The main compounds found in *L. meyenii* were phenylacetonitrile and benzyl-isothiocyanate (degradation products of glucotropaeolin), and 3-methoxyphenylacetonitrile (degradation product of glucolimnanthin). No degradation products were found using GC-MS analysis of the *M. oleifera* volatile isolates, but the FTIR analysis confirmed their presence. Oxidation processes are a widespread problem in the food industry. These days, the popularity of adding various plants or herbs to oils, in order to aromatize them or prolong their shelf life, is growing. Therefore, the effect of the investigated plants' powdered samples on the oxidation stability of fish oil was studied using the Rancimat apparatus.

Keywords: glucosinolates, oil oxidation, GC-MS, FTIR, Rancimat

INTRODUCTION

Glucosinolates (*β*-thioglucoside-*N*-hydroxysulfates) are secondary plant metabolites found in 16 families of the Brassicales order. Structural characteristics shared by all glucosinolates (GLs) are *β*-D-thioglucoside, a sulphate group connected to the rest of the molecule via the C=N bridge, and the side chain. The GL side chain is the only variable part of the molecule and it's the basis of their structural diversity and the biological activity of the compounds formed by their enzymatic, thermal, or chemical degradation (Blažević et al., 2017).

GLs are non-volatile compounds that can be found in all plant parts; usually more than one GL can be found in the same plant, but some are known to contain more than fifteen GLs. Composition, distribution, and content of the GLs depends on the plant species, the plant part, and the development status, but also on the development conditions (Fahey et al., 2012). When

the plant tissue is damaged, they come into contact with the enzyme myrosinase. Many of the volatile and biologically active compounds are formed, such as isothiocyanates, nitriles, thiocyanates, and oxazolidinthiones (Bones and Rossiter, 2006) as a result of their hydrolysis. Some of these degradation products show antioxidant (Vaglimigli and Iori, 2009), antimicrobial (Dufour et al., 2015), and even anticancerogenic (Steinbrecher et al., 2010, Fofaria et al., 2015) properties, but some have shown to be harmful (Fahey et al., 2012). Mostly aliphatic GLs, including sinigrin, gluconapin, progoitrin, glucoiberin, glucoraphanin, glucoraphasatin, glucolesquerellin, glucohesperin, as well as glucobrassicin have been investigated using different antioxidant assays (2,2-diphenyl-1-picrylhydrazyl radical, DPPH; 3-ethyl-benzylthiazoline-6-sulfonic acid radical cation, $ABTS^{\cdot+}$; oxygen radical absorbance capacity, ORAC; superoxide radical scavenging activity, SRSA) (Barillari et al., 2005; Pappi et al., 2008; Cabelo-Hurtado et al., 2012; Montaut et al., 2012; Natella et al., 2014). Studies have shown divergent results using these methods (Vaglimigli and Iori, 2009; Montaut et al., 2017).

Over 130 different GLs have been identified (Agerbirk and Olsen, 2012), but the closely related taxonomic groups contain only a small number of these compounds. The largest quantities of GLs can be found in three families of the Brassicales order: Resedaceae, Capparaceae, and Brassicaceae (Blažević et al., 2017). The *Lepidium* genus is a part of the Brassicaceae family, and one of its most notable plants is *Lepidium meyenii*, also known as maca. Maca is considered to be a superfood, due to the wealth of phytonutrients it contains and consequently its positive health effects. It contains over 20 amino acids, including 8 essential amino acids, 20 free fatty acids, vitamins B1, B2, C, and E, calcium, magnesium, potassium, copper, zinc, manganese, phosphorus, selenium, sulphur, sodium, and iron. Regular consumption of this plant leads to increased sperm production and sperm motility (Gonzales et al., 2001, Lee et al., 2016). Maca is also an adaptogen, which means that this plant helps the body to deal with stress.

Besides Brassicaceae, the Moringaceae family is also known for plants rich in GLs. *Moringa* is the only genus of this family. The most notable plant in this genus is the horseradish tree (*Moringa oleifera*), also categorized as superfood. This plant contains more than 90 phytonutrients, which makes it the most nutrient dense plant currently being investigated. 4-(α-L-Rhamnopyranosyloxy)benzyl GL (glucomoringin), a rarely found multiply glycosylated GL, is present in this plant in extremely high amounts, representing over 25% of dry weight (DW), i.e. up to 600.0 μmol/g DW (Blažević et al., 2017). Moringa leaves have also been reported to be a rich source of *β*-carotene, protein, vitamin C, calcium, and potassium, and act as a good source of natural antioxidants (Anwar et al., 2007). The horseradish tree was found to have a positive effect on the cardiovascular, gastrointestinal, immune, and nervous systems, but also on the prostate, eye health, and it's said to increase energy levels (Maldini et al., 2014).

The aim of this study was to determine the GLs present in *Lepidium meyenii* and *Moringa oleifera* plants, using indirect methods of detection of their degradation products collected by hydrodistillation in a Clevenger-type apparatus and/or extraction with an organic solvent after enzymatic hydrolysis. The volatile isolates were analysed with gas chromatography coupled with mass spectrometry (GC-MS) and/or Fourier transform infrared spectroscopy (FTIR). In addition, the effect of the investigated plants' powdered samples on the oxidation stability of fish oil was studied using the Rancimat apparatus.

MATERIALS AND METHODS

General

The solvents employed for the hydrodistillation were diethyl-ether stabilised with 6 ppm butylated hydroxytoluene (BHT) (Panreac, Barcelona, Spain) and pentane (Lach-ner, Neratovice, Czech Republic), and for the extraction, dichloromethane stabilised with 2-methylbuthene (T.T.T., Sveta Nedjelja, Croatia) and the enzyme thioglucosidase: myrosinase isolated from white mustard seeds (*Sinapis alba*; Sigma-Aldrich Chemie, Stenheim, Germany). Anhydrous Na_2SO_4 was obtained from AnalR Normapur, VWR (International), Radnor, Pennsylvania, USA). Dri-Block heater (Techne, Cambridgeshire, UK) was used for volatile sample concentration. Gas chromatography analyses were performed on a gas chromatograph (model 3900; Varian Inc., Lake Forest, CA, USA) equipped with a mass spectrometer (model 2100T; Varian Inc.), and a non-polar capillary column VF-5MS (30 m × 0.25 mm i.d., coating thickness 0.25 μm). The infrared spectra were recorded on an IRAffinity-1 spectrometer (Shimadzu, Japan). A Rancimat 743 apparatus from Metrohm A.G. (Herisau, Switzerland) was used to measure the induction time of fish oil with and without additives.

Plant material

Commercially available *L. meyenii* Walp. powders from two different manufacturers were used: Orgona superfood and Bio&bio superfood, both from Peru. Also, commercially available *M. oleifera* Lam. powder was used (Encian superfoods) from India.

Isolation of Volatiles

Volatiles were isolated from maca (powdered roots) and moringa (powdered leaves) using two methods: hydrodistillation and extraction. The plant material (50 g) was immersed directly in a flask filled with 1000 mL of water, and hydrodistillation was carried out in a Clevenger-type apparatus for 3 hours, using 3 mL pentane:ether (3:1 v/v) for trapping. The vapour carries the volatile compounds and the condensate drops onto a pentane:ether trap in the inner tube of the apparatus, where the volatiles are retained. Volatiles were also isolated from the same plant materials (10 g) after autolysis at 27 °C for 24 hours, using the added enzyme myrosinase, and finally extracted by dichloromethane. The obtained volatile fractions were dried over anhydrous sodium sulphate. The volatile samples were concentrated using a nitrogen stream in a Dri-Block at 40 °C, and 1 μL of this solution was used for GC–MS analysis.

GC-MS and FTIR Analysis

VF-5MS column temperature was programmed at 60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. Other chromatographic conditions were: carrier gas helium; flow rate 1 mL min^{-1}; injector temperature 250 °C; volume injected 1 μL; split ratio 1:20; ionization voltage 70 eV; ion source temperature 200 °C; mass scan range: 40–350 mass units. The IR spectra were recorded using a KBr transmission cell, in the spectral area 4000-400 cm^{-1} and with the resolution of 4 cm^{-1}.

Identification and Quantification of Components

The individual peaks were identified by comparing their retention indices (relative to C8-C40 n-alkanes for VF-5MS) to those from a homemade library, literature, and/or authentic samples, as well as by comparing their mass spectra with literature, Wiley 7 MS (Wiley, New York, NY, USA), and NIST02 (Gaithersburg, MD, USA) mass spectral databases. The homemade library was created from authentic compounds obtained commercially and from the main components of many essential oils obtained *during* our previous studies.

Rancimat assay (Oxidative stability testing)

The oxidative stability of the fish oil was evaluated using the Rancimat instrument to monitor the progress of the accelerated oxidation of oil at high temperatures. The fish oil samples (3 g) were tested at two different temperatures (80 and 100 °C) with the constant air flow of 20 L h^{-1}. The amounts of the added plant material were 25, 50, and 100 mg. The conductivity was measured as a function of time. All determinations were performed in duplicate, and the results are presented as mean value ± standard deviation.

RESULTS AND DISCUSSION

Glucosinolates (GLs) can degrade enzymatically, thermally, or chemically, as shown in Figure 1, with the formation of two main products, isothiocyanates and nitriles. Other products, that are possible from certain GLs in certain experimental conditions, were not observed in the present study. The structures of the GLs identified by these volatiles in *Lepidium meyenii* and *Moringa oleifera* are given in Figure 1.

Lepidium meyenii Walp.

Table 1. represents the GC-MS analysis of the *L. meyenii* powdered root hydrodistillate and root extract.

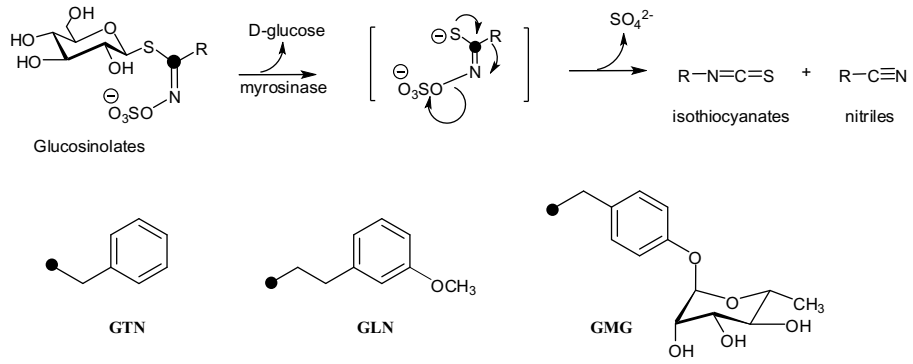

Fig. 1. Degradation of GLs and the main GLs present in *Lepidium meyenii* (glucotropaeolin, GTN; glucolimnanthin, GLN) and *Moringa oleifera* (glucomoringin, GMG)

Table 1. GC-MS analysis of the *Lepidium meyenii* root hydrodistillate and extract.

Glucosinolate / identified degradation product	**Retention time (min)**	**Hydrodistillate (%)**	**Extract (%)**
Glucotropaeolin			
Phenylacetonitrile	17.52	54.4	7.3
Benzyl-isothiocyanate	27.60	0.8	10.5
Glucolimnanthin			
3-Methoxyphenylacetonitrile	28.99	1.5	2.3
3-Methoxybenzyl-isothiocyanate	37.80	-	1.2

L. meyenii root distillate analysis showed that the most abundant compound was phenylacetonitrile (54.4%), a glucotropaeolin degradation product. The root distillate also showed a presence of benzyl-isothiocyanate (0.8%), another glucotropaeolin degradation product. Finally, 3-methoxyphenylacetonitrile (1.5%) was identified as a glucolimnanthin degradation product.
In the *L. meyenii* root extract, the following two glucotropaeolin degradation products were found: phenylacetonitrile (7.3%) and benzyl-isothiocyanate (10.5%). Besides the glucotropaeolin degradation products, the analysis showed the presence of two glucolimnanthin degradation products; 3-methoxyphenylacetonitrile (2.3%) and 3-methoxybenzyl-isothiocyanate (1.2%), which was not found in the distillate.

Moringa oleifera Lam

M. oleifera is known to be a rich source of glucomoringin (multiple glycosilated GL). Radulović et al. (2014) successfully isolated the 2-(α-L-rhamnopyranosyloxy)benzyl GL degradation product from the *Reseda lutea* autolysate and identified it using GC-MS (Papi et al., 2008). This degradation product represents a glucomoringin isothiocyanate isomer. Thus, the main focus of the research was the isolation and identification of the glucomoringin degradation products, and the identification of the aforementioned GL.
However, GC-MS analysis under given conditions showed no presence of the glucomoringin degradation products in the *M. oleifera* distillate or extract. Hence, the GC-MS analysis conditions were modified as following: at 70 °C isothermal for 3 min, and then increased from 70 °C to 290 °C at a rate of 5 °C min^{-1} and held isothermal for 10 min (chromatogram is not shown). Even under the new conditions, the same results were obtained, meaning that the expected glucomoringin degradation product was not identified. Thus, the IR spectra of the *M. oleifera* hydrodistillate and extract were recorded and are given in Figure 2.
Broad-band corresponding to O-H stretching on both spectra was observed in the range of 3400 cm^{-1} that corresponds to O-H stretching of alcohols. The peak on ~ 1265 cm^{-1} (C-O stretching) was very strong, indicating a large number of C-O bonds, which are present in sugar moieties. Peaks observed in the range of 1600 - 1400 cm^{-1} correspond to benzene C=C bonds stretching. Peaks at ~ 2054 cm^{-1} correspond to the -N=C=S group. Radulović et al. (2014) also reported the IR spectra of 2-(*α*-L-ramnopyranosyloxo)benzyl isothiocyanate, which had a peak at 2080 cm^{-1}. Also, a sharp peak observed at ~ 2304 cm^{-1} corresponds to the -C≡N group. Nitrile formation is preferable when dried material is used (which was in our case). The IR spectra of *M. oleifera* distillate and extract indicated sugar moieties, benzene ring, isothiocyanate, and

nitrile group, which suggested abundant degradation products of glucomoringin in the investigated isolates.

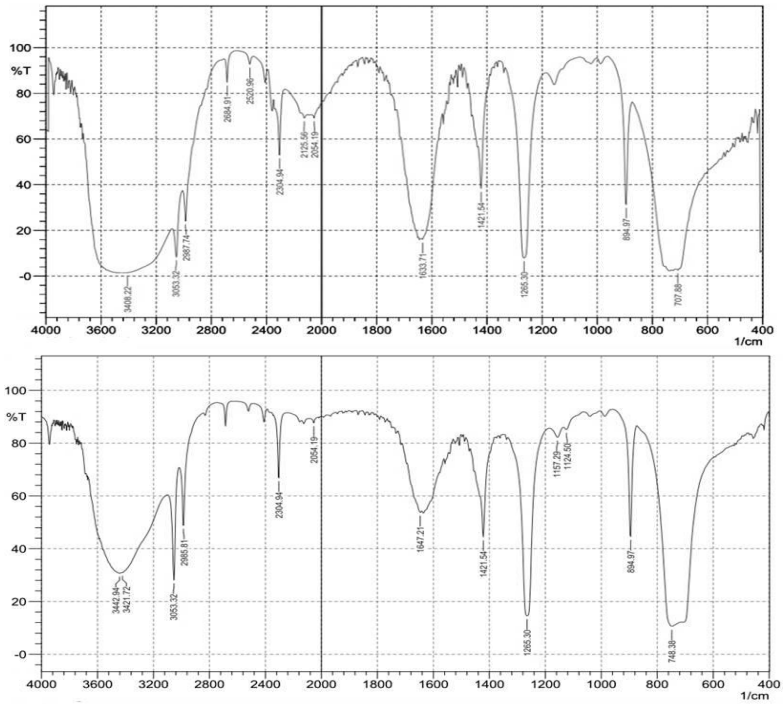

Fig. 2. Infrared spectra of the *M. oleifera* volatile samples: Obtained by hydrodistillation (above) and obtained by organic solvent extraction (bellow).

Due to the large numbers of hydrogen bonds, compounds with sugar groups are not likely to be identified using the GC-MS analysis. However, Radulović et al. (2014) successfully identified the degradation product of the *Reseda lutea* GL. The degradation of the *R. lutea* GL led to the formation of the *para*-substituted isothiocyanate and the glucomoringin degradation led to the formation of the *ortho*-substituted isothiocyanate and nitrile. The difference in the positions of the ramnopyranosyl substitutes is probably the reason why the glucomoringin degradation products were not identified, while the *R. lutea* GL degradation product was identified.

Antioxidant potential

The potential of maca and moringa to inhibit (delay) the fish oil oxidation process was tested using different amounts of plant powders at 80 °C and 100 °C, and is given in Figures 3 and 4.

According to the obtained results, the addition of moringa plant material caused prolonged oil stability and the effect was influenced by the added amount of plant material. On the other hand, maca had a lower antioxidant ability at 80 °C, which improved by increasing the temperature to 100 °C. The Protection Factor (PF), as a measure of protection, can be calculated according to the equation PF=sample induction time/control induction time.

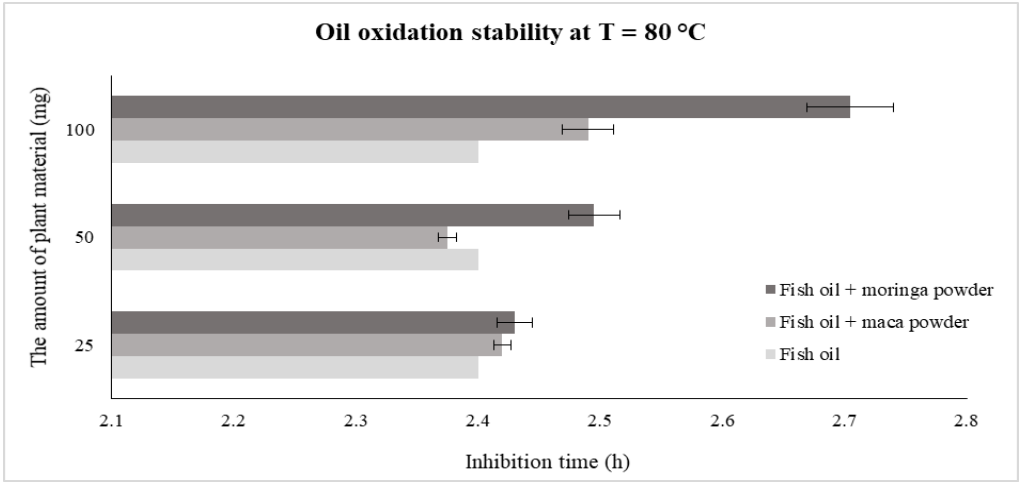

Fig. 3. Antioxidant ability of maca and moringa against fish oil oxidation at 80 °C.

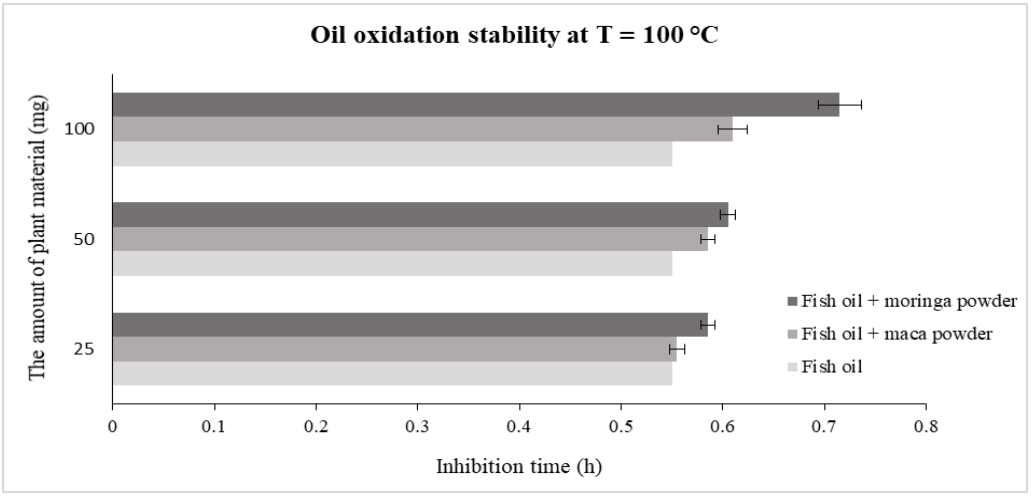

Fig. 4. Antioxidant ability of maca and moringa against fish oil oxidation at 100 °C.

The control (pure fish oil) oxidized at 80 °C after 2 h and 24 min, while at 100 °C it oxidized after only 33 min. Generally, moringa powder showed a better protective effect than maca powder. When 100 mg of moringa was used, it prolonged oil oxidation for about 17 min at 80 °C and 9 min at 100 °C, having the protection factor of 1.12 and 1.27, respectively. If we compare the obtained results for PF of maca and moringa with those for some other substances that are usually added to oil to improve its stability (synthetic antioxidants, tocophenols, herbs and their extracts, etc.), the overall results are not significant. As the chain-breaking activity of isothiocyanates (ITCs) is almost negligible (Valgimigli and Iori, 2009), the overall inhibition effect of maca and moringa is the result of the presence of other compounds (antioxidants like vitamins, phenolics, and prooxidants like minerals and pigments).

CONCLUSION

The GC-MS analysis of volatile isolates from *L. meyenii* root revealed volatile degradation products of aromatic GLs. The analysis by GC-MS was unsuccessful in detecting degradation products of glucomoringin, a known GL in the *Moringa oleifera* leaves. On the other hand, FTIR analyses showed to be useful for detecting the presence of the glucomoringin isothiocyanate and the glucomoringin nitrile. The antioxidant potential of moringa was greater than the investigated antioxidant potential of maca, when tested at elevated temperature and accelerated oxidation conditions. These plants' different aromatic GLs makes them suitable subjects for further research of their biological and pharmacological properties.

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Author Contributions: I.B. and I.G.M. designed the study. Isolation and identification was performed by P.B. and A.Đ. The Rancimat test was performed by I.G.M., and P.B. All the authors analysed the data. P.B. and A.Đ. drafted the manuscript with the input from all the authors.

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Topic: Functional food and food supplemnents / **Sekcija: Funkcionalna hrana i dodaci prehrani**

MELISSOPALYNOLOGICAL ANALYSIS OF HONEY FROM THE UNA-SANA CANTON

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ABSTRACT

Contemporary trends and trade have contributed to the great presence of different types of honey in the market, which leads to confusion and insecurity regarding its authenticity and quality. The aim of this study was to determine the botanical origin of the unifloral and multifloral honey types collected in the Una-Sana Canton. The pollen spectrum (botanical origin) was determined melissopalynologically from the collected samples. It was determined that in chestnut honey, aside from chestnut (*Castanea sativa*) pollen (87.9 ± 8.9%), the most common grains are the ones from willow (*Salix* spp.), black locust (*Robinia pseudoacacia*). and lime (*Tilia* spp.). Lime honey, aside from lime (*Tilia* spp.) pollen grains (25.8 ± 48.9%), mostly contains chestnut (*C. sativa*), plantain (*Plantago* spp.), and black locust (*R. pseudoacacia*) pollen grains, while the black locust honey, aside from the black locust (*R. pseudoacacia*) pollen (25.4 ± 29.0%), is rich in chestnut (*C. sativa*), cock's-foot (*Dactylis glomerata*), and plantain (*Plantago* spp.) pollen. Meadow honey has the highest number of chestnut (*C. sativa*) (31.1 ± 93.3%), willow (Salix spp.), black locust (*R. pseudoacacia*), plantain (*Plantago* spp.), and dandelion (*Taraxacum officinale*) pollen grains. In the samples of honeydew honey, the most common pollen is chestnut (*C. sativa*) (13.1 ± 22.1%), followed by wild privet (*Ligustrum vulgare*), oak (*Quercus* spp.), beech (*Fagus* spp.), and birch (*Betulapendula*). Mixed honey also has the highest number of chestnut (*C. sativa*) grains (61.5 ± 100.6%), followed by black locust (*R. pseudoacacia*) and lime (*Tilia* spp.), while floral honey, aside from chestnut (*C. sativa*) pollen (55 ± 91.4%), contains lime (*Tila* spp.) pollen.

Keywords: melissopalynological analysis, honey, Una-Sana Canton

INTRODUCTION

Melissopalynological analysis determines the percentage of pollen in honey, and it is one of the first methods used to determine the botanical origin of honey (Ruoff and Bogdanov, 2004; Song et al., 2012; Ponnuchamy et al., 2014), i.e., it is used to determine the purity of honey, geographic origin, and classification of honey (Kaya et al., 2005; Persano Odo et al., 2004). It is a very useful method in estimating the correlation of *in situ* climatic parameters, such as rain and temperature, which is important in the context of external factors affecting pollinators (Bilisik et al., 2008). The advantage of this method is that it does not require expensive instruments, but it is also limited. E.g., one of the constraints is that it requires highly

specialized staff, because pollen can be added later, which is characterized as fraud. In order to determine the subsequent addition of pollen, the criteria for the percentage of dominant pollen in different types of honey were determined (Louveaux et al., 1978), where the pollen grains are classified by percentage as predominant pollen (> 45%), secondary pollen (16-45%), important minor pollen (3-15%), and minor pollen (<3%). The differences in the pollen spectrum between samples of honey from different geographic and climatic zones are easily detected. However, if the geographic areas are closer, it is much more difficult to determine the differences (Bogdanov and Martin, 2002). Depending on the origin of the plant from which bees gather nectar or honeydew, honey contains pollen grains or other microscopic particles such as honeydew elements (fungal spores, hyphae fragments, and algae). The result is that the composition of pollen in honey reflects the vegetation from which the honey is harvested, which is useful for determining the geographical/botanical origin. Also, during microscopic research, the sediment of honey reveals valuable information about beekeeping practices (feeding on various substitutes and general hygiene), honey extraction techniques, fermentation, and some types of counterfeiting (Kerkvliet et al., 1995; Russmann, 1998). The identification of pollen in honey has been carried out since the beginning of the last century, but the methodology has been improved and harmonized several times (Von der Ohe et al., 2004). It is considered that samples of unifloral honey must have at least 45% of the pollen from the plant species of which the nectar originates from, but unfortunately the ratio of pollen to nectar varies significantly between plant species.

Numerous factors can affect the presence of pollen in honey. Some of the most important influences are the morphology and the physiology of the plant. Some plant species produce very little pollen or have sterile anthers and do not produce pollen - such as some *Citrus* sp. (Von der Ohe et al., 1999; Von der Ohe and Von der Ohe, 2002).

An important factor is contamination in the hive - during the processing of honey and pollen input in the hive, honeybees that perform different tasks, i.e. those that solely collect the pollen, can introduce pollen into nectar, and honey may eventually be contaminated with the pollen of other plant species (Fernandez and Ortiz, 1994). Pollen may also be inadvertently introduced into the honey during uncapping and extraction of the honeycombs (from combs with stored pollen). Despite some drawbacks, melissopalynological analysis in combination with other techniques is still an unavoidable way to determine the geographic and botanical origin of honey (Persano Oddo et al., 2004; Von der Ohe et al., 2004). In determining the uniflority in the honey, most analysts take into account the percentage of pollen grains that are more or less represented in the honey. For unifloral honey types derived from nectar of plant species with less represented pollen, the minimum representation is 10% or even lower. That is the case with strawberry tree honey, dandelion, and citrus species honey. For types of honey derived from nectar of plant species with a higher amount of pollen, such as chestnut and eucalyptus honey, they must contain more than 85% of pollen of the plant species in order for their uniflority to be declared (Persano Oddo and Piro, 2004; Piazza and Persano Oddo, 2004). According to the National Rulebook on Quality of Honey and Other Bee Products (Official Gazette of B&H, 37/09), there are differences in the case of sweet chestnut (*Castanea sativa* Mill.), which must have 85% of pollen grains, lucerne (*Maedicago sativa* L.) 30%, rosemary (*Rosmarius officinale* L.) 30%, lime (*Tilia sp*.) 25%, sage (*Salvia sp*.) 20%, black locust (*Robinia pseudoacacia* L.) 20%, and lavender (*Lavandula* sp.) 20%. Multifloral honey is a mixture of different plants. The aim of this research was to determine the type and the presence

of pollen of seven types of honey from the USC area - to classify them as unifloral or multifloral honeys.

MATERIALS AND METHODS

The area covered by this research is the Una-Sana Canton (USC), which is geographically located in the north-western part of Bosnia and Herzegovina (B&H), on a surface area of 4 125 km^2, representing 8.0% of the total area of B&H (Fig. 1). This is the border area with the south and south-eastern part of the Republic of Croatia. The administrative and economic centre is the city of Bihać, with around 60 000 inhabitants. The Canton includes the town of Cazin and six other municipalities: Bosanska Krupa, Bužim, Velika Kladuša, Bosanski Petrovac, Sanski Most, and Ključ. These areas are very different in terms of geographical characteristics and total biodiversity, which directly affects the production of different types of honey. Forests, along with water resources, are the largest natural resource of the USC and they cover approximately 47% of total USC area. The peculiarity of the landscape is made up of habitats of sweet chestnut, and with an area of around 7000 ha it is the largest habitat of this forest community in B&H. In addition to this, USC has 42.8% of arable agricultural land, which has caused a large number of around 287000 inhabitants to turn to agriculture. With these conditions in the USC area, annual honey production is recorded between 300 and 500 t, which is far below the possible amounts. Beekeeping includes about 1000 beekeepers with a capacity of about 34000 beehives (SDPUSC, 2013). According to the research objective, it was necessary to collect representative honey samples from all eight areas. Considering that each area has an association of beekeepers, it was arranged through their representatives to acquire samples from several beekeepers, along with the criteria of geographical coverage of the honeybee's area, as well as sampling the same beekeepers in both seasons of research. A total of 205 honey samples were collected, of which, 99 in the first and 106 in the second sampling season (2009 and 2010). Among the multifloral samples (108 in total), the most common is meadow honey (86%), while among the unifloral honey types (88), chestnut honey is the dominant one (49%). Samples were collected from beekeepers immediately after filling and they were stored in a dark place, in glass packaging, until analysis. Sampling was carried out in accordance with the provisions of the National Rulebook on Methods of Control of Honey and Other Bee Products (Official Gazette of B&H, 37/09). It is necessary to point out that the chestnut forests are present in only four areas (Cazin, Velika Kladuša, Bužim, and Bosanska Krupa), so there are no samples of chestnut honey from Ključ and Bosanski Petrovac, and a very small number is sampled in Bihać and Sanski Most.

Melissopalynological analysis of honey samples was performed using the microscope OLYMPUS, model Bx53F (Japan), according to the National Rulebook of Methods for the Control of Honey and Other Bee Products (Official Gazette B&H, 37/09). During the analysis, two parallel samples of the same honey were made. They are enlarged 200 to 600 times. Visible fields have been changed until 300 pollen grains have been counted. The counted pollen grains are classified according to the plant species. Plant species are determined on the basis of the shape of the pollen, the size of the grain, the wall material, and the type, shape, and the number of germination openings. Pollen grains were compared with reference preparations and the image of the atlas (Von der Ohe and Von der Ohe, 2002; Von der Ohe and Von der Ohe, 2003; Von der Ohe et al., 2004).

Fig. 1. Area of research Una-Sana Canton

RESULTS AND DISCUSSION

With melissopalynological analysis, which is based on counting the pollen grains in a honey type (in this case the honey type was established by the beekeepers), it was possible to establish the average presence of different plant species in samples, as shown in tables from 1 to 4. It was found that in chestnut honey, in addition to the dominant *C. sativa* pollen grains (87.9 ± 8.9%), the most common are *Salix* spp., *R. pseudoacacia*, and *Tilia* spp. pollen grains (Table 1.). In lime honey, in addition to the pollen grains of *Tilia* spp. (25.8 ± 48.9%), it contains mostly *Plantago* spp. and *R. Pseudo acacia* pollen grains, while black locust honey, in addition to *R. pseudoacacia* grains (25.4 ± 29.0%), is rich in *C. sativa*, *Dactylis glomerata*, and *Plantago* spp. pollen grains.

Table 1. The share of pollen grains in unifloral honeys

Type of honey	Melissopalynological parameters		x ±σ	Min.	Max.
Chestnut	Number of plant species in the samples		7±2.3	3	13
	The most common plant species (%)	*Castanea sativa*	87.9±8.9	85.0	96.7
		Salix spp.	2.9±4.5	0.3	6.3
Linden	Number of plant species in the samples		13±4.4	6	20
	The most common plant species (%)	*Tilia* spp.	25.8±48.9	2.0	53.3
		Castanea sativa	23.6±60.6	0.3	59.7
Acacia	Number of plant species in the samples		13±3.1	8	19
	The most common plant species (%)	*Robinia pseudoacacia*	25.4±29.0	7.7	62.3
		Castanea sativa	25.4±58.7	1.7	81.0

Table 2. The share of pollen grains in multifloral honeys

Type of honey	Melissopalynological parameters		x ±σ	Min.	Max.
Meadow	Number of plant species in the samples		15±4.8	3	22
	The most common plant species (%)	*Salix* spp.	4.2±6.6	0.7	12.3
		Castanea sativa	31.1±93.3	0.7	92.0
		Robinia pseudoacacia	5.0±11.5	0.3	19.0
		Plantago spp.	6.3±9.1	0.7	14.3
		Taraxacum officinale	4.0±10.6	0.3	22.7
Mixed	Number of plant species in the samples		11±6	2	20
	The most common plant species (%)	*Castanea sativa*	61.5±100.6	3	98
		Robinia pseudoacacia	5.1±15.6	1.0	16.0
		Tilia spp.	3.0±13.7	0.3	14.0
		Plantago spp.	4.2±11.6	1.7	11.7
Floral	Number of plant species in the samples		15±3.0	11	18
	The most common plant species (%)	*Castanea sativa*	55±91.4	21.3	80.7
		Tilia spp.	13.8±35.0	0.7	23
		Rumex spp.	5.7±10.0	2.3	9.0
		Dactylis glomerata	5.0±9.6	2.7	8.7
		Plantago spp.	4.9±6.6	2.3	7.3

In the case of multifloral honey, specifically meadow honey, it is determined that it has the highest number of *C. sativa* (31.1 ± 93.3%), *Salix* spp., *R. pseudoacacia*, *Plantago* spp., and *Taraxacum officinale* pollen grains (Table 2). Mixed honey also has the highest number of *C. sativa* (61.5 ± 100.6%), then *R. pseudoacacia* and *Tilia* spp. pollen grains. In floral honey, the most common are *C. sativa* (55 ± 91.4%) with *Tilia* spp. pollen grains. In honeydew samples, the most common are *C. sativa* (13.1 ± 22.1%), *Ligustrum vulgare*, *Quercus* spp., *Fagus* spp., and *Betula pendula* pollen grains (Table 3).

Table 3. The share of pollen grains in honeydew honey

Type of honey	Melissopalynological parameters		x ±σ	Min.	Max.
Honeydew honey	Number of plant species in the samples		17±3	13	22
	The most common plant species (%)	*Castanea sativa*	13.1±22.1	2.3	21
		Ligustrum vulgare	5.4±13.3	2.0	16.0
		Quercus spp.	5.4±6.4	2.7	8.3
		Fagus spp.	6.0±10.1	2.3	10.7
		Betula pendula	4.4±4.9	2.3	6.7

According to the National Rulebook on Methods for Controlling Honey and Other Bee Products (Official Gazette B&H, 37/09), unifloral honeys have met the requirements considering product marking by honey type, with the share of 90.9%. Six samples of honey, labelled as lime honey, did not satisfy the required share of pollen grains (min. 25% of *Tilia* spp. pollen grains): one sample from Bosanska Krupa (6.3%), two from Bihać (2.0% and 5.6%), and three from Sanski Most (6.3%, 5.6% and 5.0%). Also, two samples of honey, labelled as black locust honey, do not satisfy the required share of pollen grains (min. 20%) – one sample is from Velika Kladuša (7.6%) and one from Bužim (10%).

Table 4. Pollen types and their distribution in the types of honey

Pollen type	Honey types						
	Chestnut	Lime	Black locust	Meadow	Mixed	Floral	Honeydew honey
	(N=43)	(N=18)	(N=22)	(N=93)	(N=11)	(N=4)	(N=9)
Castanea sativa	87.9*	23.6•	25.4•	31.1•	61.5*	55.0*	13.1
Tilia spp.	2.1	25.8*	5.3	6.3	3.0	13.3	4.8
Robinia pseudoacacia	2.3	5.9	25.4*	5.0	5.1	2.3	2.4
Brasica napus var. oleifera	1.2	14.7	1.0	2.0	-		21.3•
Salix spp.	2.9	4.1	5.3	5.2	4.0	3.3	4.5
Lamiaceae	2.9	8.0	8.7	11.0	4.7	5.1	10.0
Fagus spp.	2.0	2.1	3.6	3.7	3.0	4.9	6.0
Rumex spp.	1.8	4.2	3.4	5.2	3.1	5.7	5.0
Quercus spp.	1.7	3.0	3.3	3.5	2.9	2.8	5.5
Planatago spp.	1.7	5.4	4.9	6.3	4.2	2.2	5.5
Betula pendula	1.5	2.6	1.7	3.8	2.3	4.9	4.4
Corylus avellana	1.3	3.4	4.4	3.4	1.8	1.7	3.8
Ligustrum vulgare	1.2	-	-	3.8	1.9	-	5.5
Cupressus sempervivens	1.2	1.9	3.0	2.7	2.3	2.7	3.6
Dactylis glomerata	1.1	5.0	5.4	5.9	2.8	5.0	4.7
Chenopodium album	-	3.4	3.7	3.6	2.7	2.9	5.1
Ligustrum ovalifolium	-	3.1	2.9	-	-	1.3	-
Taraxacum officinale	-	2.3	2.0	4.0	4.2	1.7	4.6
Ambrosia artemisifolia	-	2.2	1.7	2.7	2.7	1.2	3.9
Populus spp.	-	2.0	4.7	1.8	1.8	-	5.5
Calluna vulgaris	-	1.9	1.7	2.0	2.1	2.2	2.8
Helianthus annuus	-	1.8	1.6	3.3.	-	1.3	1.9
Pinus spp.	-	-	2.0	1.2	-	-	3.7
Fraxinus spp.	-	-	1.8	4.4	4.3	-	2.0
Secale cereale	-	-	-	2.9	-	1.3	-
Alnus glutinosa	-	-	-	1.0	-	-	2.3

*Predominant pollen (>45%); •Secondary pollen (16-45%); according to (Louveaux et al., 1978).

In honey samples (chestnut, mixed, and floral), the predominant type of pollen (> 45%) was *C. sativa* pollen. It is also the secondary pollen (16-45%) in all other honeys

(lime, black locust, and meadow), except for honeydew honey, where *Brassica napus* var. *oleifera* pollen is the secondary pollen (Table 3). However, the dominance of *B. napus* var. *oleifeera* pollen has been determined only in one sample of honeydew honey, while in all other honey samples, *C. sativa* pollen had the highest share. As a dominant pollen, *C. sativa* appears in Croatian regions - in the Varaždin area with a 97% share, and Našice with the share of 84.41% (Sabo et al., 2011; Sabo et al., 2013), then in north-western Spain, León and Palencia provinces - in recent research from the same area with the share of 70.4-90.2% (Herrero et al., 2002; Rodríguez-Flores et al., 2016). It also appears as a dominant pollen in the samples of chestnut honey from Italy (75-90%) (Perna et al., 2014). These results are associated geographically with chestnut forests as the most widespread type. However other present types of pollen differ depending on the plant cover. In the group with an important minor pollen (3-15%), in all honey samples (except for chestnut), there is a presence of the pollen from the Lamiaceae family (4.7-11.0%), *Salix* spp. (4.0-5.3%), *Rumex* spp. (3.1-5.2%), *Plantago* spp. (4.2-6.3%), while the pollen grains of *Fagus* spp., *Quercus* spp., *Betula pendula*, *Corylus avellana*, Cupressus sempervirens, and *D. glomerata* are present in different proportions. The pollen of *T. officinale* and *Plantago* spp. are also found.

The presence of 26 plant species in total was detected in the investigated area, with the share of unidentified species ranging from 2.5% in chestnut honey to a maximum of 17.9% in black locust honey. A similar number of plant species (20-28) were detected by Sabo et al. (2011; 2013), while 71 species of pollen grains (Rodríguez-Flores et al., 2016) were noticed in north-western Spain. In the north-western district of Turkey, Adapazari, there were 42 detected species of pollen grains (Erdoğan et al., 2009). *C. sativa* pollen was present with the amount of 0.3% to 57.9% in lime honey, and from 1.7% to 81% in black locust honey, which is similar to the presence of *R. pseudoacacia* pollen in Slovenian black locust honeys (0-87%). In multifloral honeys, the presence of *C. sativa* pollen grains in Slovenian honeys was between 1-94% (Kregar and Rutar, 2011), while in this study it was 0.3-98%.

CONCLUSION

Out of the 88 analysed samples that were classified as unifloral, eight honey samples do not comply with the requirements of the national rulebook on the quality of unifloral honey in terms of melissopalynological analysis (two samples of black locust honey and six samples of lime honey). Uniflority was confirmed in all other samples. In all honey samples (unifloral and multifloral), the pollen that was most represented was the pollen from *C. sativa*, followed by *Tilia* spp., *R. pseudoacacia, Salix* spp., and the pollen from the Lamiaceae family. Slightly lower shares of *Rumex* spp., *Plantago* spp., and *D. glomerata* pollen grains are present.

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***Topic: Functional food and food supplemnents /* Sekcija: Funkcionalna hrana i dodaci prehrani**

PRODUCTION AND USAGE OF HONEY BASED PRODUCTS AND MEDICINAL HERBS IN NORTH-WESTERN BOSNIA AND HERZEGOVINA, WITH THE PROPER PRODUCT LABELLING REVIEW

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ABSTRACT

Honey has many components that have a positive biological effect in small amounts. That puts it in the position of a highly prized grocery. In addition to honey, beekeeping markets offer products with added value, i.e. products containing honey and other bee products, fruits, vegetables, or medicinal, aromatic, and spice herbs. When these products are used in preventing or treating diseases, they are referred to as apitherapeutic. Scientific medicine increasingly accepts them today as an aid in the fight against chronic diseases. The aim of this research paper was to investigate the value-added product range in the Beekeeping Production of the Una Sana Canton and to classify them according to the number and type of the product, geographical distribution, and the application place on the body for medicinal effects. Additionally, by reviewing the statement on the declaration, it was possible to probe the knowledge of beekeepers on the healing properties of the product and to examine their insight regarding the usage of food labelling rules.
The obtained results have shown that 36 investigated beekeepers, aside from the 6 types of unifloral and 4 types of multifloral honey, have over 80 different types of products placed on the market. Among value-added products, honey in combination with other bee products is dominant (48%), and it is followed by 39.5% of products combined with medicinal and spice herbs. 12.5% are products that are combined with food. The most common plants combined in the preparations are houseleek, pine needles, elecampane, ginger, nettle seeds and leafs, spruce, black cumin, linseed, sesame, cinnamon, and sage. Most commonly used food: walnut, figs, almonds, chestnuts, and cranberries. Given the place of application for treatment, most products are used to treat the immune system. The investigated api products have instructions regarding health effects on the label that are confirmed by the literature, but they are not labelled in accordance with food labelling regulations.

Keywords: api products, bee products, honey, value-added products

INTRODUCTION

Apitherapy is the practice of using bee products such as honey, pollen, propolis, royal jelly, and bee poison for disease prevention or for treatment purposes. It is also described as "science (and art) of the use of bee products in maintaining health and helping the individual in recovery from

illness or disability" (Trumbeckaite et al., 2015). Since chronic diseases are one of the biggest public health problems today (King et al., 2015), and medicine often doesn't have a proper response to chronic illnesses, more and more people are opting for Complementary and Alternative Medicine (CAM) in addition to official medicine, including apitherapy. Examining the results of several studies conducted in several countries (USA, Canada, Australia, Austria, Germany, Switzerland, Denmark, Italy, UK, South Korea) which are processing data on CAM use (Frass et al., 2012), it has been determined that between 5% and 75% of the population use some alternative form for the prevention or treatment of diseases depending on geographical areas (mostly in the German speaking countries). The most common forms of CAM are homeopathy and acupuncture. CAM is mostly used to treat back pain, depression, insomnia, and intestinal diseases. Among carcinoma patients in the European continent, the prevalence of CAM is higher than 39.5% (Molassiotis et al., 2007), when taking into consideration that herb treatments and homeopathy are the most common forms. Research also shows similar data for cardiovascular disease (CVD). The healing properties of honey and bee products are known to mankind since the beginning of history, but due to the increasing scientific evidence of their nutritional and biological values, these products are becoming more and more appealing on the world market. It well known that honey has antimicrobial, antioxidant, antiviral, antiparasitic, anti-inflammatory, antimutagenic, antitumor, and immunosuppressive effects. According to the research, a daily consumption of 50 to 80 g of honey is required in order to have a healthy effect (Bogdanov et al., 2008). If the honey is combined with other bee products, food, or medical herbs, it becomes a value-added product that has even more beneficial effects on the body, especially if it is a product combined with herbs (Muharemagić et al., 2016). An overview of literature and medical effects of pine needle honey, honey with nettle seeds or leafs, and black cumin honey are shown below, as these are the three most common combinations of honey with medicinal herbs in the investigated area.

Pine needle honey

It is believed that various parts of the pine tree, including needles, bark, cloves, and pollen, have a healing effect and prevention effects for some chronic diseases associated with aging of the body (Kwak et al., 2006). There is an increasing amount of evidence showing that pine needles have antioxidative, antimutagenic, and antiproliferative effects on cancer cells (Lee et al., 2007). Pine needle methanol extracts and essential oil show potential as chemoprevention or chemotherapeutic agents for breast cancer that does not respond to endocrine treatment (Hoai et al., 2015). Antimicrobial (Rauha et al., 2000) and antibacterial activity of pine needles has been identified and pine needles are also considered to be natural antiseptics (Feng et al., 2010). In the older literature, it is stated that honey with the addition pine needles has extreme antibacterial and antimycotic properties, strengthens the immune system, stimulates circulation, and improves the vision and function of the digestive and respiratory tract. It is used for bronchitis treatment, which is stated in the declaration of this product, and it has been confirmed to have health effects on rhinitis, sinusitis, tonsils, throat, and it relieves cough ailments. It helps in the treatment of fungal and atrophic wounds, and gynaecological diseases such as trichomonas colitis (Mladenov and Radosavović, 1999).

Honey with nettle seeds or leaves

Nettle (*Urtica dioica*) is a type of plant rich in biologically active substances that have antioxidant properties (Otles and Yalcin, 2012; Shailajan et al., 2014). This plant has been used for centuries in traditional medicine to treat a wide range of diseases or disorders, such as arthritis, rheumatism, and eczema (Upton, 2013). The product declaration states that this product effects arthritis and rheumatism of the respiratory organs, stimulates circulation and purifies the blood, stomach, liver, and intestines. It also claims treatment of urinary tract infections, gastric ulcer, duodenal ulcer, and gout. Indicated medicinal instructions on the product have references in the literature (El Haouari et al., 2006; Daher et al., 2006). It is also stated that it treats anaemia and strengthens immunity. In addition to the healing effect on the circulatory system, nettle extract has many benefits for skin health and it is widely used in cosmetic products, particularly anti-aging products. These properties are based on the antioxidant activity of nettle (Bourgeoisa et al., 2016). Also, the aqueous extract inhibits the deaminase of adenosine in prostate tissue (Durak et al., 2004) and generally has antioxidative and antimicrobial properties. Nettle is considered to be good for the treatment of ulcers and has pain alleviating properties (Gülcin et al., 2004). The seed was identified as a tool in treating kidney and genitourinary diseases (Treasure, 2003). The effect for lowering blood sugar levels (Farzami et al., 2003) and allergic rhinitis (Sayin et al., 2013) has also been confirmed. It has been shown to reduce inflammation, it assists elimination functions, arthritis, and possesses antirheumatic properties (Riehemann et al., 1999; Treasure, 2003).

Black cumin honey

Black cumin (*Nigella sativa*) is a spice that grows in the Mediterranean region and in the countries of Western Asia. The declaration on this product indicates that black cumin is a natural antibiotic, with no adverse consequences for the human body. It helps with colds, infections, and strengthens the immune system. These health effects are confirmed by numerous literature sources (Ramadan, 2007; Khan et al., 2011; Padhye et al., 2008; Bhatti et al., 2013; Abdel Azeiz et al., 2013). It is also stated that it can maintain healthy gastric and intestinal microflora and simulate the activity of the kidneys and liver. Research by El Gazzara et al. (2006), Halawani et al. (2009), Al Ameen et al. (2011), Bhatti et al. (2013), Abdel Azeiz et al. (2013) supports the mentioned statement. The health effect in the treatment of asthma and pollen allergies - since it has antihistamine properties - is confirmed in the literature (Zaoui et al., 2000; Amin and Hosseinzadeh, 2015). Anti-tumour properties (i.e. healthy cells protection, stimulation in the creation of a natural interferon body that inhibits tumour cell proliferation, as well as the effect on the prevention of all cancers through their experiments) have been confirmed by Khan et al. (2003), El Gazzara et al. (2006), Ramadan (2007), Halawani et al. (2009), and Abdel Azeiz et al. (2013). In addition to already mentioned health effects of black cumin, the literature also confirms cumin's beneficial properties against suppression; for muscle relaxation; as a diuretic (Padhye et al., 2008); for the treatment of dermatitis (Zedlitz et al., 2002); for the treatment of hypertension, diabetes, fever, eczema, digestive problems (Amin and Hosseinzadeh, 2015); against bad digestion (especially stomach acid); increases the strength and improves the blood image (Bhatti et al., 2013); helps in the regulation of blood pressure and lipid levels, hyperlipidemia, and cholesterol HDL and LDL (Bhatti et al., 2013; Sarina et al., 2013; Sarina et al., 2014).

Other plants that are combined with honey are mostly houseleek, coltsfoot, and ginger. Houseleek (*Sempervivum tectorum*), in combination with honey or as syrup, is stated in the declaration as a detoxifying agent that accelerates metabolism and stimulates vomiting. It is also stated that it can be used for strengthening the immune system, fighting constipation, treating myomas and cysts in the uterus, and thyroid problems. The literature has confirmed the use of a houseleek extract in the treatment of inflammatory infections, insect bites, burns and ulcers (Abram and Donko, 1999), and skin diseases (González-Tejero et al., 2008). It has an important role in regulating lipid metabolism, decreasing triglyceride levels, and increasing HDL cholesterol levels (Blázovics et al., 2000). It contains quercetin and kaempferol glycosides – it is believed that they have antioxidant properties (Alberti et al., 2008). The water extract also has good antioxidant properties. It is a protection against free radicals produced by aluminium exposure (Florin et al., 2014), but it is also enhancing the excretion of toxic elements of Ba, Ni, and Ti from the liver (Szentmihályi et al., 2004). No scientific findings for the treatment of thyroid or mycotic and cystic problems has been found and there is a lack of information about the combination of houseleek and honey. Coltsfoot (*Tussilago farfara*) is recommended as syrup in the Declaration for the Treatment of Cancer. In the traditional medicine context - it is used to treat asthma in Kashmir (Dogra et al., 2015), as well as for the treatment of cough and asthma in Pakistan (Shah et al., 2015). In some studies, it has been confirmed that the leaf extract is used to treat lung cancer (Liang et al., 2011; Yin et al., 2015). Ginger (*Zingiber officinale*), in combination with honey, is labelled as anticancerogenic (Hakim et al., 2014). Also, it is efficient in treating high cholesterol, improves immunity, alleviates swelling, prevents chronic inflammation, and helps in losing weight. Ginger has a scientific confirmation as a product that stimulates the production of interferon and has direct anti-inflammatory properties. It helps with the common cold and expectoration. It is excellent for improving circulation, helping women in menopause, etc. The Chinese have been using it for more than 2500 years as a tool against the common cold, fever, headache, and muscle ache (Kundu et al., 2009).

The production of honey and bee products is a tradition in the north-western part of Bosnia and Herzegovina. Like in other beekeeping areas, there are more and more products that combine honey and bee products with food or herbs and they are becoming products with a “healing” tag. There is no scientific data on these products, so the aim of this paper was to explore and classify them in terms of multiple parameters, especially regarding labelling.

MATERIALS AND METHODS

The area covered by this study is the Una Sana Canton (USC), which is geographically located in the distant north-western part of Bosnia and Herzegovina and it is traditionally a beekeeping area. Analysis was conducted in 2016, in six administrative-defined municipalities/cities, with 36 beekeepers and with the help of a survey questionnaire prepared for this research. Survey questions, in addition to general data, included an overview of all the products that beekeepers produce and a review of the product declaration. The products are classified according to the type (products combined with herbs and products combined with food), number, frequency of usage of herbs, geographical distribution, and the target site for medicinal effects (organ or system of organs). The comparison with literature sources was carried out by reviewing the label of the product's healing effect on the declaration, during which it was established whether product labelling is compatible with the regulations.

The highest number of surveyed beekeeping families is from Bihac (47.2%), followed by Cazin (16.7%), Bužim (13.9%), Ključ (11.1%), and 5.5% from Sanski Most and Bosanska Krupa. The total number of mentioned families have 151 members (average of 4.15 members per family). Most of them have secondary education (51.9%), and an interesting fact is that 44.4% of them have a high level of education. Among children, 44.4% are high school or college students. Only 27.1% are employed, meaning that the production is an important source of income. The average monthly income among families is higher than 800 € in 44.5%, 25.9% has between 400 and 800€, and 29.6% has an income below 400 €, which means that all families can be considered as a social category with a low-income per person (below 192 €).

RESULTS AND DISCUSSION

One hive of honey produces 14.9 kg of honey on average (Table 1), which is within the quantities that, for example, are produced by beekeepers in Croatia (NPP RH, 2016), in the continental region (11.4 - 21.8 kg / honey for the period 2013 - 2015), but significantly below the highest possible result. Most of the beekeepers (70.4%) produce acacia honey, 59.3% of them produce chestnut honey, which is specific for this area, and 26.0% of them produce linden honey. Considering multifloral honeys, the most common is the production of meadow honey (85.2%). On average, three types of honey are produced per family - stationary beekeeping is more frequent (63%).

In addition to eight types of honey, the production of 81 different api products in total has been identified. 39 of them are standard bee products (pollen, propolis, royal jelly, wax) and their combinations. The other 42 products (Table 2) are combinations of honey with food (19%) or with herbs (81%). Sales most commonly take place at the producers' houses (91.5%) and at fairs (28.2%). Product control is regularly done by 19 (52.8%) out of the 36 studied beekeepers. Others conduct product controls occasionally or never.

Four types of products have been identified: honey with food, honey with herbs, syrups, and one juice (fresh juice made from milfoil and nettle with pollen and propolis in the honey). Nut, fig, almond, chestnut, and cranberry were used as food. Combinations with herbs (34 products), including 20 species of plants, have also been identified. Pine needles, nettle leaves, and black cumin are used most frequently.

Table 1. General data about beekeeping

Description of beekeeping		The share of honey types in beekeeping practices	
Number of hives/family (average)	73.5	**Unifloral**	**(%)**
Yield per hive (kg)	14.9	*Chestnut*	59.3
Type of beekeeping	**(%)**	*Acacia*	70.4
Stationary beekeeping	63.0	*Linden*	26.0
Mobile beekeeping	18.5	*Sage*	7.4
Mixed	18.5	***Multifloral***	**(%)**
Agricultural subsidies	**(%)**	*Meadow*	85.2
Yes	74.0	*Mixed*	7.4
No	26.0	*Floral*	3.7
The average subsidies (€)	300	*Forest*	59.3

Table 2. Types and quantities of value added api products

Total number of products	**81**	**Value-added products**	**42**
Areas	(%)	*With food (%)*	19
Bihać	57.1	*With plants (%)*	81
Cazin	14.3	**Type of selling**	**39**
Bužim	9.5	*House sale*	91.5
Sanski Most	18.0	*Trade shows*	28.2
Bosanska Krupa	7.1	Markets	14.1
Quality control	(%)	Pharmacy	14.1
Yes	52.8	Wholesale	8.5
No	47.2	EU export	2.8

Aside from those, houseleeks, elecampane, ginger, aloe vera, nettle seeds, spruce, flax seeds, plantain, sesame, cinnamon, cranberries, sage, thyme, coltsfoot, chestnut, chia seeds, and milfoil were also commonly used. Given the principles of healing indicated on the declaration (Figure 1), the majority of the products are intended for the preservation or treatment of the immune system (14), followed by the circulatory (12), respiratory, and digestive system (10); five products are labelled as having detoxification and anticancer properties. When it comes to other systems, urinary and bone systems are mentioned often, and one product is claimed to have a healing effect on the endocrine system, anti-allergic properties, weight reduction effect, and that it is beneficial for pregnancy. Three products do not have instructions on the declarations, but instead, the beekeepers transmit them orally. It was noted on all the products that instructions refer to multiple health problems, i.e. organ or organ systems. None of the products were labelled in accordance with the Ordinance on General Declaration or Labelling of Packaged Food (Official Gazette of B&H, 87/08) and the Ordinance on Labelling Nutrient Value of Packed Food (Official Gazette of B&H, 85/08).
Analysis of the beekeepers' knowledge on the healing properties of their products was carried out by reviewing the scientific data, i.e. seeking the confirmation of the medicinal effect of the plant that was added to the honey in literature.

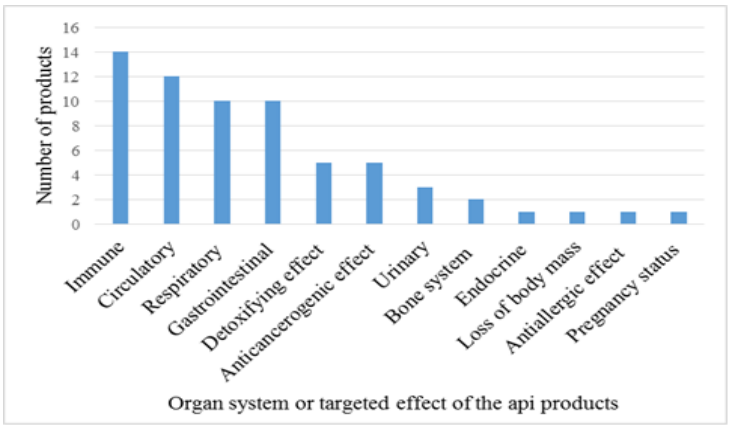

Fig. 1. The frequency of detected api products regarding the target organ system or health effect

Table 3. Declaration of api products in terms of medicinal instructions, compared to literature

Products	Health effects marked on the product	Scientific proof
Honey with pine needles	Lumber problems, colds, bronchitis, bronchial asthma, for coughing. It strengthens immunity.	Mladenov and Radosavović, 1999; Rauha et al., 2000; Kwak et al., 2006; Lee et al., 2007; Feng et al., 2010; Hoai et al., 2015.
Honey with nettles	It cures anaemia, strengthens the immune and respiratory systems, stimulates circulation, cleanses blood, stomach, liver and intestines. It helps with urinary tract infections, gastric ulcer, ulcer on the twin-bowel gut, gout, and rheumatic diseases.	Riehemann et al., 1999; Treasure, 2003; Farzami et al., 2003; Durak et al., 2004; Gülçin et al., 2004; El Haouari et al., 2006; Daher et al., 2006; Otles and Yalcin, 2012; Upton, 2013; Sayin et al., 2013; Shailajan et al., 2014; Bourgeoisa et al., 2016.
Honey with black cumin	A natural antibiotic with no harmful effects for the human body. It helps with colds, infections and strengthens the immune system. Combined with honey, it maintains a healthy gastric and intestinal microflora, and simulates kidney and liver function. It treats asthma and pollen allergies. It has anti-tumour properties.	Zaoui et al., 2000; Zedlitz et al., 2002; Khan et al., 2003; El Gazzara et al., 2006; Ramadan, 2007; Padhye et al., 2008; Halawani et al., 2009; Khan et al., 2011; Al Ameen et al., 2011; Bhatti et al., 2013; Abdel Azeiz et al., 2013; Sarina et al., 2013; Sarina et al., 2014; Amin and Hosseinzadeh, 2015.

Table 3 shows the three most common combinations of honey with medicinal herbs, instructions about healing properties on the producers' declaration, and literature sources that confirm these instructions. In all three cases where honey is combined with herbs, claims about health effects on the product declaration have been confirmed by literature, which could be expanded with previously mentioned proven health effects. Honey with nettle seeds or leaves, chestnut honey, and meadow honey are preferred in products in general.

CONCLUSION

By reviewing the labels of the api products produced in the Una Sana Canton and the instructions for treatments made by the producers, it has been established that in most cases - facts do support the inscriptions on the products. This indicates the existence of solid education among the producers. However, there is a lot of space for improvement in the context of their education, since their findings are based on the traditional preparation and usage of api products, as well as on the information coming from a not so wide range of literature. The range of products and quantities they are offering are quite humble, and the consumption of api culture products with healing effects is below the European average - even in beekeeping families. The question is: what is the actual api culture products consumption for an average citizen? This can be an interesting topic for further research.

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Topic: Food safety

Sekcija: Zdravstvena sigurnost hrane

HORMONI U HRANI ŽIVOTINJSKOG PODRIJETLA - PRIRODNA POJAVNOST ILI ZLOUPORABA?

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SAŽETAK

Hormoni su organsko-kemijske tvari čijom se uporabom u stočarskoj proizvodnji mogu postići značajniji prinosi, a time i veći profit. U farmskih životinja hormoni imaju anabolički učinak, odnosno djeluju na način da povećavaju sintezu proteina i razgradnju masti čime se ujedno dobivaju i senzorski prihvatljiviji proizvodi. Međutim, primjenom na životinjama ove tvari se *carry over* efektom prenose u jestiva tkiva i tjelesne tekućine te, u konačnici, i u gotove proizvode (mlijeko, iznutrice, meso, jaja), a termičkom obradom uglavnom ih nije moguće inaktivirati ili ukloniti. Na taj način potrošači, konzumacijom namirnica životinjskog podrijetla, mogu biti izloženi hormonskom djelovanju. S obzirom da su dokazane brojne intoksikacije i hormonski učinci u ljudi, njihova primjena kao promotora rasta u životinja je u Europskoj uniji zabranjena. Zlouporaba ovih tvari predstavlja potencijalni rizik za potrošače, a kako bi se spriječio mogući štetan utjecaj na zdravlje, važno je sustavno pridržavanje zakonskih odrednica definiranih za njihovu primjenu i kontrolu. Budući da su spolni hormoni prisutni u tkivima i tjelesnim tekućinama farmskih životinja u fiziološkim razinama, a pojavnost može biti i posljedica liječenja odnosno terapeutske primjene kako bi se dokazala njihova ilegalna uporaba, od velikog je značaja utemeljena interpretacija utvrđenih razina. U posljednjem desetljeću se u ovom području provode brojna istraživanja budući da za veliki broj tvari s hormonskim djelovanjem još uvijek nisu istraženi toksični učinci. Stalan je i razvoj novih sintetskih hormona i njihovih mješavina, stoga je u cilju proizvodnje zdravstveno ispravne hrane i zaštite zdravlja potrošača potreban kontinuirani nadzor pojavnosti hormona u svim kritičnim točkama proizvodnje hrane od farme do potrošača, kao i razvoj novih suvremenih analitičkih metoda u identifikaciji njihove zlouporabe.

Ključne riječi: hormoni, anabolički učinak, ostaci u hrani životinjskog podrijetla, intoksikacija, sigurnost hrane

UVOD

U medijima se posljednjih desetljeća sve češće spominju slučajevi pojavnosti hormona u hrani životinjskog podrijetla. Budući da je svakim danom svijest potrošača sve veća, razumljiva je njihova zabrinutost o mogućem unosu ovih tvari putem hrane i posljedičnom štetnom utjecaju na zdravlje. Hormoni su organsko-kemijske tvari koje po sastavu mogu biti steroidi, prostaglandini, amini, peptidi i proteini. Budući da u organizmu cirkuliraju krvlju, dolaze u doticaj s gotovo svim stanicama te su zaslužni za regulaciju brojnih

fizioloških procesa u organizmu, poput metabolizma, rasta i razvoja, djeluju i na raspoloženje, a ujedno i na stres.
Hormoni u farmskih životinja direktnim ili indirektnim mehanizmom djeluju anabolički, što rezultira pojačanim zadržavanjem dušika i sintezom proteina te razgradnjom masnog tkiva (Lone, 1997). Upravo zbog takvog načina djelovanja još 50-ih godina u stočarskoj se proizvodnji započelo s uporabom ovih tvari s ciljem dobivanja senzorski prihvatljivijih svojstava proizvoda, a ujedno i povećanih proizvodnih prinosa i ostvarenja većeg profita (Armstrong i sur., 2004; Pleadin i sur., 2011; Pleadin i Bogdanović, 2016). Efikasnost unaprjeđenja rasta životinja ovisi o vrsti, odnosno pasmini životinje, dobi, reproduktivnom statusu i načinu davanja hormona, a smatra se da se njihovom uporabom može unaprijediti rast životinja i za više od 20% (Meyer, 2001). Osim estrogenih, androgenih i progestagenskih spojeva, poznati su i stilbenski, tireostatički, kortikosteroidni i beta-adrenergički spojevi koje se koristilo pojedinačno ili u učinkovitim kombinacijama (Courtheyn i sur., 2002; Reig i Toldrá, 2009; Stephany, 2010).
U nekim zemljama svijeta dozvoljena je primjena pojedinih hormona kao promotora rasta u farmskih životinja, dok je njihova uporaba u iste svrhe zabranjena u zemljama Europske unije (EU). Pronalazak ovih tvari u hrani životinjskog podrijetla na području EU razlogom je brojnih međunarodnih prijepora i trgovinskih sporova vezanih za zdravstvenu ispravnost hrane koje potječe od životinja tretiranih ovim tvarima. Također, podatci dobiveni u okviru zakonom propisanih državnih programa nadzora u zemljama EU pokazuju da učestalost nezakonite primjene tvari s hormonskim učinkom iznosi 5 - 15% (Stephany, 2010).
Primjenom na životinjama ove tvari se prenose u jestiva tkiva i tjelesne tekućine te u konačnici i u gotove proizvode (Hartmann i sur., 1998). Budući da se unosom u organizam putem hrane ostvaruje isti hormonski učinak kao da je riječ o endogeno prisutnim hormonima, potrošači konzumacijom namirnica životinjskog podrijetla mogu biti izloženi dodatnom hormonskom djelovanju što može utjecati na brojne fiziološke procese u organizmu te u konačnici utjecati i na njihovo zdravlje.
Ovaj rad daje pregled spoznaja o primjeni prirodnih i sintetskih tvari s hormonskim djelovanjem na farmskim životinjama, pojavnosti ostataka ovih tvari u hrani životinjskog podrijetla te o zakonodavstvu i mjerama koje se poduzimaju u kontroli, odnosno suzbijanju zlouporabe s ciljem osiguranja zdravstvene ispravnosti hrane i sigurnosti potrošača.

Primjena hormona u farmskih životinja

U svrhu postizanja povećanog prirasta i iskoristivosti hrane u farmskih životinja, osim prirodnih spolnih hormona (17β-estradiol, progesteron i testosteron), tri sintetske kemijske tvari s estrogenim (zeranol), gestagenim (melengestrol acetat) i androgenim (trenbolon acetat) djelovanjem, u prošlosti su se često koristile u poticanju rasta stoke aplikacijom putem ušnih implantata te vode i stočne hrane (Galbraith, 2002; Jeong i sur., 2010). Međutim, u EU je u stočarstvu uporaba bilo kojeg aktivnog promotora rasta iz skupine hormona, osim primjene prirodnih hormona u terapeutske svrhe, u potpunosti zabranjena. Budući da se prirodni hormoni nalaze fiziološki prisutni u organizmu te s obzirom da njihova količina varira ovisno o brojnim čimbenicima, prisutnost u tkivima i tekućinama ne upućuje automatski na nelegalnu uporabu u farmskih životinja (Pleadin i sur., 2011; Pleadin i sur., 2013).

Unatoč zabrani primjene ovih tvari u anaboličke svrhe u EU, poznato je da se danas u svijetu visok postotak farmskih životinja uzgaja uz uporabu hormona (Stephany, 2010). Za razliku od europskih zemalja, kontrolirana uporaba određenih hormona kao anabolika je ozakonjena, primjerice, u SAD-u, Kanadi, Australiji, Novom Zelandu te nekim državama Južne Amerike, Azije i Afrike. 17ß-estradiol, testosteron, progesteron, trenbolon i zeranol se primjenjuju u obliku malih kompaktnih ušnih implantata. Nadalje, dozvoljeno je koristiti melengestrol acetat, a u svinja raktopamin, u obliku dodataka krmivu za hranidbu tovnih junica. Trenutno su sve tvari iz skupine beta-agonista zabranjene za korištenje u svrhu poticanja rasta, a jedine iznimke čini raktopamin čija je uporaba zakonita, npr. u SAD-u, te zilpaterol čija je uporaba zakonita, primjerice, u Južnoj Africi. Istraživanja anaboličkog učinka i nadzor zlouporabe spolnih hormona, pokazuju da je od svih hormona najznačajnija primjena 17β-estradiola i to zbog utvrđene najizraženije anaboličke aktivnosti ovog hormona (Meyer, 2001; Pleadin i sur., 2013). Popis najznačajnijih predstavnika hormona po skupinama tvari koje imaju anabolički učinak u organizmu prikazan je u tablici 1.

Tablica 1. Popis tvari s hormonskim učinkom u organizmu (Direktiva Vijeća 96/23/EZ)

Skupina tvari	**Najznačajniji predstavnici**
Stilbeni, derivati stilbena i njihove soli i esteri	Dietilstilbestrol, dienestrol, heksestrol
Antitiroidni agensi	Tiouracil, metiltiouracil, propiltiouracil, tapazol
Steroidi	17β-Estradiol*, progesteron*, testosteron*, trenbolon, 19-nortestosteron, boldenon, metiltestosteron, stanozolol
Laktoni rezorcilne kiseline, uključujući zeranol	Zeranol, zearalanon
Beta-agonisti	Klenbuterol, brombuterol, mabuterol, cimaterol, izoksuprin, raktopamin, salbutamol, zilpaterol

*predstavljaju prirodne hormone; ostale navedene tvari s hormonskim djelovanjem su sintetskog podrijetla

Ostaci hormona u hrani

Slučajevi pojavnosti hormona u hrani životinjskog podrijetla započeli su tijekom 1980. – 1981. godine kada su u različitim europskim državama u dječjoj hrani nađeni ostaci izrazito toksičnog sintetskog hormona dietilstilbestrola (DES-a) (Payne i sur., 1999), a pronalazak ovog humanog karcinogena u dječjoj hrani izazvao je niz političkih, trgovačkih i komercijalnih problema. Podatci o količinama hormona u hrani plasiranoj na tržište općenito su vrlo rijetki i u većini slučajeva su dobiveni kao rezultat pojedinačnih nasumičnih akcijskih mjera koje su provela inspekcijska tijela ili potrošačke organizacije (tablica 2).
Budući da su spolni hormoni prisutni u tkivima i tjelesnim tekućinama farmskih životinja u fiziološkim razinama, a njihova pojavnost u organizmu može biti i posljedica liječenja odnosno terapeutske primjene, razumljivo je da su ove tvari u hrani životinjskog podrijetla prisutne u fiziološkim, ali i nešto većim razinama ukoliko su primijenjene u svrhu liječenja i ako nije došlo do njihovog potpunog izlučivanja iz organizma.

Tablica 2. Slučajevi nelegalne primjene tvari s hormonskim učinkom u organizmu dokazani na uzorcima iz mesnica i supermarketa na području Europske unije (Stephany, 2010)

Godina uzorkovanja	Zemlja	Vrsta uzorka	Broj uzoraka	Nesukladni nalazi (%)	Pronađeni hormoni
1989-1993	Nizozemska	mljeveno meso	249	3,2	medroksiprogesteron, nortestosteron
1991-1992	Belgija	mljeveno meso	51	9,8	klortestosteron, nortestosteron
1993-1999	Nizozemska	mast	430	0,5	klortestosteron, medroksiprogesteron
1994	EU	meso	1183	1,6	17 vrsta androgena i 2 gestagena
1994	EU	jetra	936	10	klenbuterol

Međutim, ukoliko se hormoni apliciraju na farmskim životinjama u dozama, npr. 10-15 puta većim od terapeutskih, kod životinja se ostvaruje anabolički učinak, a ove tvari se prenose u jestiva tkiva i tjelesne tekućine te bivaju prisutne u većim količinama u hrani (Hartmann i sur., 1998; Anderson i sur., 2005; Andrée i sur., 2010; Pleadin i sur., 2010). Osim mesa, iznutrica i mlijeka, utvrđeno je da i kokošja jaja predstavljaju glavni izvor 17β-estradiola, a kao namirnica se također koriste u svakodnevnoj prehrani potrošača (Stephany, 2010).
Time potrošači konzumacijom namirnica životinjskog podrijetla mogu biti izloženi pojačanom hormonskom djelovanju. Ujedno, istraživanja pokazuju da ostatke hormona u hrani uglavnom nije moguće inaktivirati ili ukloniti postupcima termičke obrade (Rose i sur., 1995). U razmatranju izloženosti potrošača ne treba zanemariti ni unos hormona u organizam putem hrane biljnog podrijetla te moguće sinergističke učinke kao posljedice unosa hormona u organizam iz više izvora. Usporedni prikaz unosa prirodnih hormona u organizam putem hrane te njihovog relativnog doprinosa kod potrošača prikazan je u tablici 3.

Toksični učinci u organizmu

Prisutnost ostataka hormona u hrani uzrok je brojnih slučajeva alimentarnih intoksikacija i hormonskih učinaka u ljudi kao posljedice konzumacije kontaminirane hrane (Martinez-Navarro, 1990). Istraživanja učinka tvari s hormonskim djelovanjem na različite životinjske vrste pokazuju različitu osjetljivost, a stupanj toksičnosti ovisi o genetskim (vrsta i pasmina) te fiziološkim čimbenicima (dob, spol, prehrana, opće stanje organizma). Pojedinim tvarima s hormonskim učinkom u organizmu pripisuju se citotoksični, hepatotoksični, genotoksični, imunosupresivni, mutageni, teratogeni i karcinogeni učinci (FAO/WHO, 2000).
Istraživanja su pokazala da, npr. klenbuterol kao često zlouporabljena sintetska tvar iz skupine beta-agonista u ljudi, uzrokuje ubrzan rad srca, drhtanje, nervozu, opću slabost, vrtoglavicu i glavobolju (Ramos i sur., 2003; Woodward, 2005; Pleadin i sur., 2012). Najveći broj istraživanja toksičnosti prirodnih steroidnih hormona odnosi se na

17ß-estradiol i povezuje s njegovim hormonskim djelovanjem, vjerojatno stoga jer je od svih prirodnih spolnih hormona bio najviše primjenjivan i zlouporabljen.

Tablica 3. Usporedni prikaz dnevnog unosa prirodnih hormona u organizam i njihovog relativnog doprinosa putem različitih vrsta hrane (Adolf i sur., 1994)

	Meso i riba	**Mliječni proizvodi**	**Jaja**	**Hrana biljnog podrijetla**
17β-Estradiol i estron				
Muškarci (µg/dan)	0,02	0,06	0,02	0,00
Žene (µg/dan)	0,01	0,05	0,02	0,00
Dječaci (µg/dan)	0,01	0,06	0,01	0,00
Djevojčice (µg/dan)	0,01	0,05	0,01	0,00
Relativni doprinos (cca, %)	15-20	60-70	15-20	< 10
Progesteron				
Muškarci (µg/dan)	0,63	8,18	0,92	0,86
Žene (µg/dan)	0,45	7,06	0,76	0,71
Dječaci (µg/dan)	0,37	7,27	0,62	0,61
Djevojčice (µg/dan)	0,33	6,57	0,60	0,57
Relativni doprinos (cca, %)	5	80	10	10
Testosteron				
Muškarci (µg/dan)	0,02	0,02	0,01	0,02
Žene (µg/dan)	0,01	0,02	0,01	0,02
Dječaci (µg/dan)	0,01	0,02	0,01	0,01
Djevojčice (µg/dan)	0,01	0,02	0,01	0,02
Relativni doprinos (cca, %)	20-30	30-40	15-20	20-40

Mutageni, kancerogeni i teratogeni učinci ovog hormona dokazani su u brojnih životinjskih vrsta. Oralna, a osobito parenteralna primjena 17ß-estradiola, ovisno o dozi i trajanju izloženosti, može prouzročiti povećanu pojavnost tumora kod tretiranih životinja i to u tkivima s visokom koncentracijom specifičnih hormonskih receptora (uterus, vagina, cerviks, dojka), uključujući tumore hipofize, kostiju i jetre (Zimmerman, 1998). Rast mliječnih žlijezda, orožnjavanje vaginalnog epitela i slični toksični učinci jasni su pokazatelji morfoloških promjena. Biokemijske promjene uzrokovane 17ß-estradiolom (promjene u genskoj ekspresiji, prijenos signala i regulacija staničnog ciklusa) mnogo su suptilnije i samim time manje primjetljive od morfoloških promjena, iako su zasigurno jednako važne. 17ß-estradiol, kao i ostali steroidni hormoni, u mliječnim žlijezdama prolaze barijeru krv-mlijeko, a podatci pokazuju da je dnevni unos ovog hormona, naročito konzumacijom mlijeka i mliječnih proizvoda, vrlo značajan (Hartmann i sur., 1998).
Koncentracija estrogena u mlijeku povezana je s količinom mliječne masti i fazi gravidnosti krave te je u drugoj polovici graviditeta veća nego u fazi ranog graviditeta.
Novija istraživanja pokazuju trend ranijeg spolnog sazrijevanja djevojčica (Andersson i Skakkebæk, 1999) te pojavu menarhe s povećanjem indeksa tjelesne mase (IBM) uslijed izloženosti egzogenim estrogenima (Maruyama i sur., 2010). Pojedina istraživanja pokazuju da povećane razine estrogena i njegovih metabolita mogu prouzročiti karcinogene učinke reproduktivnog sustava (FAO/WHO, 2000). Ispitivanjem

prehrambenih navika i drugih rizičnih faktora za razvoj karcinoma kod žena u menopauzi utvrđeno je da se rizik pojavnosti karcinoma jajnika može dovesti u vezu s konzumacijom mliječnih proizvoda (Asif, 2013). Istraživanja ujedno pokazuju da je u većini slučajeva meso liječenih životinja, kada su ove tvari pravilno korištene, beznačajan izvor hormona u usporedbi s fiziološkom endogenom produkcijom u čovjeka. Međutim, istraživanja razina spolnih hormona u tkivima su još uvijek nepotpuna i nedostatna za mnoge životinjske vrste i pasmine te su ovisna o brojnim čimbenicima, stoga su potrebna daljnja istraživanja toksičnih učinaka ovih tvari i njihovih metabolita, budući da i metaboliti u organizmu imaju biološku aktivnost (Andersson i Skakkebæk, 1999).

Nadzor zlouporabe hormona

Potaknuta nizom skandala vezanih uz pronalazak ostataka hormona u goveda i svinja, Europska komisija je razvila cjelovitu strategiju utvrđivanja prisutnosti ostataka (rezidua) ovih tvari u hrani (Heitzman, 1994). Direktiva Vijeća 86/469/EEZ koja je stupila na snagu 1986. godine propisala je način pregledavanja životinja i svježega mesa u svrhu utvrđivanja prisutnosti ostataka hormona, a u cilju osiguravanja istovjetne primjene propisanih mjera kojima se sprječava da u hrani bude neželjenih ostataka. Zakonske regulative koje su trenutno važeće u ovom području su: Direktiva Vijeća 96/22/EZ kojom se zabranjuje primjena hormona na farmskim životinjama u anaboličke svrhe u Europskoj uniji, Direktiva Vijeća 96/23/EZ kojom se propisuje obavezno praćenje ostataka ovih tvari putem godišnjih planova monitoringa i to tijekom tova životinja i na klaonici te Uredba Komisije (EU) br. 37/2010 kojom se definiraju najveće dopuštene količine (NDK) farmakološki aktivnih tvari u hrani životinjskog podrijetla.

Svrha inspekcijskoga programa u zemljama u kojima je uporaba pojedinih tvari dozvoljena je ispitati sukladnost utvrđene razine hormona s propisanom NDK u hrani. U Europi ove tvari nemaju propisane NDK vrijednosti, a njihova pojavnost u materijalu uzorkovanom od farmskih životinja po pitanju prirodnih hormona smije biti isključivo fiziološke prirode, dok su sintetske tvari potpuno zabranjene. S obzirom da su spolni hormoni prisutni u tkivima i tjelesnim tekućinama farmskih životinja u fiziološkim razinama, a pojavnost može biti i posljedica liječenja odnosno terapeutske primjene, za interpretaciju rezultata nužno je poznavati fiziološke razine, vodeći računa i o karenciji lijeka (vremensko razdoblje izlučivanja iz organizma životinje). Inspekcijski programi baziraju se pritom na uzorkovanju bioloških materijala podobnijih za ispitivanje prisutnosti ovih tvari još na farmama (urin, krv i životinjska dlaka) te na klaonici (jetra i mišićno tkivo). Međutim, prema zapažanjima Nacionalnih referentnih laboratorija koji provode istraživanja u ovom području, broj aktivnih spojeva stalno raste, odnosno kontinuirano se identificiraju nove tvari s hormonskim učinkom u organizmu, bilo kao pojedinačno primijenjene ili u tzv. „pametnim“ kombinacijama (Stephany, 2010).

Analitičke metode kojima se u službenim laboratorijima u različitim materijalima ispituje prisutnost tvari s hormonskim učinkom mogu se podijeliti na *screening* i potvrdne metode. Od *screening* metoda najviše se koristi imunoenzimska ELISA metoda budući da omogućuje brzi *screening* ili kvantifikaciju vrlo niskih koncentracija hormona. Karakterizira je i lakoća provedbe, točnost i dostupnost reagenasa, a nedostatak ove metode je moguća *cross* reaktivnost pojedinih supstancija, odnosno pojavnost lažno pozitivnih rezultata (Pleadin i Bogdanović, 2016). Također, manjkavost ELISA metode predstavlja i određivanje samo pojedinih tvari, za

razliku od potvrdnih tehnika koje omogućavaju određivanje većeg broja tvari pri jednoj analizi. Budući da je uglavnom riječ o zabranjenim tvarima, te uzimajući u obzir činjenicu da se analitički postupci provode s ciljem nadzora moguće zlouporabe hormona na farmskim životinjama, završna identifikacija, odnosno potvrđivanje ovih tvari, provodi se primjenom najzahtjevnijih analitičkih tehnika (Stolker i Brinkman, 2005). Od potvrdnih metoda najveću primjenu imaju tekućinska (LC) i plinska kromatografija (GC) u kombinaciji s dvostrukom masenom spektrometrijom (MS-MS) koje omogućavaju nedvojbenu identifikaciju putem omjera mase i naboja iona karakterističnih za svaku pojedinu tvar te njihovu preciznu kvantifikaciju, a čime se ujedno i dokazuje fiziološka pojavnost ili zlouporaba hormona na farmskim životinjama.

ZAKLJUČCI

Problem hormona u hrani, zbog mogućih sinergističkih učinaka, potrebno je sagledavati uzimajući u obzir njihov ukupni unos putem hrane, uključujući meso i mesne proizvode, ribu i riblje proizvode, mlijeko i mliječne proizvode, jaja, ali i hranu biljnog podrijetla. Potrebna je provedba daljnjih toksikoloških istraživanja, budući da za veliki broj tvari s hormonskim djelovanjem još uvijek nisu istraženi toksični učinci u organizmu, a stalan je i razvoj novih sintetskih hormona i njihovih mješavina koje sadrže veći broj različitih aktivnih tvari u niskim dozama. Također, potreban je stalni razvoj suvremenih analitičkih metoda u identifikaciji novih spojeva te uzorkovanje i analiza novih pigmentiranih materijala (dlaka, retina oka) koji nakon aplikacije hormona na farmskim životinjama tijekom značajno dužeg vremenskog razdoblja mogu ukazati na njihovu zlouporabu te pomoći u pronalasku ovih tvari na tzv. crnom tržištu. Stoga je u cilju zaštite zdravlja potrošača nužan kontinuirani nadzor pojavnosti hormona u svim kritičnim točkama proizvodnje hrane životinjskog podrijetla od farme do potrošača.

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Topic: Food safety / Sekcija: Zdravstvena sigurnost hrane

HORMONES IN FOOD OF ANIMAL ORIGIN - NATURAL OCCURRENCE OR ABUSE?

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Summary

Hormones are organic-chemical substances whose use in livestock production can achieve higher yields and thus higher profits. In a farm animal, hormones have anabolic effect, that is, they act to increase protein synthesis and fat degradation, which also yields more sensory-responsive products. However, when applied to animals, these substances are transmitted to edible tissues and body fluids, and ultimately to finished products (milk, offal, meat, eggs), and cannot be inactivated or removed by thermal treatment. In this way, consumers by consumption of food of animal origin can be exposed to hormonal action. Given that numerous intoxications and hormonal effects have been demonstrated in humans, their use as an animal growth promoters are prohibited in the European Union. Abuse of these substances poses a potential risk to consumers, and to prevent possible harmful effects on health, it is important to systematically comply with the legal definitions given for their application and control. Because sex hormones are present in the tissues and body fluids of the farm animals at physiological levels, and the occurrence may be the consequence of therapeutic application, to prove their illegal use interpretation of established levels is of great significance. In the last decade, numerous studies have been carried out in this area, since many of the substances with hormonal action have not yet explored for their toxic effects, and the development of new synthetic hormones and their mixtures is ongoing. Therefore, in order to produce health-safe food and consumer health protection, continuous monitoring of the occurrence of hormones in all critical points of food production from farm to consumer is required, as well as the development of new, modern analytical methods in identifying their abuses.

Keywords: hormones, anabolic effect, residues in food of animal origin, intoxication, food safety

FAKTORI RIZIKA U LANCU SNABDIJEVANJA HRANOM

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SAŽETAK

U lancu snabdijevanja hranom niz je faktora koji utječu na zdravstvenu sigurnost hrane nakon što se ona počne distribuirati iz proizvodnih pogona u distributivne centre. U prvom redu to je transport, potom prijem proizvoda, njegovo izlaganje, rukovanje proizvodima, kao i primjena Dobre higijenske (DHP) i Dobre proizvođačke prakse (DPP) u pogonima gdje se taj proizvod proizvodi. Cilj ovog rada bio je na osnovi ispitivanih parametara napraviti „Rejting shemu higijene hrane“ za odabrane trgovačke centre u rasponu od 1 do 5. Najveća ocjena '5' znači da je utvrđeno da objekt ima *'veoma dobre'* higijenske standarde, a niže ocjene znače da procedure samokontrole nisu na zadovoljavajućem nivou. Također, izvršena je i mikrobiološka analiza briseva uzetih s različitih površina. Istraživanje je provedeno u periodu od 30 dana. Rejting shemom utvrđeno je da se u prodajnom objektu III postupci higijene najadekvatnije primjenjuju, i nivo higijene ocijenjen je prosječnom ocjenom 3,35 dok je nivo higijene u trgovačkom centru II ocijenjen prosječnom ocjenom 3, što je najniža prosječna ocjena za sva tri prodajna objekta. Kako nijedan objekt nije dobio najveću ocjenu 5, neophodna su određena poboljšanja. Mikrobiološkom analizom utvrđeno je da je čistoća sva tri objekta zadovoljavajuća jer je broj nezadovoljavajućih briseva bio znatno manji od onog koji je dozvoljen Pravilnikom.

Ključne riječi: trgovački centri, rejting shema higijene hrane, mikrobiološka čistoća

UVOD

U vremenu kada ljudska populacija postaje sve brojnija, a raspoloživost obradivih površina sve manja, proizvodnja i distribucija hrane za ljudsku upotrebu postaje sve teži i zahtjevniji proces. Kako bi „od polja do stola“ stigao proizvod koji je siguran za ljudsku upotrebu, a ujedno ispunio sva očekivanja potrošača u pogledu organoleptičkih svojstava, neophodno je stalno usavršavanje proizvodnje, prerade i transporta hrane.

U lancu snabdijevanja hranom svatko je odgovoran za svoj dio posla, pa tako trgovci moraju utvrditi da je hrana koju su primili od proizvođača ili dobavljača ispravna. Niz faktora utječe na zdravstvenu sigurnost hrane nakon što se ona počne distribuirati iz proizvodnih pogona u distributivne centre. U prvom redu, to su uvjeti samog transporta od mjesta proizvodnje do mjesta prodaje. Nakon što se hrana transportira do prodajnih objekata sljedeći korak je prijem proizvoda u prodajne objekte koji moraju ispunjavati određene uvjete u pogledu infrastrukture.

Različite vrste prehrambenih proizvoda imaju različite zahtjeve u pogledu temperature i načina skladištenja i izlaganja. Da bi se očuvala zdravstvena ispravnost namirnica, kao i njihova prvobitna kvaliteta, potrebno je osigurati odgovarajuće mikroklimatske uvjete. To se posebno odnosi na toplo i hladno izlaganje proizvoda (proizvodi koji zahtijevaju održavanje hladnog lanca te kuhana jela koja se prodaju topla). Hrana koja se kupuje u supermarketima izložena je na osvijetljenim policama, zapakirana u atraktivna pakiranja i na prvi pogled sve izgleda u redu. Mnogi faktori u lancu prehrane mogu utjecati na zdravstvenu ispravnost hrane, a samim tim i na zdravlje potrošača koji tu hranu konzumiraju.

Postoji bezbroj načina kojima se hrana u supermarketima predstavlja kupcu, od svježeg voća i povrća, preko minimalno procesiranih proizvoda, sirovog (svježeg) mesa, pekarskih proizvoda, kuhanih, konzerviranih i drugih proizvoda. Svaki od tih proizvoda zahtijeva posebne uvjete čuvanja (temperaturni režimi), kao i održavanje maksimalnog nivoa higijene prostora i zaposlenika kako bi do kupca došla samo zdravstveno ispravna hrana. Osim temperature čuvanja namirnica, sljedeći bitan faktor je higijena radnika i objekta koji posluje s hranom, kao i zdravstveno stanje radnika. Kada je u pitanju higijena radnika u prodajnim objektima, velika pažnja pridaje se čistoći ruku, upotrebi jednokratnih rukavica, nošenju čiste i prikladne odjeće za određenu namjenu (ovo se posebno odnosi na situacije gdje prodavač ima direktan kontakt s nezapakiranim proizvodima kao što su svježe meso, voće, povrće te kruh, peciva i mesne prerađevine, mliječni proizvodi).

Zdravstvena ispravnost namirnica može biti ugrožena i od kupaca i to najčešće prilikom same kupovine (kada kupac bira „najbolji“ komad voća ili peciva) ili nakon kupovine (prilikom pripreme, kuhanja, zagrijavanja i slično). Dakle, bez obzira što je prodavač ispravno obavio svoj dio posla, zadnji nivo odgovornosti pripada kupcu, pa je dobra praksa u domaćinstvima (Good Housekeeping) neizostavan dio kada se govori o zdravstvenoj sigurnosti hrane. Gotovo neizostavan dio svake kupovine u trgovačkim centrima je upotreba trgovačkih korpi i kolica. Pored naprijed navedenog, vrlo je važna stavka pri odabiru prehrambenog proizvoda i deklaracija. Deklaracija predstavlja svojevrstan način komunikacije između proizvođača i potrošača hrane.

MATERIJAL I METODE

U ovom radu praćeni su parametri koji mogu ugroziti zdravstvenu sigurnost hrane od trenutka kada ona postaje dostupna za kupovinu od strane krajnjeg potrošača. Ispitivanje higijensko – sanitarnih uvjeta izvršeno je u tri trgovačka centra na području grada Sarajeva. Ispitivanje je ukupno trajalo mjesec dana i to od 18. 10. 2016. do 17. 11. 2016. godine.

Mikrobiološka analiza provedena je u akreditiranom laboratoriju za mikrobiologiju Poljoprivredno-prehrambenog fakulteta, Univerziteta u Sarajevu. Kao materijal za provođenje eksperimenta korišteni su brisevi. Ukupno je uzeto 120 briseva iz tri trgovačka centra. Brisevi su uzeti sa sljedećih površina: opreme i pribora za rad (vage, radni stolovi, noževi, vješalice za meso, aparat za rezanje, žlice za kolače), korpi i kolica za kupovinu, radne odjeće zaposlenika, vrećica i folija te rashladnih vitrina.

Istraživanje je napravljeno prema FDA (Food and Drug Administration) i EFSA (European Food Safety Authority) uputama *„Retail Foodborn Illness Risk Factors“ 2016.*

Tablica 1. Kriteriji za ocjenu higijene u prodajnim objektima
Table 1. Criteria for hygiene evaluation in sales premises

Kriterij	**Ocjena**					
Nivo higijene prilikom rukovanja hranom	0	5	10	15	20	25
Uvjeti i struktura prodajnog prostora	0	5	10	15	20	25
Kako se vrši upravljanje i vođenje zapisnika u postupcima postizanja sigurnosti hrane	0	5	10		20	30
Ukupna ocjena	0	→				80
Nivo sukladnosti	Visok	→				Nizak

Ukupan broj mikroorganizama određivao se na uobičajeni način i preračunavao na 1 cm^2 površine *(Pravilnik o mikrobiološkim kriterijima za hranu (Sl. list BiH 11/13).* Interpretacija rezultata vršena je na osnovi graničnih vrijednosti za aerobne mezofilne bakterije i enterobakterije predstavljenih u *Normativima mikrobiološke čistoće za predmete, površine i ruke, koje dolaze u dodir s hranom (Sl. novine FBiH, broj 101/12).*

REZULTATI I RASPRAVA

Prema podacima prikazanim u tablici 2 mikrobiološkom analizom briseva, odnosno određivanja prisustva aerobnih mezofilnih bakterija, utvrđeno je da je razina higijene odgovarajuća za sve površine osim za stroj za mljevenje mesa gdje taj broj prelazi dozvoljene granice. Pri analiziranju mikrobiološke ispravnosti površina uzimanjem briseva, odnosno utvrđivanja prisustva bakterija roda *Enterobacteriaceae*, utvrđeno je da su svi brisevi ispravni, tj. da nema porasta *Enterobacteriaceae* izuzev briseva uzetih s plastičnih pladnjeva u trgovačkom centru I u kojima se izlaže svježe pileće meso. S obzirom na to da je svježe pileće meso idealna podloga za razvoj mikroorganizama, potrebno je redovno prati pladnjeve u kojima se ono izlaže za prodaju, a po mogućnosti koristiti i posude za meso od nehrđajućeg čelika.
Osim analize prisustva ukupnih aerobnih mezofilnih bakterija i enterobakterija, napravljena je analiza na prisustvo bakterije *Escherichia coli*. Analizom mikrobiološke ispravnosti briseva utvrđeno je da ni na jednoj ispitivanoj površini nije dokazano prisustvo *Escherichia coli*.
Nakon obavljene analize higijenske ispravnosti površina u sva tri trgovačka centra utvrđeno je sljedeće:

- u trgovačkom centru I utvrđen je samo jedan neispravan bris od ukupno 120 testiranih, što je 1,66 %,
- u trgovačkim centrima II i III pojavljuje se po jedan neispravan bris što je izraženo u postocima 0,83 %.

Prema *Pravilniku o normativima mikrobiološke čistoće i metodama njenog određivanja* (Narodne novine 46/94), čistoća objekata je prihvatljiva ako je manje od 30 % ispitivanih uzoraka nezadovoljavajuće čistoće. Tijekom provedenog istraživanja u trgovačkim centrima utvrđeno je da je udio briseva koji su ispod zadovoljavajuće razine znatno manji od 30 %. Na osnovi toga može se zaključiti da se mjere higijene uspješno provode

te da su zaposlenici savjesni i dovoljno educirani i o važnosti osobne higijene, kao i o higijeni opreme i prostorija.

Tablica 2. Rezultati analize briseva na aerobne mezofilne bakterije
Table 2. Swab analysis results for aerobic mesophilic bacteria

AEROBNE MEZOFILNE BAKTERIJE						
	Odgovara			**Ne odgovara**		
Predmeti, površine, ruke	I	II	III	I	II	III
Radna odjeća	2	2	2	0	0	0
Ruke zaposlenika	2	2	2	0	0	0
Stroj za mljevenje	1	1	1	1	1	1
Radne površine	2	2	2	0	0	0
Noževi	2	2	2	0	0	0
Sudoper	2	2	2	0	0	0
Stol za rasijecanje	2	2	2	0	0	0
Žlica za kolače	2	2	2	0	0	0
Vrećice	2	2	2	0	0	0
Folije	2	2	2	0	0	0
Stroj za rezanje	2	2	2	0	0	0
Police	2	2	2	0	0	0
Kutije za kolače	2	2	2	0	0	0
Vješalice za meso	2	2	2	0	0	0
Metalni pladnjevi	2	2	2	0	0	0
Plastični pladnjevi	2	2	2	0	0	0
Rashladne vitrine	2	2	2	0	0	0
Površine na zamrzivačima	2	2	2	0	0	0
Korpe	2	2	2	0	0	0
Kolica za kupovinu	2	2	2	0	0	0

Rejting shema

Najniža ocjena dana je za izlaganje i prodaju kruha i ostalih pekarskih proizvoda. Razlog tome je što se drugi proizvodi u kućnim uvjetima ponovo peru ili termički obrađuju dok se proizvodi poput kruha konzumiraju u onakvom stanju u kakvom su i kupljeni, bez prethodne obrade u domaćinstvima. Kao što je ranije navedeno, svi pekarski proizvodi trebaju biti adekvatno zaštićeni od naknadne kontaminacije. Ocjenom 2 također je ocijenjen nivo higijene skladišnih prostora u trgovačkim centrima I i II, kao i nivo higijene zaposlenika u trgovačkom centru II.
Najvišom ocjenom 4, ocijenjen je nivo higijene izlaganja proizvoda koji se mogu čuvati na sobnoj temperaturi, kao i nivo higijene izlaganja proizvoda u rashladnim vitrinama. Ocjena 4 je dana također i za nivo higijene trgovačkih korpi i kolica za kupovinu.
Na osnovi podataka prikazanih u tablici 3 može se zaključiti da niti jedan parametar nije ocijenjen ocjenom 5 te da postoji potreba za poboljšanjem u svim segmentima koji su bili predmet istraživanja.

Iz podataka u tablici može se vidjeti da su najniže ocjene dane za nivo higijene u trgovačkom centru II, dok su najviše ocjene dane za nivo higijene u trgovačkom centru III.

Tablica 3. Ocjena nivoa higijene trgovačkih centara
Table 3. Evaluation of hygiene levels at shopping centres

ISPITIVANI PARAMETRI	OCJENE ZA ISPITIVANE PARAMETRE		
	Trgovački centar I	**Trgovački centar II**	**Trgovački centar III**
Skladišni prostor	2	2	4
Izlaganje proizvoda na sobnoj temperaturi	4	4	4
Izlaganje i prodaja svježeg mesa i proizvoda od mesa	3	3	3
Izlaganje mesa u rashladnim vitrinama i zamrzivačima	4	4	4
Izlaganje i prodaja kruha i ostalih pekarskih proizvoda	2	2	2
Trgovačke korpe i kolica za kupovinu	4	4	4
Odjel voća i povrća	3	3	3
Nivo higijene zaposlenika	3	2	3
Ukupno	**25**	**24**	**27**

Da bi se napravila „Rejting shema higijene hrane“, neophodno je bilo ocijeniti na koji se to način rukuje proizvodima koji nisu zapakirani jer je vjerojatnost za njihovu kontaminaciju veća zbog toga što nisu zaštićeni ambalažom. Tu se ponajprije misli na svježe meso, voće, povrće, kruh i peciva.
Uobičajeno je da se provjera higijene u objektima koji posluju s hranom usmjerava na površine koje su najčešće u kontaktu s hranom (Watnick i Kolter, 2000). Briseve treba uzimati i s teško dostupnih površina koje su najčešće najviše kontaminirana mjesta. Kod mikrobiološkog ispitivanja briseva, procjena higijene površina se uglavnom zasniva na određivanju ukupnog broja bakterija i enterobakterija po cm^2 (Aarnisalo i sur., 2006.).
Kao što je ranije navedeno, mikrobiološkom analizom utvrđena je pojava neispravnih briseva na ukupan broj bakterija, kao i enterobakterija, na površinama koje dolaze u kontakt sa svježim mesom (panj, stroj za mljevenje mesa, posude za izlaganje svježeg mesa).
Prema istraživanju koje su proveli Ivanović i sur. (2013.) broj neispravnih briseva, za ukupan broj bakterija i enterobakterija, na stolu za rasijecanje (panju) iznosio je 66,6 %, za stroj za rezanje 18,6 %, te na stroju za mljevenje 20,59 %.
U istraživanju koje je provela Đurić (2014) utvrđeno je da je na drvenim površinama za rezanje mesa statistički značajno veća učestalost organskih tvari u odnosu na plastične površine (daske).
Tijekom praćenja nivoa higijene rukovanja hranom, utvrđeno je da su radnici koji su u izravnom kontaktu s hranom (na odjelu svježeg mesa i pekarskih proizvoda) nosili nakit ili nisu imali odgovarajuće kape na glavi za zaštitu od kontaminacije. Radnici koji dolaze u kontakt s hranom moraju biti educirani i informirani o značaju pravilnog rukovanja hranom (Nel i sur., 2004). Njihova higijena i radne navike moraju biti primijenjene na pravilan način. Radnici se

moraju pridržavati svih uputa koje se odnose na higijenu opreme, higijenu ruku i zaštitne odjeće. Neprihvatljivi rezultati u pogledu zaštitne odjeće, kao i nošenja nakita, vjerojatno su rezultat nedovoljne upućenosti radnika o pravilnom rukovanju hranom u objektima koji posluju s hranom.

Osim dijela u kojem se vrši prodaja prehrambenih proizvoda, tijekom provedenog istraživanja praćen je i ocijenjen nivo higijene skladišnog prostora, te dijela za odlaganje iskorištene ambalaže i ostalog otpada.

Hrana se treba skladištiti odignuta od poda, a hranu namijenjenu neposrednoj konzumaciji (svježe voće i povrće) treba odvojeno skladištiti od ostale hrane postavljanjem određenih pregrada, čuvanjem na odvojenim policama, u zasebnim uređajima i sl.

Pored navedenog, potrebno je voditi računa o slijedu ulaza robe u skladište, odnosno da se prodaje prvo ona hrana koja je prva uskladištena kako ne bi došlo do isteka roka trajanja proizvoda prije njegove prodaje. Mjesto na kojem se pohranjuje hrana s oštećenom ambalažom ili vidljivim znakovima kvarenja mora biti odvojeno od ostale hrane (Bačelić Grgić i sur. 2011).

U objektima u kojima je istraživanje provedeno utvrđene su različite nepravilnosti u pogledu skladištenja prehrambenih proizvoda. U trgovačkim centrima II i III prehrambeni proizvodi nisu odignuti od poda, a vreće brašna i šećera koje su puknute i vlažne nisu odvojene od onih koje su ispravne.

Utvrđeno je neadekvatno odlaganje iskorištene ambalaže u trgovačkom centru III iako je *Pravilnikom o uslovima minimalne tehničke opremljenosti poslovnih prostora za obavljanje trgovine i trgovinskih usluga (Službene novine FBiH, broj: 49/12)* propisan način odlaganja istog.

ZAKLJUČCI

Na osnovi rezultata istraživanja mogu se iznijeti sljedeći zaključci:

- mikrobiološkom analizom briseva utvrđeno je da se u trgovačkom centru I pojavljuje 1,66 %, dok se u trgovačkim centrima II i III javlja 0,83 % neispravnih briseva od ukupno 120, koliko je analizirano u svim tržnim centrima,
- najvišom prosječnom ocjenom (3,35) za ukupan nivo higijene ocijenjen je trgovački centar III, a najnižom prosječnom ocjenom 3, ocijenjen je nivo higijene u trgovačkom centru II,
- svi sudionici u proizvodnji, distribuciji i potrošnji hrane imaju određene odgovornosti za očuvanje njene zdravstvene ispravnosti,
- na očuvanje zdravstvene ispravnosti hrane u prodajnim objektima utječe nivo znanja zaposlenika o važnosti osobne higijene i pravilnom načinu rukovanja prehrambenim proizvodima,
- sve površine koje dolaze u izravni kontakt sa svježim prehrambenim proizvodima neophodno je redovito prati, čistiti i po potrebi dezinficirati,
- neophodno je svakodnevno održavanje, čišćenje i dezinfekcija opreme i pribora za manipuliranje svježim mesom kako bi do kupca dolazio samo zdravstveno ispravan proizvod,
- svi pekarski proizvodi u prodajnim objektima moraju biti izloženi na način koji će spriječiti kontaminaciju istih,

- nedovoljno razvijena svijest potrošača o primjeni higijenskih mjera prilikom pripreme hrane u domaćinstvima može narušiti zdravstvenu sigurnost prehrambenih proizvoda, te negativno utjecati na zdravlje potrošača.

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RISK FACTORS IN THE FOOD SUPPLY CHAIN

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ABSTRACT

A number of factors in a food chain influences food safety after it is distributed from the production facilities to retail centres. Primarily, it is transportation, followed by its entrance into retail centre, showing, manipulation, as well as application of Good Hygiene (GHP) and Good Production Practices (GPP) in facilities. The aim of this research was to establish „Rating scheme of food hygiene“ in selected retail centres in range 1 – 5. The highest grade (5) marks „very good“ hygiene standard sin retail centres, while lower grades indicate that self-control procedures are not well established. In addition, microbial analyses of swabs taken from different surfaces were made. Research lasted 30 days. Rating scheme showed that retail centre III had the best hygiene practice (average grade 3.35), while retail centre II had the lowest average grade (3) among three evaluated. Since none of analysed centres received the grade 5, it is evident that improvements in hygiene practices are necessary. Microbial analyses showed that cleanliness of all three retail facilities was adequate since the number of unsatisfactory swabs was lower than maximum permitted levels. nezadovoljavajućih briseva bio znatno manji od onog koji je dozvoljen Pravilnikom.

Keywords: retail centre, rating scheme of food hygiene, microbial quality

IDENTIFICATION OF *Listeria* spp. IN FOODSTUFFS IN THE CITY OF BIHAĆ

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ABSTRACT

This study included the identification of the potential presence of *Listeria* spp. bacteria, which are a significant public health problem and a potential human health risk. These bacteria have the ability to survive in many adverse conditions, compared to most other microorganisms. *L. monocytogenes* can contaminate various types of food products, and it can reproduce at temperatures between 2 and 4°C. It is most commonly detected in raw meat and cured meat products, various salads, and milk and dairy products. The number of food poisoning cases is low compared to others, but it emphasizes the need for constant monitoring, because of the high mortality rate of patients (30%). The present study was undertaken to determine the presence of *L. monocytogenes* in meat and meat products, milk and dairy products, cakes, and fruit and vegetables. From the total of 300 examined samples, this species was not isolated in either of the samples, which is, certainly, a good result. However, a number of samples were positive to the presence of other species from the of the genus *Listeria*. The most common species, *L. innocua,* was found in twenty-five samples (8.33%). Out of these twenty-five samples, its presence is not allowed in fifteen of the samples (5%) and there were also ten samples (3.33%) in which a certain amount is allowed. It was dominant in dairy products and meat products. *L. seeligeri* was identified in four samples (1.33%). *L. grayi* was identified in four samples (1.33), in three of these samples (1%) its presence is not allowed. *L. welshimeri* was identified in one sample (0.33%).

Keywords: Listeria monocytogenes, bacterium, foodstuffs, sample

INTRODUCTION

Genus *Listeria* comprises a group of Gram positive, stumpy, asporogenic bacteria that stand out due to their exceptional adaptability and environmental resistance, especially to temperature fluctuations. Within the genus, there are fifteen species: *L. monocytogenes* (Pirie, 1940), *L. ivanovii* (Seeliger et al., 1984), *L. seeligeri* (Rocourt & Grimont, 1983), *L. innocua* (Seeliger, 1981), *L. welshimeri* (Rocourt & Grimont, 1983), *L. grayi* (Errebo Larsen & Seeliger, 1966), *L. marthii* (Graves et al., 2010), *L. rocourtiae*

(Leclercq et al., 2010), *L. fleischmannii* (Bertsch et al.,2013), *L. weihenstephanensis* (Lang Halter et al., 2013), and the newly described *L. floridensis, L. aquatica, L. cornellensis, L. riparia,* and *L. grandensis* (den Bakker et al., 2014). Of the previously identified species, two are considered pathogenic, *L. monocytogenes* and *L. ivanovii*. Only *L. monocytogenes* is a human pathogen and it causes listeriosis, while *L. ivanovii* is an animal pathogen (Batz et al., 2005; Gillespie et al., 2010; Painter et al., 2013, Weler et al., 2015). Listeriosis is transmitted after the ingestion of food products that contain a large number of bacteria, and generally occurs in high-risk groups, such as pregnant women, newborns, elderly, and immunocompromised persons. Although listeriosis outbreaks are low compared to other pathogenic diseases caused by bacteria in foodstuffs, listeriosis is a significant risk to human health, since the invasive form may have a mortality rate of 30% (Leong et al., 2014). This disease is mostly associated with eating contaminated raw or insufficiently pasteurized milk and cheese, ice cream, raw vegetables, fermented sausages, raw or insufficiently thermally processed meat, and raw and smoked fish. The smallest infectious dose of *L. monocytogenes* for humans is unknown because it depends on the host immunity and the concentration of pathogens in the consumed food. The data collected after several major epidemics of listeriosis indicate the values of 10^7 to 10^{11} CFU/g of food (Dalton et al., (1997), but also minimal doses of this pathogen, may cause the development of the disease (Ooi & Lorber 2005). The Ordinance on Microbiological Criteria for Foodstuffs lays down a standard for *L. monocytogenes*, which must not be present in food (0/25 g), except in raw minced meat and portioned meat, where 10^2 CFU/g is allowed, and frozen products prepared for culinary processing such as fillets, crumbed products, seafood etc. (Ordinance on Microbiological Criteria for Foodstuffs, "Službeni glasnik BiH" (Official Gazette of Bosnia and Herzegovina) no. 79/16 and Guidelines 2011). Food safety criteria must be respected up to the food expiration date and it is an obligation of food manufacturers to ensure quality and safety of the food they put on the market. In prepared foodstuffs, in which *L. monocytogenes* growth can occur (except infant formulas and ready-to-eat foods for special medical purposes), the presence of 100 cfu/g is permitted; these are the products placed on the market at the time of the expiration date. The manufacturer can establish temporary limit values during the process, which must be low enough to ensure that the limit of 100 CFU/g will not be exceed by the expiration date. Worldwide public interest for *L. monocytogenes* has not ceased since the epidemic in Southern California in 1985, which was caused by the consumption of fresh cheese prepared with improperly pasteurized milk (Markov et al., 2009). *L. monocytogenes* can be isolated from unpasteurised milk or different kinds of cheese in which the number of bacteria can be up to 10^7 CFU/g (Kozačinski and Hadžiosmanović, 2001; Markov et al., 2009). In the research by Aureli et al. (2000), *L. monocytogenes* was isolated from cold tuna and corn salad. An outbreak of febrile gastroenteritis occurred and the symptoms were reported by 1566 people (72%); 292 (19%) of them were hospitalized, while one type of sour milk was responsible for 34 cases of listeriosis, including 8 fatalities in Austria, Germany, and the Czech Republic in 2009 and 2010 (Fretz et al., 2010). According to the research by Kovačević (2010) on the presence of *L. monocytogenes* in the minimally processed and refrigerated vegetables, which was carried out in the period from March of 2008 to April of 2009, 100 samples from Osijek supermarkets were analysed for the presence of *Listeria* spp. Lettuce, delicatessen salads and cabbage salads, salads from mixed vegetables, leafy vegetables, and salads from root

vegetables were analysed. *Listeria* spp. was found in 20% of the samples, and *L. monocytogenes* in 1% of the analysed samples. According to the data of the Centres for Disease Control and Prevention, one of the major epidemics of listeriosis in the United States was in 2011; there were 147 cases, 33 deaths, and one spontaneous miscarriage associated with eating fresh cantaloupes from a farm (CDC, 2016). Numerous epidemic cases of listeriosis have been reported, and 24 cases of listeriosis were confirmed in the USA, in the period from 1998 to 2008 (Cartwright et al., 2013). EU members reported 1,763 cases of listeriosis in 2013, and a total of 191 deaths. In France alone, 64 deaths were reported (Anon., 2015). The foods most commonly involved in recent *Listeria* epidemics in the EU and the US were dairy products and ready-to-eat food. Many of these epidemics are linked to ready-to-eat food from restaurants (EFSA, 2015, Lomonaco et al., 2015). During 2014/2015, the epidemic associated with caramelized apples caused 35 diseases in 12 states, including 7 deaths (CDC, 2015).

MATERIALS AND METHODS

The study was carried out on 300 samples, from February to October this year (fresh meat and meat products, milk and dairy products, cakes, fruits, and vegetables), obtained from retail shops. The samples were delivered to the laboratory of the Veterinary Institute in Bihać, which is an accredited body (BAS EN ISO/IEC 17025:2006), and subjected to microbiological analysis to qualitatively determine the bacteria of the genus *Listeria* spp., i.e. *L. monocytogenes* in 25 g of sample according to the norm.

Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products (in our case, these included samples of pastry, fruit, and vegetables).

The detection of *L. monocytogenes* can be broken down in successive stages, by primary and secondary enrichment, and by streaking selective plates of Aloe and Oxford agar. Typical colonies on Aloa agar are green-blue in colour and surrounded by an opaque halo (Figure 1a), while on Oxford agar, the typical colonies were small (1 mm) and greyish with a black halo, which become darker with greenish sheen, after 48 h they had a black halo and a dotted centre (Figure 1b).

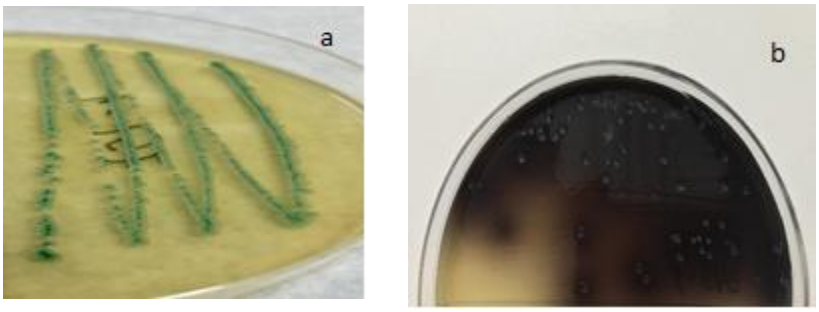

Fig. 1. *L. innocua* colonies on an Aloa plate (a) and on an Oxford plate (b)

The colonies suspected of being *Listeria* spp. were tested further, which includes the catalase test, Gram staining, the hemolysis test, the carbohydrate fermentation test and the Camp test, and the API *Listeria* spp. test. Determination of biochemical properties of the studied bacteria and their more precise detection. Figure 3 shows the API identification of bacteria from the genus *Listeria* spp.

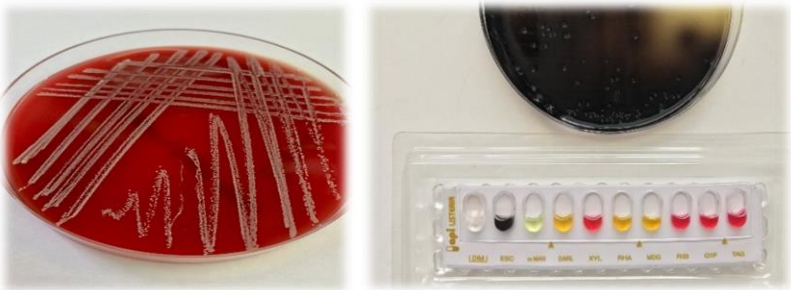

Fig. 2. API readings for the identification of bacteria of the genus *Listeria* spp.

RESULTS AND DISCUSSION

The present study was undertaken on a total of 300 samples of meat and meat products, milk and dairy products, cakes, and fruits and vegetables, to determine the presence of *Listeria* spp. The results of bacterial screening for the presence of bacteria of the genus *Listeria* are shown in Table 1 and 2. *L. monocytogenes* was not found in any of the samples analysed. However, biochemical identification revealed the bacteria *L. innocua, L. grayi, L. welshimeri,* and *L. seeligeri*.

Table 1. The results of the microbiological testing of the samples for the presence of bacteria of the genus *Listeria*

Sample	**n**	**% of sample**	**Species identified**
Meat and meat products	120	13 (10.83 %)	*L. innocua*
Milk and dairy products	60	6 (10 %)	*L. innocua, L. Welshimeri*
Cakes	60	7 (11.67 %)	*L. welshimeri, L. Grayi*
Fruits and vegetables	60	8 (13.33 %)	*L. innocua, L. seeligeri, L. grayi.*

n-number of samples tested

In the research by Bouayad et al. (2015), 212 samples of broilers from three broiler abattoirs were sampled for bacteria of the genus *Listeria.* Sampling was performed at the evisceration stage and at the end of processing, after packaging and refrigerating at 4°C for 24 h. In terms of microbiological quality of all broiler meat samples, 99 (46.7%) were positive for *Listeria*, including *L. monocytogenes* 19 (8.9%), *L. innocua* 69 (32.5%), *L. grayi* 10 (4.7%), and *L. welshimeri* 1 (0.5%).
The prevalence and level of *L. monocytogenes* in retail sale, which covers most provincial capitals in China, were studied by Wu et al., (2015). 1036 samples of

vegetables, edible mushrooms, crude meat, and frozen products were analysed from September of 2012 to January of 2014.

Table 2. The testing results of the samples which were positive for the presence of bacteria of the genus *Listeria*

Sample	**n**	***L. monocytogenes*/25 g**	**Remark**
Fresh cheese 3,8 % milk fat	3	Neg	*L. innocua*/25 g = pos.
Farm cheese	1	Neg	*L. innocua*/25 g = pos.
Ready-to-eat cake	5	Neg	*L. innocua*/25 g = pos.*
Ready-to-eat cake	2	Neg	*L. grayi*/25g = pos.*
Ice cream	1	Neg	*L. innocua*/25 g = pos.
Ice cream	1	Neg	*L. welshimeri*/25 g = pos.
Meat product	8	Neg	*L. innocua*/25 g = pos.
Minced meat	5	Neg	*L. innocua*/25 g = pos.*
Vegetables	2	Neg	*L. innocua*/25 g = pos.
Vegetables	3	Neg	*L. seeligeri/25 g* = pos.
Vegetables	2	Neg	*L. grayi*/25 g = pos.
Fruit	1	Neg	*L. seeligeri*/25 g = pos.

*n-the number of positive samples; *-allowed in certain amounts (Ordinance on Microbiological Criteria for Food, "Službeni glasnik BiH" no 79/16)*

The overall prevalence of *L monocytogenes* was 20.0% (207/1036). Taguchi et al., (2017) examined the samples of lightly pickled vegetables manufactured at 55 processing factories. 12 samples manufactured at five of the factories were positive for *L. monocytogenes*. Microbiological surveillance at two factories (two surveys at factory A and three surveys at factory B between June of 2014 and January of 2015) identified the areas predominantly contaminated with *L. monocytogenes:* refrigerators and packaging rooms. Genotyping provided further evidence that the contaminants found in these areas were linked to those found in the final products. Microbiological surveillance at the manufacturing factories further clarified the sources of contamination in the retail products. In their research, Caggiano et al. (2015) examined 108 samples of cooked ready-to-eat foods sampled from catering services: 16 rice dishes, 28 pasta dishes, 22 meat products, 12 egg products, 22 potato portions, and 8 samples of mixed cooked vegetables. *Listeria* spp. was detected in 9 products (9/108, 8.3%). In particular, *L. monocytogenes* was detected in 7 samples (7/9; 77.7%), with the highest prevalence in potato samples (6/9; 66.6%), followed by rice dishes (1/9; 11.1%), and *Listeria innocua* was isolated from potato puree (1/9; 11.1%) and cooked vegetables (1/9; 11.1%). Nayak et al., (2015) conducted a research study on 200 food samples of animal origin; 18 (9%) were found positive for *Listeria* spp., which were identified as *L. seeligeri* (6, 33.3%), L. *innocua* (5, 27.7%), *L. welshimeri* (4, 22.2%), and *L. monocytogenes* (3, 16.6%). Dalzini et al. (2014) found out that a salami sample, containing up to 30% less fat than the traditional one and packed in modified atmosphere, does not allow the growth of *L. monocytogenes* and cannot reach or exceed the limit of 100 CFU/g, even if the storage temperature is between 8 and 12°C. Goh et al., (2012) have directed their research on the

detection of *L. monocytogenes* in raw chicken meat samples at hypermarkets and wet markets. The occurrence of *L. monocytogenes* was the highest in breast samples (42.03%), and samples from hypermarkets showed a higher occurrence (25.71%) of *L. monocytogenes* compared to wet markets (14.29%). Oxaran et al., (2017) determined the incidence of *L. monocytogenes* in five dairies and retail products in the Southeast and Midwest regions of Brazil over eight months. Of 437 samples, three samples (0.7%) from retail shops and only one sample (0.2%) from the dairies were positive for *L. monocytogenes*. Sharma, et al., (2017) analysed 457 samples of raw milk and five samples tested positive for *L. monocytogenes.* After that, multiplex serotyping was performed on positive samples. Compared to this literary data, we can say that our results are satisfactory due to the absence of *L. monocytogenes*.

CONCLUSION

Although *L. monocytogenes* was not isolated in any of the analysed samples, the results of the investigation indicated the presence of a number of other species of *Listeria*, the most common being the species of *L. innocua*. This does not diminish the real danger to human health because *L. monocytogenes* is widely distributed in the environment. For these reasons, and on the basis of our results, we recommend mandatory regular control of the microbiological quality of raw foods and foodstuffs from production and distribution, which would result in improved hygiene conditions and wholesome food.

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Topic: Food safety / Sekcija: Zdravstvena sigurnost hrane

BIOTOKSINI U DAGNJAMA I KAMENICAMA IZLOVLJENIM U PODRUČJU ISTOČNE I ZAPADNE OBALE ISTRE

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SAŽETAK

Školjkaši su značajan izvor hranjivih tvari, ne sadrže aditive, lako su probavljivi i konzumiraju se minimalno procesuirani ili sirovi, što ih čini nutritivno visokovrijednom namirnicom poželjnom u prehrani. Zbog načina hranidbe živih školjkaša moguće je nakupljanje kontaminanata u njihovom tkivu, poput biotoksina, zbog čega predstavljaju potencijalno toksične namirnice. Tijekom 2015. i 2016. godine prisustvo biotoksina ispitano je u 30 uzoraka dagnji (*Mytilus galloprovincialis*) i 30 uzoraka kamenica (*Ostrea edulis*) podrijetlom iz područja za izlov živih školjkaša smještenih na istočnoj i zapadnoj obali Istarskog poluotoka. Količine biotoksina (skupina koje uzrokuju gubitak pamćenja, proljev i paralizu) ispitivane su metodom tekućinske kromatografije visokog učinka u kombinaciji s DAD, FLD i masenom detekcijom. Njihovo prisustvo dokazano je u 27 % ispitivanih uzoraka, u rasponu od 46,8 do 117,5 µg/kg za okadaičnu skupinu, te 0,1 do 0,3 mg/kg za jesotoksine, a utvrđeni maseni udjeli bili su ispod od najviših dopuštenih vrijednosti definiranih trenutno važećim propisima u Republici Hrvatskoj. Lipofilni biotoksini identificirani su u uzorcima dagnji, dok su u kamenicama maseni udjeli svih istraživanih skupina biotoksina bili ispod granice određivanja. S obzirom na toksičnost morskih biotoksina koji su određeni u ispitivanim uzorcima, zbog zaštite zdravlja potrošača, potreban je kontinuirani monitoring zdravstvene ispravnosti živih školjkaša koji se stavljaju na tržište.

Ključne riječi: školjkaši, biotoksini, kontaminanti, sigurnost hrane

UVOD

Školjkaši su lako probavljiva, nutritivno visokovrijedna namirnica te, s obzirom na veliki broj uzgajališta u Republici Hrvatskoj, dostupna potrošačima. Ukoliko se uzgajaju u čistom moru te izlovljavaju i stavljaju na tržište u skladu s dobrom proizvođačkom praksom, poželjni su kao dio uravnotežene prehrane.
Meso školjkaša bogato je bjelančevinama koje sadrže esencijalne aminokiseline, sadrže mali udio masti u kojima prevladavaju mono i polinezasićene masne kiseline, značajan su izvor omega-3-masnih kiselina, posebno zbog toga što se školjkaši konzumiraju sirovi ili minimalno procesuirani. Siromašni su kolesterolom, sadrže biljne sterole i stanole koji

smanjuju njegovu apsorpciju (Dong, 2009; FAO, 2016; Phillips i sur., 2012; Rittenschober i sur., 2013).

Živi školjkaši zbog načina prehrane mogu biti uzročnici bolesti koje se prenose hranom. Filtrirajući velike količine morske vode, u njihovom mekom tkivu mogu se nakupljati bakterije, virusi, paraziti, anorganske tvari te morski biotoksini (fikotoksini) (Oliveira i sur., 2011). Ukoliko rastu u moru u kojem su prisutne toksične vrste mikroalgi, poput nekih vrsta dinoflagelata i diatoma, u njihovom mekom tkivu nakupljaju se biotoksini (Huss i sur., 2000).

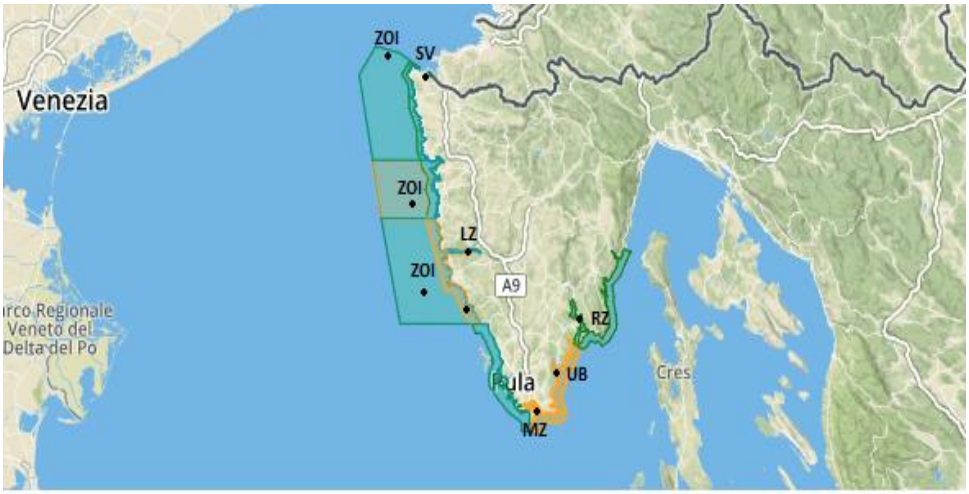
U svrhu sprječavanja bolesti koje mogu uzrokovati kontaminanti iz školjkaša, Ministarstvo poljoprivrede Republike Hrvatske je donijelo Plan praćenja kakvoće mora i školjkaša na proizvodnim područjima i područjima za ponovno polaganje živih školjkaša, kojim se definiraju proizvodna područja za uzgoj ili izlov živih školjkaša, vrste školjkaša koje se uzorkuju, parametri, učestalost i metode ispitivanja, način uzorkovanja i dostave u laboratorije te izvješćivanja o rezultatima ispitivanja (MP, 2017). S ciljem zaštite zdravlja ljudi u Republici Hrvatskoj Zakonom o veterinarstvu utvrđena su određena higijenska pravila za hranu životinjskog podrijetla koja definiraju granice najvećih dopuštenih količina (NDK), kao i referentne metode određivanja pojedinih skupina morskih biotoksina u živim školjkašima. Propisano je ispitivanje biotoksina koji uzrokuje gubitak pamćenja (Amnesic shellfish poison - ASP), koji uzrokuju paralizu (Paralytic shellfish poison - PSP) i koji uzrokuju probavne smetnje (Diarrheic shellfish poison - DSP) (HS, 2013).

ASP trovanje izazivaju domoična kiselina (DA) i njeni analozi koji se zbog strukturne sličnosti s glutaminskom kiselinom vežu na glutamatne receptore. Povećavajući koncentraciju kalcijevih iona, aktiviraju enzime koji uzrokuju oštećenje i smrt neurona, osobito u području hipokampusa, što uz gastrointestinalne i neurološke simptome izaziva gubitak kratkoročnog pamćenja, po čemu je trovanje dobilo ime (Lefebvre i Robertson, 2010; Mos, 2001).

PSP čini skupina više od trideset neurotoksina topivih u vodi čiji je glavni predstavnik saksitoksin (STX). Dijele se u četiri skupine: karbamat, dekarbamoil, N-sulfokarbamoil i deoksidekarbamoil toksine, od kojih se STX, koji pripada skupini karbamat toksina, koristi za izražavanje toksičnosti ostalih toksina (Zhuo i sur., 2013). Trovanje može izazivati blage neurološke simptome poput trnjenja prstiju, peckanja u usnoj šupljini te jače poput glavobolje, mučnine i povraćanja, a u najtežim slučajevima nastupa smrt uslijed paralize respiratornih mišića (EFSA, 2009).

DSP toksičnost uzrokuju lipofilni biotoksini podijeljeni u četiri skupine: okadaična kiselina (OA) i njeni derivati dinofizistoksini (DTX), pektenotoksini (PTX), azaspiracidi (AZA), yesotoksini (YTX) i njihovi derivati. Uzrokuju povećanje propusnosti epitelnih stanica crijeva što izaziva proljev koji je, uz povraćanje, najčešći simptom ovog trovanja. Simptomi se javljaju u kratkom vremenu nakon konzumacije školjaka i nestaju nakon nekoliko dana bez posljedica (Botana i sur., 2010; Van Dolah, 2000). Iako za YTX nije dokazano izazivanje DSP toksičnosti u ljudi, svrstani su u ovu skupinu zbog svojih lipofilnih svojstava prije nego je objašnjeno njihovo toksično djelovanje (Luckas i sur., 2012). Zbog spomenutih opasnosti, cilj ovog rada je prikazati podatke o pojavnosti i masenim udjelima prisutnih skupina biotoksina u školjkašima izlovljenim u području istočne i zapadne obale Istre (Slika 1), usporediti ih s podacima dosadašnjih istraživanja

biotoksina na području sjevernog Jadrana te utvrditi postoji li opasnost za potrošače s obzirom na utvrđene razine.



Slika 1 Područja za uzgoj ili izlov živih školjkaša na Istarskom području (CAPS 2 Web GIS Application, 2014)
Fig. 1 Areas for breeding or harvesting live shellfish in Istria (CAPS 2 Web GIS Application, 2014) LZ (Lim Bay); MZ (Medulin Bay); RZ (Raša Bay); SV (Savudrija Gulf); UB (Budava Cove); ZOI (western coast of Istria)

MATERIJALI I METODE

Od siječnja 2015. do prosinca 2016. godine prikupljani su uzorci školjkaša te je prisustvo biotoksina ispitano u 30 uzoraka dagnji (*Mytilus galloprovincialis*) i 30 uzoraka kamenica (*Ostrea edulis*) podrijetlom iz područja za uzgoj i izlov živih školjkaša smještenih na istočnoj i zapadnoj obali istarskog poluotoka. Uzorci su analizirani unutar 24 h od trenutka prijema u laboratorij, a ukoliko to nije bilo moguće, u tom je vremenu provedena ekstrakcija biotoksina, ekstrakti su adekvatno pohranjeni i naknadno ispitani. Određivani su domoična kiselina (ASP), skupina toksina koja uzrokuje PSP toksičnost (izraženo kao ekvivalent STXdiHCl) te skupine koja uzrokuje DSP toksičnost (izraženo kao ekvivalent okadaične kiseline, azaspiracida i jesotoksina pojedinačno).
Meko tkivo živih školjkaša (najmanje 100 g) odvojeno je od ljušture, isprano i homogenizirano pri 22000 okretaja/min (Waring 8011EG, Stamford, SAD). Određivanje domoične kiseline provedeno je metodom visoko učinkovite tekućinske kromatografije s UV detekcijom (HPLC-DAD) koju je opisao Quilliam (2003). Biotoksini koji uzrokuju PSP toksičnost ispitani su metodom visoko učinkovite tekućinske kromatografije s fluorescentnom detekcijom uz prethodnu oksidaciju PSP toksina (AOAC, 2005). Lipofilni toksini ispitani su metodom visoko učinkovite tekućinske kromatografije s masenom detekcijom, primjenom kiselih kromatografskih uvjeta (EU-RL-MB, 2011).
Za ekstrakciju i pripremu mobilnih faza korištena su organska otapala HPLC i LCMS čistoće, ostale kemikalije korištene u pripremi uzoraka za analizu bile su analitičke čistoće. Određivanje

ASP i PSP toksina provedeno je na instrumentu HPLC proizvođača Agilent Technologies 1200 s DAD odnosno FLD detektorom, a lipofilnih na instrumentu UHPLC Agilent Technologies 1290 Infinity, s trostrukim kvadrupolom (QQQ) masenim detektorom. Za kvantifikaciju su korišteni kalibracijski pravci koncentracijskog raspona 1 do 25 µg/mL za ASP toksine, 1,5 do 30 ng/mL za OA i AZAs, 3,8 do 75 ng/mL za YTX i 1,4 do 174,7 ng/mL za PSP toksine. Za pripremu kalibracijskih otopina korišteni su certificirani referentni materijali proizvođača National Research Council, Institute for Marine Biosciences, Kanada. Prilikom svake analize provjeravalo se iskorištenje istovremenim ispitivanjem certificiranih referentnih materijala: CRM-ASP-Mus-d, CRM-DSP-Mus-c, CRM-AZA-Mus (NRC CNRC, Halifax, Kanada) i PO PST CRM 1101 (Cefas, Weymouth, UK).

REZULTATI I RASPRAVA

Primijenjene metode su validirane u skladu s Pravilnikom o provođenju analitičkih metoda i tumačenju rezultata (MPŠVG, 2005). Vrijednosti granice određivanja (LOD) i granice kvantifikacije (LOQ) metoda za pojedine skupine biotoksina prikazane su u Tablici 1. Kao LOD metode za PSP toksine je uzeta najmanja koncentracija koja se mogla pouzdano odrediti u obogaćenim uzorcima, dok je LOQ određen računski, uvećavanjem LOD za šest standardnih devijacija (σ) iskorištenja obogaćenih uzoraka. LOD i LOQ za ASP i DSP toksine određeni su analizom sigurno negativnih uzoraka čija se σ površina u RT analita uvećala tri, odnosno šest puta.
Prisustvo biotoksina dokazano je u 27 % ispitivanih uzoraka i to u uzorcima dagnji, dok su u kamenicama maseni udjeli svih skupina biotoksina bile niže od LOD za pojedini toksin ili skupinu toksina. U nijednom uzorku obje vrste školjkaša maseni udio nije bio viši od NDK definiranih u trenutno važećim propisima Republike Hrvatske. Utvrđeni su OA i YTX, koji pripadaju skupini lipofilnih biotoksina, a koncentracije AZA, ASP i PSP bile su niže od LOD. Najniži maseni udio OA u uzorcima iznosio je 46,8, a najviši 117,5 µg OA ekv./kg, što je ispod NDK (160 µg OA ekv./kg za OA, DTX i PTX ukupno) definirane Uredbom EZ br. 853/2004 o utvrđivanju određenih higijenskih pravila za hranu životinjskog podrijetla (Tablica 2).

Tablica 1. Granica određivanja (LOD) i granica kvantifikacije (LOQ) metoda za ispitivanje pojedinih skupina biotoksina
Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) methods for testing individual groups of biotoxins

SKUPINA BIOTOKSINA	LOD	LOQ
ASP (mg/kg)	0,10	0,14
AZA (µg AZA ekv./kg)	31,70	32,14
OA, DTXs, PTXs (µg OA ekv./kg)	46,50	50,45
YTXs (mg YTX ekv./kg)	0,05	0,07
PSP (µg STXdiHCl ekv./kg)	202,00	240,13

ASP (biotoksini koji uzrokuju gubitak pamćenja); AZA (azaspiracidi); OA (okadaična kiselina); DTXs (dinofizistoksini); PTXs (pektenotoksini); YTXs (yesotoksini); PSP (biotoksini koji uzrokuju paralizu); STX (saksitoksin)

Tablica 2. Najveće dopuštene količine (NDK) biotoksina
Table 2. Maximum permissible quantity (NDK) of biotoxins (HS, 2013)

SKUPINA BIOTOKSINA	NDK
PSP (µg STXdiHCl ekv./kg)	800
AZA (µg AZA ekv./kg)	160
OA, DTXs, PTXs ukupno (µg OA ekv./kg)	160
YTXs (mg YTX ekv./kg)	3,75
ASP (mg/kg)	20

NDK – najveća dopuštena količina; ASP (biotoksini koji uzrokuju gubitak pamćenja); AZA (azaspiracidi); OA (okadaična kiselina); DTXs (dinofizistoksini); PTXs (pektenotoksini); YTXs (jesotoksini); PSP (biotoksini koji uzrokuju paralizu); STX (saksitoksin)

Maseni udjeli YTX bili su od 0,1 do najviše 0,3 mg YTX ekv./kg, što je znatno ispod 3,75 mg YTX ekv./kg koliko iznosi NDK za ovu skupinu biotoksina. Srednja i vršna vrijednost te standardna devijacija koncentracija pojedinih skupina biotoksina određenih u uzorcima, navedeni su u Tablici 3.
U 17 % ispitanih uzoraka određen je YTX, a u 10 % uzoraka OA. Utvrđeni maseni udjeli YTX bili su zanemarivi, dok su maseni udjeli OA u pojedinim uzorcima, iako ispod NDK, bili značajni dovoljno da mogu predstavljati opasnost za osjetljivu populaciju. Prema mišljenju EFSA CONTAM Panela, trenutno definirane vrijednosti NDK za OA, AZAs i PSP skupinu te DA ne štite dovoljno potrošače (EFSA, 2009). Ukoliko osoba tjelesne mase 60 kg pojede 400 g školjaka, koliko prosječno iznosi velika porcija, u organizam će unijeti 0,8 µg OA ekv. po kg tjelesne mase. Ako je u uzorku prisutno 117,5 µg OA ekv./kg, kao što je utvrđeno u uzorku s najvećom koncentracijom (Slika 2), za 2,6 puta premašuje se akutna referentna doza (ArfD) od 0,3 µg OA ekv. po kg tjelesne mase koju je definirala EFSA (2009). Dobiveni rezultati u skladu su s rezultatima dosadašnjih istraživanja pojavnosti biotoksina u sjevernom Jadranu, prema kojima su YTX i OA najčešće izolirani biotoksini u školjkašima izlovljenima u tom području te da su maseni udjeli OA blizu NDK i viši (Arapov, 2013; Arapov i sur., 2015; Ciminello i sur., 2003; 2010; Pistocchii sur., 2012).

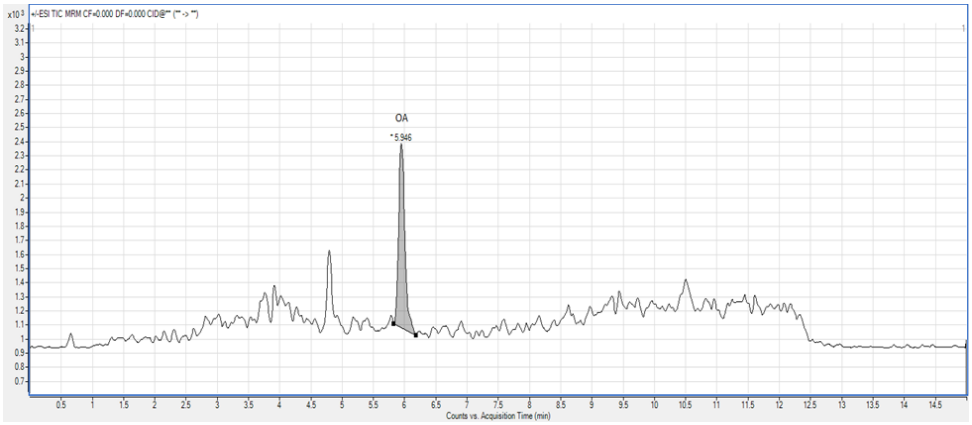
Tablica 3. Koncentracije biotoksina određene u dagnjama
Table 3. Mass fractions of biotoxins detected in mussels

SKUPINA BIOTOKSINA	SREDNJA VRIJEDNOST	SD	MIN	MAX	MASENI UDJELI IZNAD LOD %
ASP (mg/kg)	ND	ND	ND	ND	ND
AZA (µg AZA ekv./kg)	ND	ND	ND	ND	ND
OA (µg OA ekv./kg)	75.4	26,1	46,8	117,5	10,0
YTX (mg YTX ekv./kg)	0.2	0,1	0,1	0,3	17,0
PSP (µg STXdiHCl ekv./kg)	ND	ND	ND	ND	ND

ND-nije detektirano; ASP (biotoksini koji uzrokuju gubitak pamćenja); AZA (azaspiracidi); OA (okadaična skupina); YTX (jesotoksini); PSP (biotoksini koji uzrokuju paralizu); STX (saksitoksin)

Iako u analiziranim uzorcima nisu izolirane ostale skupine biotoksina, ali s obzirom da su u posljednjih dvadesetak godina u školjkašima izlovljenim na području sjevernog Jadrana više

puta određeni maseni udjeli OA i DTX iznad granica definiranih legislativom te da je utvrđena i pojavnost PSP i DA (Arapov, 2013; Ciminello i sur., 2010; Pistocchi i sur., 2012), postoji opravdana potreba kontinuiranog praćenja pojavnosti biotoksina u školjkašima, s ciljem osiguranja zaštite zdravlja potrošača.



Slika 2. Kromatogram uzorka s najvećom koncentracijom OA (117,5 µg OA ekv./kg)
Fig. 2. Mussel sample chromatogram with the highest mass fraction OA (117.5 µg OA equiv/kg)

ZAKLJUČCI

Utvrđeni maseni udjeli istraživanih skupina morskih biotoksina u uzorcima dagnji i kamnenica bili su niži od NDK te su svi uzorci bili sukladni važećim propisima zakonodavstva. Biotoksini su određeni u dagnjama, dok su u kamenicama maseni udjeli bili ispod granice određivanja. U najvećem broju uzoraka određen je YTX, u manjem postotku OA, dok ostale skupine nisu određene. Rezultati rada pokazuju da su školjkaši izlovljeni u području istočne i zapadne obale Istre zdravstveno ispravni, ali i da pojedini uzorci mogu predstavljati opasnost za ljudsko zdravlje budući da trenutno definirane NDK ne uzimaju u obzir osjetljiviju populaciju potrošača. Stoga je potreban kontinuirani nadzor pojavnosti biotoksina u različitim vrstama školjkaša te daljnja toksikološka ispitivanja kojima bi se utvrdila nužnost revizije zakonskih propisa u ovom području.

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OCCURRENCE OF BIOTOXINS IN MUSSELS AND OYSTERS HARVESTED ON THE EASTERN AND WESTERN COAST OF ISTRIA

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ABSTRACT

Bivalves, as a significant source of nutrients, are an additive free and easily digestible nutritionally high-value food desirable in the diet, if consumed minimally processed or crude. Due to filter feeding, there is a possibility of accumulation of contaminants, such as biotoxins, in their meat, which makes them potentially hazardous. During 2015 and 2016, the presence of biotoxins in 30 samples of blue mussels (*Mytilus galloprovincialis*) and 30 samples of oysters (*Ostrea edulis*) originating from the harvesting area located on the eastern and western coast of the Istrian peninsula was investigated. The concentrations of biotoxins (groups that cause memory loss, diarrhoea, and paralysis) were analysed by high performance liquid chromatography in combination with DAD, FLD, and mass detection. Their presence was demonstrated in 27% of the examined samples, ranging from 46.8 to 117.5 µg / kg for the ocadaic group and 0.1 to 0.3 mg / kg for the yesotoxins, and all the determined concentrations were below the maximum permitted levels defined by the legislation. Biotoxins determined in the samples belong to a group of lipophilic biotoxins and were identified in samples of blue mussels, while the concentrations of all biotoxin groups in the oysters were below the limit of determination. Given the toxicity of marine biotoxins and their presence in the tested samples, focusing on consumer protection, there is a need for continuous monitoring of the safety of live bivalve molluscs placed on the market.

Keywords: shellfish, biotoxins, contaminants, food safety

Topic: Food safety / Sekcija: Zdravstvena sigurnost hrane

UČINKOVITOST DEZINFEKCIJE I MIKROBIOLOŠKA ISPRAVNOST VODE ZA LJUDSKU POTROŠNJU VODOOPSKRBNOG SUSTAVA GRADA ŠIBENIKA

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professional paper/stručni rad

SAŽETAK

Kakvoća vode za ljudsku potrošnju u vodoopskrbnom sustavu uvjetovana je nizom čimbenika, a prije svega kakvoćom vode na mjestu zahvaćanja u prirodi (arteški bunar, jezero, vodotok), procesu prerade te sanitarno-tehničkim i higijenskim uvjetima u vodoopskrbnim objektima i pratećoj infrastrukturi. Zdravstveno ispravnom vodom za ljudsku potrošnju smatra se voda koja: (i) ne sadrži mikroorganizme, parazite i njihove razvojne oblike u broju koji predstavlja opasnost za zdravlje ljudi, (ii) ne sadrži štetne tvari u koncentracijama koje same ili zajedno s drugim tvarima predstavljaju opasnost za zdravlje ljudi, (iii) ima vrijednosti parametara zdravstvene ispravnosti vode za ljudsku potrošnju u skladu s odredbama Zakona o vodi za ljudsku potrošnju (MZ HR, NN 56/13, 65/15, 104/17) i Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju(MZ HR, NN 125/13, 141/13, 128/15).
Cilj ovog rada bio je utvrditi učinkovitost dezinfekcije i mikrobiološku kakvoću vode za ljudsku potrošnju koja se putem vodoopskrbnog sustava Jaruga isporučuje stanovnicima grada Šibenika. Analizirani su podaci o koncentraciji slobodnog rezidualnog klora te pojavnosti mikrobioloških parametara (broj kolonija na 22 °C i 37 °C, ukupni koliformi, *Escherichia Coli, Closridium Perfringens, Pseudomonas aeruginosa* i enterokoki) tijekom petogodišnjeg razdoblja (2011.-2015.). Grupiranjem pojedinih podataka, ovisno o dijelu godine, utvrdila se i učinkovitost dezinfekcije vode o godišnjem dobu s obzirom da se voda koja se isporučuje stanovnicima grada Šibenika zahvaća iz podzemnih izvora, a koji su, uslijed geološkog sastava tla, podložni utjecaju padalina.

Ključne riječi: voda za ljudsku potrošnju, dezinfekcija, kloriranje, Šibenik

UVOD

Prema odredbama Zakona o vodi za ljudsku potrošnju (HS, NN 56/13, 64/15, 104/17) vodom za ljudsku potrošnju smatra se voda koja je u svojem izvornom stanju ili nakon obrade namijenjena za piće, kuhanje, pripremu hrane ili druge potrebe kućanstava, a može potjecati iz sustava javne vodoopskrbe, cisterni ili iz boca odnosno posuda za vodu. Dostupnost i dostatnost zdravstveno-ispravne vode za ljudsku potrošnju osnovni je preduvjet osiguranja zdravlja populacije na određenom području, a omogućuje i podizanje općeg životnog

standarda. Osnovni preduvjet isporuke zdravstveno-ispravne vode stanovništvu gradova i naselja, te pratećoj industriji, je postojanje vodovoda ili vodoopskrbnog sustava kojeg čini skup građevinskih objekata na samom mjestu zahvaćanja vode, odnosno izvorištu, glavni dovodni cjevovod, objekti u kojima se provode tehnološki postupci kondicioniranja vode, spremnici prerađene vode (vodosprema) te vodoopskrbna mreža grada ili naselja. Vodoopskrbni sustavi mogu biti javni ili lokalni. Javni vodoopskrbni sustav isporučuje vodu za više od 50 ljudi ili isporučuje više od 10 m^3 vode krajnjem korisniku, a djelatnost isporuke obavlja privredni subjekt registriran za obavljanje javne vodoopskrbe, dok lokalni vodoopskrbni sustav podrazumijeva zahvaćanje vode i distribuciju vode do krajnjih korisnika putem građevinskih objekata i vodoopskrbne mreže za manji broj potrošača, a kojim ne upravlja registrirani privredni subjekt. Kakvoća vode koja se isporučuje krajnjim potrošačima u oba slučaja mora udovoljavati odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15), a ovisi prije svega o kakvoći vode zahvaćenoj na izvorištu. Izvorište je lokacijski definiran dio prostora na kojem se zahvaćaju određene količine vode namijenjene vodoopskrbi, a odabir izvorišta jedan je od najsloženijih i najodgovornijih zadataka kod izgradnje vodoopskrbnog sustava, naročito u pogledu investicijskih i pogonskih troškova. Izvorište vode mora osigurati adekvatne količine kvalitetne vode za trenutnu i potrošnju u budućnosti, neprekidnost vodoopskrbe, sanitarno–higijensku sigurnost kvalitete vode, optimalne investicijske i pogonske troškove te se mora uklapati u vodno gospodarenje šireg područja. Ovisno o porijeklu vode, izvorište može biti atmosfersko, površinsko ili podzemno (Vuković, 1996.). Atmosferska izvorišta vode se koriste u nedostatku drugih izvorišta i to uglavnom za opskrbu vodom manjih naselja, a ova vrsta izvorišta vodom se snabdijeva zahvaćanjem oborina (kiše ili snijega). Površinska izvorišta podrazumijevaju zahvaćanje vode iz rijeka, jezera ili mora, dok podzemna izvorišta podrazumijevaju zahvaćanje vode iz vodnog tijela sa slobodnim vodnim licem, vodnog tijela pod tlakom ili zahvaćanjem izvorske vode (Vuković, 1996).

Vodoopskrbni sustav grada Šibenika vodom za ljudsku potrošnju snabdijeva stanovništvo i privredne subjekte gradova Šibenika, Skradina i Vodice te stanovništvo i privredne subjekte obalnog dijela županije od mjesta Murtera, Pirovca do Rogoznice i Ražnja, otoke Zlarin i Prvić te naselja Dubrava, Danilo, Perković, Mirlović Zagora, Goriš, Pokrovnik, Pakovo Selo te dio naselja Unešića. Na šibenski vodoopskrbni sustav priključeno je ukupno 75 naselja, nešto više od sedamdeset i četiri tisuće stanovnika. U sklopu vodoopskrbne mreže, ukupne dužine približno 800 km, nalazi se i 55 vodosprema. Kontrolu zdravstvene ispravnosti vode iz vodoopskrbnog sustava grada Šibenika provodi Služba za ekologiju Zavoda za javno zdravstvo Šibensko-kninske županije uzorkovanjem vode u javnim objektima kao što su škole, vrtići i ugostiteljski objekti (Zavod za javno zdravstvo Šibensko-knisnke županije, 2017).

Osnovni zadatak pri snabdijevanju stanovništva vodom za piće je primjena procesa prerade vode u cilju postizanja zdravstveno-ispravne vode i kakvoće koja udovoljava odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15). Pri tome je dezinfekcija neizostavan postupak obrade. Cilj dezinfekcije je uništiti, odnosno smanjiti broj mikroorganizama na razinu koja u danim okolnostima i u uobičajenim uvjetima upotrebe nije opasna.

Danas su dostupne brojne metode dezinfekcije vode. U praksi se dezinfekcija vode najčešće provodi različitim klornim preparatima, a u novije vrijeme ultraljubičastim zrakama i ozonom. Pri odabiru načina dezinfekcije vode koja se potrošačima isporučuje putem vodoopskrbnog

sustava, važno je imati u vidu tehničke uvjete i cijenu postupka te mogućnost rezidualnog djelovanja dezinfekcijskog sredstva nakon što voda izađe iz pogona za preradu, a prije isporuke potrošačima (Nalko, 2005). Učinkovitost pojedinog dezinfekcijskog sredstva ovisi o nizu čimbenika, od kojih su najvažniji: (i) vrsta i doza dezinfekcijskog sredstva (i) mutnoća i kemijski sastav sirove vode, (ii) temperatura i pH vrijednost vode, (iv) vrsta i količina mikroorganizama u sirovoj vodi i (v) vrijeme kontakta dezinfekcijskog sredstva i vode (Mijatović i Matošić, 2008, Lee i sur., 2015)

Cilj ovog rada bio je analizom analitičkih izvješća monitoringa kakvoće vode uzorkovane iz vodoopskrbnog sustava grada Šibenika utvrditi učinkovitost dezinfekcije i mikrobiološku ispravnost vode tijekom petogodišnjeg razdoblja (2011. – 2015. godine). Učinkovitost dezinfekcije i mikrobiološka ispravnost vode određene su praćenjem koncentracije slobodnog rezidualnog klora te određivanjem vrijednosti mikrobioloških parametara zdravstvene ispravnosti vode za ljudsku potrošnju: broj kolonija na 22 °C i na 37 °C, ukupni koliformi, *Escherichia coli, Clostridium perfringens, Pseudomonas aeruginosa* i enterokoki. Grupiranjem pojedinih podataka utvrdila se povezanost kakvoće vode u vodoopskrbnom sustavu i pojedinog dijela godine.

MATERIJALI I METODE

Vodoopskrbni sustav Jaruga

Voda za šibenski vodoopskrbni sustav kojim upravlja tvrtka Vodovod i odvodnja d.o.o. iz Šibenika, zahvaća se na pet izvorišta i to: Jaruga, Torak, Jandrići, Kovča i Miljacka. Prema količini zahvaćene vode, odnosno s kapacitetom crpljenja od 1000 L/s, glavno izvorište je izvorište Jaruga koje se nalazi na području Nacionalnog parka Krka (Slika 1), a sastoji od tri crpne stanice, Jaruga I, Jaruga II i Jaruga III. Ostala navedena izvorišta s ukupnim kapacitetom crpljenja vod od 235 L/s koriste se samo u vrijeme povećane potrošnje vode, odnosno tijekom ljetnih mjeseci (Marguš, 2002; Jurković, 2016). Nakon zahvaćanja, voda se glavnim cjevovodom dovodi u pogon tvrtke Vodovod i odvodnja d.o.o. Šibenik gdje se kondicionira metodom filtracije i dezinfekcije kako bi kakvoća vode bila u skladu s odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15). Prosječna količina isporučene vode za ljudsku potrošnju dnevno iznosi 55000 m^3/dan.

Slika 1. Objekti izvorišta Jaruga na području Nacionalnog parka Krka
Fig. 1. Facilities at the source Jaruga in the National Park Krka

Određivanje učinkovitosti dezinfekcije

Dezinfekcija vode je postupak koji se provodi doziranjem klora u vodu u cilju uklanjanja i/ili smanjenja broja mikroorganizama. Pri doziranju klora u vodu, dio se klora troši na oksidaciju organskih i drugih tvari prisutnih u vodi. Kako koncentracije tvari koje reagiraju s klorom, uslijed krškog terena, tijekom godine osciliraju, i količina klornog preparata koju je potrebno dozirati se mijenja. Pojava viška klora nakon obavljene dezinfekcije vode ukazuje da je dezinfekcija uspješno provedena, a višak klora koji pri tome zaostaje u vodi, naziva se slobodni rezidualni klor. Učinkovitost dezinfekcije vode koja se distribuira potrošačima putem vodoopskrbnog sustava grada Šibenika praćena je tijekom petogodišnjeg razdoblja, odnosno tijekom 2011., 2012., 2013., 2014. i 2015. godine, pri čemu su određene vrijednosti sljedećih parametara: koncentracija slobodnog klora, broj kolonija na 22 °C i broj kolonija na 37 °C. Tijekom ispitivanog razdoblja ukupno je analizirano 1835 uzoraka, odnosno 2011. godine uzorkovano je 418, 2012. godine ukupno 426 uzoraka, 2013. godine 357 uzorkata, 2014. godine 309 uzoraka te 2015. godine uzorkovano je 325 uzoraka vode iz vodoopskrbnog sustava Jaruga. Odmah po uzorkovanju, na mjestu uzorkovanja, određene su koncentracije slobodnog rezidualnog klora. Svi uzorci su uzorkovani u polietilenske boce, prethodno ispirane deioniziranom vodom te pohranjeni u hladnjak na temperaturu 4 °C. Slobodni rezidualni klor određen je kolorimetrijskom DPD (N,N-dimetil-p-fenilendiamin) metodom (HRN EN ISO 7393-2:2001). Mjerenje se provodilo na način da se u uzorak vode dodao jastučić s DPD reagensom pri čemu je, u slučaju prisutnosti slobodnog rezidualnog klora, došlo do pojave crvene boje. Intenzitet boje bio je proporcionalan koncentraciji slobodnog rezidualnog klora, a određen je kolorimetrom. Prema Pravilniku o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 56/13, 64/15) maksimalno dozvoljena koncentracija slobodnog rezidualnog klora u vodi za ljudsku potrošnji iznosi 0,5 mg/L.
Broj kolonija na 22 °C i 37 °C obvezni su indikatorski parametri kakvoće vode koji su određeni metodom HRN ISO 6222:2000. Pojava aerobnih mikroorganizama u prirodnim vodama je uobičajena, a navedeni parametri pomažu pri procjeni prisustva i drugih vrsta mikroorganizama u analiziranom uzorku vode. Broj kolonija na 22 °C i 37 °C koristi se za procjenu učinkovitosti postupka dezinfekcije vode i ukazuje na brojnost bakterija u vodoopskrbnom sustavu.
Ukupni koliformi i Escherichia coli određeni su metodom HRN EN ISO 9308-1, enterokoki metodom HRN EN ISO 7899-2, a Clostridium perfringens i Pseudomonas aeruginosa metodom HRN EN ISO 16266. Prema Pravilniku o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15), maksimalno dozvoljen broj kolonija na 22 °C iznosi 100/1 mL, odnosno 20/1 mL na 37 °C, dok pojavnost ukupnih koliforma, Escherichia coli, enterokoka te Clostridium perfringens i Pseudomonas aeruginosa u 100 mL ispitivanog uzorka nije dozvoljena, odnosno maksimalno dozvoljen broj za prethodno navedene parametre iznosi 0/100 mL.

REZULTATI I RASPRAVA

Tijekom petogodišnjeg razdoblja (2011.-2015.) praćena je učinkovitost dezinfekcije na mikrobiološku ispravnost vode vodoopskrbnog sustava grada Šibenika. Učinkovitost dezinfekcije određena je mjerenjem vrijednosti sljedećih parametara kakvoće vode: koncentracija slobodnog rezidualnog klora, broj kolonija na 22 °C i broj kolonija na 37 °C,

ukupni koliformi, Escherichia coli, enterokoki, Clostridium perfringens i Pseudomonas aeruginosa. Ukupno je analizirano 1835 uzoraka vode iz vodoopskrbnog sustava Jaruga.
Slobodni rezidualni klor (SRK) je koncentracija klora izraženu u mg/L koja zaostaje u vodi kao višak nakon reakcije klora s tvarima koje mogu oksidirati u vodi i nakon završenog procesa dezinfekcije vode. Prisutnost slobodnog rezidualnog klora u uzorcima vode iz vodoopskrbnog sustava je obvezna jer sprječava naknadnu kontaminaciju vode i osigurava mikrobiološku ispravnost vode tijekom njene distribucije putem vodoopskrbnog sustava. Prisutnost rezidualnog klora također je i indikator učinkovite dezinfekcije vode, a ravnotežna koncentracija slobodnog rezidualnog klora u vodi ovisi prije svega o kemijskom i mikrobiološkom sastavu i temperaturi vode (Habuda-Stanić i sur., 2013). Tijekom ispitivanog petogodišnjeg razdoblja zabilježene koncentracije slobodnog rezidualnog klora bile su u rasponu od 0,03 do 0,4 mg/L. U Tablici 1 prikazane su prosječne vrijednosti koncentracija slobodnog rezidualnog klora tijekom pojedinih godišnjih doba u razdoblju od 2011. do 2015. godine. Vrijednosti su se kretale od 0,15 do 0,24 mg/L tijekom zimskih mjeseci, od 0,22 do 0,25 mg/L tijekom proljetnih mjeseci, od 0,14 do 0,22 mg/L tijekom ljetnih mjeseci te od 0,18 do 0,25 mg/L slobodnog rezidualnog klora tijekom jesenjih mjeseci.

Tablica 1. Prikaz prosječnih vrijednosti koncentracija slobodnog rezidualnog klora u razdoblju od 2011. do 2015. godine tijekom pojedinog godišnjeg doba
Table 1. Average concentration levels of free residual chlorine in the period from 2011 to 2015 during specific seasons

godina	**SLOBODNI REZIDUALNI KLOR (mg/L)**			
	zima	**proljeće**	**ljeto**	**jesen**
2011.	0,19	0,25	0,14	0,21
2012.	0,17	0,24	0,17	0,25
2013.	0,15	0,22	0,21	0,18
2014.	0,24	0,23	0,18	0,19
2015.	0,21	0,25	0,22	0,21
prosječna vrijednost	**0,192**	**0,238**	**0,184**	**0,208**

Iz prikazanih vrijednosti prosječnih vrijednosti koncentracija slobodnog rezidualnog klora u vodi uzorkovanoj iz vodoopskrbnog sustava po pojedinim godišnjim dobima (Tablica 1) uočava se da se najmanje vrijednosti slobodnog rezidualnog klora bilježe tijekom zimskih i ljetnih mjeseci. Navedena povezanost smanjenja koncentracije SRK i dijela godine uzrokovana je promjenom u kemijskom i mikrobiološkom sastavu sirove vode tijekom zimskih mjeseci, odnosno porastom temperature vode tijekom ljetnih mjeseci. Naime, uslijed krškog tla sastavljenog od mikroporoznih stijena kalcijeva i magnezijeva karbonata, tijekom zimskih mjeseci dolazi do intenzivnijeg procjeđivanja padalina u podzemne vodonosnike što se najčešće manifestira u obliku povećanja koncentracije organskih tvari i broja mikroorganizama, odnosno zamućenja sirove podzemne vode (Bonacci, 2017). Navedena promjena kakvoće sirove vode povećava utrošak klora, jer, osim što se povećava ukupni broj mikroorganizama, uslijed intenzivnijeg procjeđivanja voda dolazi i do povećanja količine organskih tvari koje reagiraju s klorom te smanjuju ravnotežnu koncentraciju slobodnog rezidualnog klora. S druge

strane, tijekom ljetnih mjeseci, prosječna temperatura vode u vodoopskrbnom sustavu povećava se prosječno za 2 do 3 °C što utječe na povećanu reaktivnost i potrošnju klora (Nouri i sur., 2015).

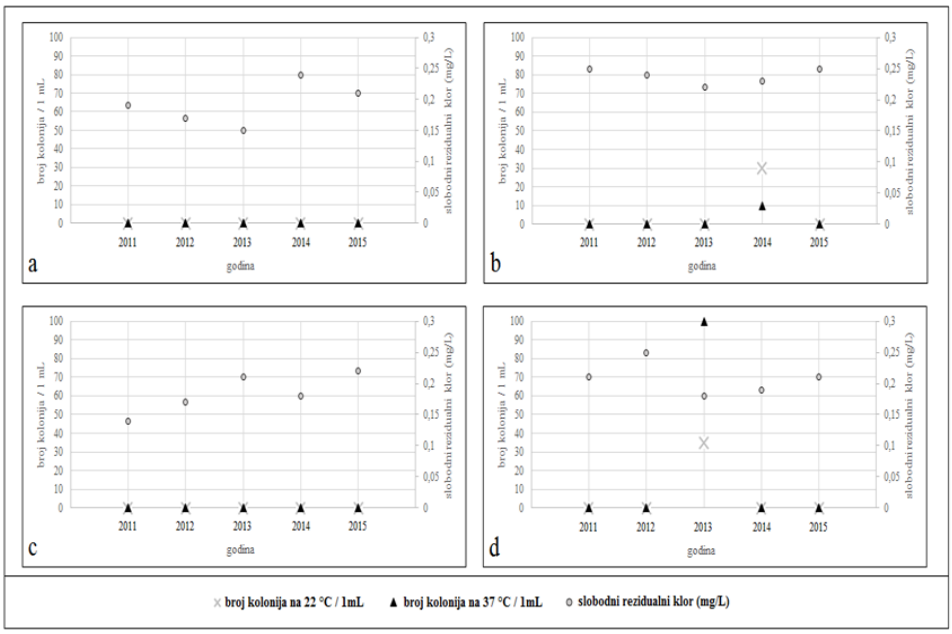
Tablica 2. Prikaz vrijednosti mikrobioloških parametara uzoraka vode iz vodoopskrbnog sustava Jaruga koji nisu bili u skladu s odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15) tijekom razdoblja 2011.-2015. godine.
Table 2. The values of microbiological parameters of water samples from the water supply system Jaruga that failed to comply to the provisions of the Ordinance on the Compliance Parameters and Analysis Methods for Water Used for Human Consumption (MZ HR, Official Gazette of the Republic of Croatia 125/13, 141/13, 128/15) during the period 2011-2015

Uzorak	**Broj kolonija na 22°C /100 mL**	**Broj kolonija na 37°C/ 100 mL**	**Ukupni koliformi /100 mL**	***E. coli* /100 mL**	**Enterokoki /100 mL**	***C. perfringens* /100 mL**	***P. aeruginosa* /100 mL**
M.D.K.*	**100**	**20**	**0**	**0**	**0**	**0**	**0**
2011. godina							
1	800	80	16	2	5	3	25
2	200	1000	10	-	-	-	26
3	750	800	13	-	-	-	-
4	15	800	-	-	-	-	-
5	180	40	-	-	-	-	-
6	-	200	-	-	-	-	-
2012. godina							
1	-	80	10	2	-	-	37
2	-	-	22	-	-	-	15
2013. godina							
1	410	400	47	17	35	12	4
2	150	60	8	9	12	-	20
3	190	250	25	-	-	-	8
4	120	50	31	-	-	-	-
2014. godina							
1	130	>300	5	1	-	-	8
2	>300	-	5	9	-	-	24
3	-	-	56	5	-	-	40
4	-	-	15	-	-	-	-
2015. godina							
1	-	50	-	-	-	-	27

** maksimalno dozvoljen broj prema Pravilniku o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15)*

Uz koncentracije slobodnog rezidualnog klora, učinkovitost dezinfekcije vode iz šibenskog vodoopskrbnog sustava tijekom ispitivanog razdoblja praćena je i određivanjem mikrobioloških parametara: broj kolonija na 22 °C i broj kolonija na 37 °C, ukupni koliformi, Escherichia coli, enterokoki, Clostridium perfringens i Pseudomonas aeruginosa. Rezultati analiza pokazuju mikrobiološku ispravnost 99,07 %, od ukupno ispitanih 1835 uzoraka. Pri tome je 2011. godine, od ukupno 418 ispitanih, 6 uzoraka vode bilo zdravstveno neispravno u

pogledu mikrobioloških parametara. 2012. godine od 426 uzoraka, odredbama Pravilnika u pogledu mikrobioloških parametara nisu odgovarala 2 uzorka, 2013. godine od 357 ispitana uzorka nisu odgovarala 4 uzorka, a identičan broj nesukladnih uzoraka utvrđen je i 2014. godine kada je ukupno analizirano 309 uzoraka vode. 2015. godine samo jedan analiziran uzorak vode iz vodoopskrbnog sustava Jaruga nije bio mikrobiološki ispravan.



Slika 2. Usporedba prosječnih koncentracija slobodnog rezidualnog klora i prosječnih broja kolonija na 22 °C i 37 °C tijekom pojedinih godišnjih doba u razdoblju od 2011. do 2015. godine (a - zima, b - proljeće, c - ljeto, d - jesen).
Fig. 2. Comparison of average concentrations of free residual chlorine and the average number of colonies at 22 °C and 37 °C during specific seasons, in the period from 2011 to 2015 (a - winter, b - spring, c - summer, d - autumn)

U Tablici 2 prikazane su utvrđene vrijednosti pojedinih mikrobioloških parametara uzoraka vode za ljudsku potrošnju uzorkovane iz vodoopskrbnog sustava Jaruga koji nisu bili u skladu s odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15) tijekom ispitivanog petogodišnjeg razdoblja (2011.-2015. godina).
Grupiranjem prosječnih vrijednosti koncentracije slobodnog rezidualnog klora i prosječnog broja kolonija na 22 °C i 37 °C ovisno o dijelu godine, odnosno o godišnjem dobu tijekom ispitivanog petogodišnjeg razdoblja, praćena je učinkovitost dezinfekcije vode iz šibenskog vodoopskrbnog sustava Jaruga (Slika 2).
Rezultati analiza pokazuju mikrobiološku ispravnost i odsutnost aerobnih mikroorganizama u 99,07%, od ukupno ispitanih 1835 uzoraka. Povišene vrijednosti broja kolonija na 22 °C i 37 °C zabilježene su tijekom jesenskih mjeseci 2013. i proljetnih mjeseci 2014. godine. Tijekom

ostalih mjeseci i godišnjih doba u razdoblju 2011.-2015., ispitani uzorci vode za ljudsku potrošnju, uzorkovani iz vodoopskrbnog sustava Jaruga, imali su vrijednosti broja kolonija na 22 °C i 37 °C manje od maksimalno dozvoljenog broja prema Pravilniku o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15). Odsutnost aerobnih mikroorganizama u većini ispitanih uzoraka vode ukazuje na visoku kakvoću sirove vode, učinkovit proces dezinfekcije vode te pravilno održavanje vodoopskrbnog sustava Jaruga. Kako je kod uzoraka uzorkovanih tijekom jesenskih mjeseci 2013. i proljetnih mjeseci 2014. godine mjeseci, kada je zabilježena nesukladnost uzoraka s Pravilnikom, zabilježena i prisutnost slobodnog rezidualnog klora, može se zaključiti kako je pojava mikroorganizama posljedica naknadne kontaminacije vode u vodoopskrbnoj mreži uslijed oštećenja cjevovoda.

ZAKLJUČAK

U ovom radu prikazana je učinkovitost dezinfekcije vode za ljudsku potrošnju koja se vodoopskrbnim sustavom Jaruga isporučuje stanovnicima grada Šibenika tijekom razdoblja 2011.-2015. godine. Uzorkovano je ukupno 1835 uzoraka s ciljem utvrđivanja učinkovitosti dezinfekcije i mikrobiološke ispravnosti vode. Pri tome su praćene koncentracije slobodnog rezidualnog klora te vrijednosti mikrobioloških parametara zdravstvene ispravnosti vode za ljudsku potrošnju. Grupiranjem pojedinih podataka utvrdila se i povezanost kakvoće vode u vodoopskrbnom sustavu i pojedinog dijela godine.

Na osnovi obrade rezultata mogu se izvesti sljedeći zaključci:

- Vrijednosti koncentracija rezidualnog slobodnog klora u analiziranim uzorcima bile su u skladu s odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15) i nisu prelazile Pravilnikom zadanu MDK vrijednost od 0,5 mg/L.
- Od ukupno 1835 ispitanih uzoraka, sedamnaest uzoraka (<0,1%) nije bilo u skladu s odredbama Pravilnika (NN 125/13, 141/13, 128/15). Pri tome je najčešći uzrok nesukladnosti kakvoće vode s navedenim Pravilnikom bio povišeni broj kolonija na 37 °C (13 uzoraka) te pojavnost ukupnih koliforma (13 uzoraka).
- U jedanaest, od ukupno 1835 analiziranih uzoraka, zabilježena nedozvoljena pojavnost Pseudomonas aeruginosa, u 10 uzoraka nedozvoljeni broj kolonija na 22 °C, dok je u 7 uzoraka zabilježena pojavnost Esherihia coli. Pojavnost enterokoka i Clostridium perfringens bio je najrjeđi razlog nesukladnosti uzoraka, u tri, odnosno dva analizirana uzorka.
- Koncentracije slobodnog rezidualnog klora tijekom razdoblja 2011.-2015. ovisile su o dijelu godine. Najmanje prosječne koncentracije slobodnog rezidualnog klora zabilježene su, uslijed promjene kakvoće vode, tijekom ljetnih i zimskih mjeseci.

Usprkos oscilacijama ravnotežnih koncentracija slobodnog rezidualnog klora i zabilježenim nesukladnim uzorcima vode iz vodoopskrbnog sustava Jaruga, može se zaključiti da je učinkovitost dezinfekcije dobra, a mikrobiološka kakvoća vode za ljudsku potrošnju vodoopskrbnog sustava Jaruga iznimna jer je više od 99% ispitanih uzoraka tijekom ispitivanog petogodišnjeg razdoblja bilo u skladu s odredbama Pravilnika o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju (MZ HR, NN 125/13, 141/13, 128/15).

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THE EFFICIENCY OF DISINFECTION AND THE MICROBIOLOGICAL QUALITY OF DRINKING WATER IN THE TOWN OF ŠIBENIK

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Quality of drinking water is influenced by many factors, primarily by the source (well, lake, river), processing and sanitary conditions in water-supply system and infrastructure.
Safe drinking water (a) is free of presence of mycroorganisms, parasites and their developmental forms in numbers that pose a risk for human health, (b) is free of harmful compounds in concentrations that individually or in combination pose a risk for human health, (c) i sin accordance with parameters of quality defined by Croatian laws (MZ HR, NN 56/13, 65/15, 104/17) and by-laws (MZ HR, NN 125/13, 141/13, 128/15).
The aim of this research was to evaluate efficiency of disinfection and microbial quality of drinking water supplied by water-supply system Jaruga to Šibenik residents. The data on free and bound chlorine and microbial parameters (colony count at 22 °C and 37 °C, total coliforms, *Escherichia coli, Closridium perfringens, Pseudomonas aeruginosa, enterococci)* collected during 5-year period (2011-2015) were analysed. Disinfection efficiency was established by grouping of values, depending on season, since water is collected from the wells that are influenced by rainfall due to geological properties.

Keywords: water for human consumption, disinfection, chlorination, town of Šibenik

Topic: Food safety / Sekcija: Zdravstvena sigurnost hrane

KONTROLA ZDRAVSTVENE ISPRAVNOSTI PITKE VODE PRIVATNIH BUNARA

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SAŽETAK

Za stanovnike Slavonije izvor vode od davnina su bili bunari u vlastitom dvorištu. Bogatstvo podzemne vode na relativno malim dubinama, kao i pogodni sastav tla, omogućavao je iskapanje bunara. Razvojem tehnologije i podizanjem standarda stanovništva, u Hrvatskoj je oko 80 % naselja pokriveno vodoopskrbnim sustavima. Nažalost, visoka cijena priključka na javni vodovod i teške materijalne prilike dijela stanovnika, i dalje su razlog isključivog korištenja vode iz vlastitih bunara. Jedno od takvih naselja je i selo Pavlovci u Požeško-slavonskoj županiji, gdje oko 95 % domaćinstava i dalje koristi jedino vodu iz vlastitih bunara.
U radu se analizirala mikrobiološka ispravnost vode iz privatnih bunara, u dva sela na području općine Brestovac, u Požeško-slavonskoj županiji, odnosno njena zdravstvena ispravnost. Analizirana bunarska voda koristi se za sve potrebe u domaćinstvu, kao i za piće. Analize su obavljene u ovlaštenom laboratoriju Zavoda za javno zdravstvo Požeško-slavonske županije. Uzorci vode su analizirani na ukupne koliformne bakterije, *Escherichiu coli,* enterokoke, *Pseudomonas aeruginosa* te ukupan broj kolonija na 37 ºC i 22 ºC. Analizom uzetih uzoraka, utvrđeno je da niti jedan uzorak vode nije zdravstveno ispravan, odnosno ne odgovara zakonski propisanim maksimalno dopuštenim koncentracijama (MDK).

Ključne riječi: mikrobiološka ispravnost vode, bunar, *Escherichia coli*, enterokoki, *Pseudomonas aeruginosa.*

UVOD

Život na nekom planetu uvjetovan je postojanjem vode. Tako možemo reći da cjelokupan život na Zemlji ovisi o raspoloživim količinama vode. Kolika je ovisnost čovjeka o vodi govori činjenica da su sve civilizacije nastale u područjima s dovoljnom količinom pitke vode. Stoljećima je količina i kakvoća vode bila dostatna za ljudske potrebe. Eksponencijalnim rastom broja stanovnika na Zemlji povećale su se potrebe za vodom, a porastom industrijske i poljoprivredne proizvodnje, povećale su se i količine otpadnih voda. Problem otpadnih voda je što sadrže otrovne tvari čijim ispuštanjem u prirodne vodne prijemnike dolazi do poremećaja prirodnog eko-sustava (Tedeschi, 1997).
Danas je voda postala prirodni resurs za koji se vode ratovi, ponekad paradigmatski ratovi, jer voda je postala resurs koji definira ekonomsku i političku moć (Shiva, 2006).

Vode su kao i šume i poljoprivredno zemljište, dobra od posebna značaja za Republiku Hrvatsku. Hrvatska je zemlja koja raspolaže velikim vodnim resursima, kako nadzemnih tako i podzemnih voda. Hrvatska po vodnom bogatstvu spada u skupinu zemalja relativno bogatih vodom, a prema istraživanju UNESCO-a iz 2003. godine, na 5. je mjestu u Europi i na 42. mjestu u svijetu (Hrvatske vode, 2009).

U Hrvatskoj je za oko 80 % stanovništva dostupna voda iz vodoopskrbnih mreža, a 74 % stanovništva je priključeno na vodoopskrbni sustav. 14 % stanovništva opskrbljuje se iz vlastitih zdenaca (Plišić, n.d.).

U hrvatskoj općini Brestovac u Požeško-slavonskoj županiji postoje naselja s vodovodnom mrežom, ali mali broj stanovnika je priključen na istu. U selima gdje je provedeno istraživanje, Pavlovci i Vilić Selo, oko 95 % domaćinstava nije priključeno na vodovodni sustav. Razlog tomu možemo potražiti u visokoj cijeni priključka, ali i sve većoj cijeni vode, te niskom standardu stanovništva. Kako stanovnici koriste vodu iz vlastitih bunara za sve svoje vlastite potrebe, između ostalog i za piće, cilj rada je bio provesti uzorkovanje bunarske vode s više lokacija općine Brestovac i pomoću analitičkih metoda utvrditi, je li bunarska voda uzetih uzoraka mikrobiološki ispravna i zadovoljava li parametre koji su određeni zakonskom regulativom o zdravstvenoj ispravnosti vode.

Zdravstvena ispravnosti vode za ljudsku potrošnju u Hrvatskoj definirana je Zakonom o vodi za ljudsku potrošnju te izmjenama i dopunama (NN 56/13, 64/15, 104/2017), Pravilnikom o parametrima sukladnosti i metodama analize vode za ljudsku potrošnju te izmjenama i dopunama (NN 125/13, 141/13, 128/15).

Uloga vode u čovjekovom organizmu je nezamjenjiva u razmjeni nutrijenata, u održavanju osobne i opće higijene, pripremi hrane, odnosno održanju zdravlja i života čovjeka. Uz sve dobrobiti što donosi voda, ona je medij prenošenja ne samo veoma teških zaraza, već i opasnih kemikalija, kancerogenih, radioaktivnih i drugih tvari.

Jedan od osnovnih pokazatelja zdravstvene ispravnosti vode, njena je mikrobiološka ispravnost. Osim mikroorganizma koji su u vodama dio normalne vodene mikroflore, u vodi mogu biti prisutni i patogeni mikroorganizmi koji dospijevaju u vodu putem različitih zagađenja. Oni se u vodi hrane prisutnim hranjivim tvarima dospjelim iz zraka, ispiranjem zemljišta ili najčešće ispuštanjem različitih otpadnih voda (Tušar, 2009).

Osiguranje zdravstvene ispravnosti vode za piće jedna je od osnovnih mjera zaštite zdravlja stanovništva. Zdravstveno neispravna voda može uzrokovati razne bolesti. Vodom prenosivi organizmi su bakterije, virusi, protozoe i helminiti. U vodi se mogu naći i drugi organizmi kao što su alge i srodni planktoni koji su uzročnici okusa i mirisa vode (Gulić, 2003).

Bakterije i virusi su najčešći uzročnici različitih bolesti kojima se čovjek može zaraziti kako putem zdravstveno neispravne različite hrane tako i vode. Poznato je da opasnost ne predstavljaju samo mikroorganizmi već i njihovi toksini koji zaostaju u namirnicama.

Nije niti praktično, a niti potrebno analizirati vodu na prisutnost svih potencijalno patogenih mikroorganizama u vodi. Postoji nekoliko vrsta mikroorganizama koji se nalaze u probavnom traktu sisavaca, a koji su indikator fekalnog zagađenja vode. Njihova prisutnost ili prelaženje maksimalno dopuštenih koncentracija pokazatelj su kontaminacije vode. Ta skupina uključuje različite vreste *Escherichia coli, Enterobacter aerogenes i Klebsiella pneumoniae.* Ovi mikroorganizmi u vodi mogu preživjeti nekoliko tjedana, pa čak i mjeseci (Duraković, 1996).

Bakterije su jednostanični organizmi koji imaju goleme razlike u obliku i veličini. Većina je duljine od 2 do 8 μm, a promjera od 0,2 do 2,0 μm. Postoji nekoliko osnovnih

oblika bakterija: kugličaste – oblik *coccus* (pl. *cocci,* u značenju bobice), oblik štapića – *bacillus* (pl. *bacilli*, u značenju mali štapići), oblik spirale (pl. *spirilla*), oblik kvadrata – *arcula* (pl. *arculae*, u značenju kocke), i oblik zvijezde – *astra* (pl. *astrae*, u značenju zvijezde). Bakterija kao jednostanični organizam obavlja sve reakcije metabolizma za koje viši organizmi trebaju milijune specijaliziranih stanica. To omogućuje složena struktura prokariotskih stanica. Sve bakterije posjeduju nukleotid, ribosome i citoplazmene (stanične) membrane. Mnoge bakterije imaju staničnu stijenku koja im osigurava stalan oblik stanice (Duraković i Duraković, 2001).

Razmnožavanje bakterija počinje kada one postignu veličinu svojstvenu određenoj vrsti, u povoljnim hranidbenim, energetskim, atmosferskim i temperaturnim uvjetima. Povoljni uvjeti podrazumijevaju dovoljne količine hranjivih tvari, izvora energije, ugljika i dušika, prisutnost ili odsutnost kisika, što ovisi jesu li bakterije aerobne ili anaerobne. Također, potrebna je optimalna temperatura za razvoj bakterija, povoljna vlaga, kao i pH okoline. Najveći broj bakterija razmnožava se binarnom diobom pri kojoj od jedne bakterijske stanice nespolnim načinom nastaju dvije nove stanice (Volner i sur., 2005).

Pijući i koristeći za pripremu hrane vodu koja je mikrobiološki neispravna, može doći do pojave različitih bolesti . Uglavnom su to bolesti probavnog sustava, a ponekad se radi o teškim bolestima kao što su hepatitis, tifus, dizenterija, infekcije urinarnog trakta, upale pluća, meningitis i druge, kada može doći i do smrtnih posljedica.

Dezinfekcijom vode osigurava se njezina mikrobiološka ispravnost, odnosno uništavaju se zarazne bakterije intestinalnog ili fekalnog porijekla, do razine koja isključuje mogućnost zaraze istima (Gulić, 2003).

Dezinfekcija vode u vodoopskrbnim sustavima je obavezan postupak, dok kod privatnih bunara to ovisi o brizi vlasnika. Osim dezinfekcije vode radi očuvanja njene zdravstvene ispravnosti, potrebno je zaštititi izvorišta vode i redovno ispitivati njenu kvalitetu.

Najrašireniji način dezinfekcije pitke vode je korištenje spojeva na bazi klora kao što je natrijev hipoklorit ili izocijanurat koji inaktiviraju prione, a polagano otpuštanje slobodnog klora rezultira produženim djelovanjem. Klor je dezinficijens s baktericidnim učinkom koji inhibira enzimske reakcije u stanici, denaturira bjelančevine i inaktivira nukleinske kiseline. Djeluje na neke spore, inaktivira neke viruse te djeluje fungicidno (Volner i sur., 2005).

Kontrolu zdravstvene ispravnosti vode za piće u Republici Hrvatskoj provodi hrvatski Zavod za javno zdravstvo sa svojim županijskim ispostavama.

MATERIJALI I METODE

U radu se analizirala voda iz šest seoskih bunara sela Pavlovci i Vilić Sela, na području Općine Brestovac, u razdoblju od dvije godine. Bunari se nalaze u dvorištima privatnih kuća i izvor su vode za potrebe domaćinstava koja se koristi i kao pitka voda. Prije izgradnje vodoopskrbne mreže gotovo svako domaćinstvo na području Slavonije imalo je vlastiti bunar za osobne potrebe. U selima koja su obuhvaćena u radu postoji javna vodoopskrbna mreža, no tek pet posto domaćinstava je priključeno na javni vodovodni sustav te je iz tih razloga potrebna česta kontrola kvalitete bunarske vode od strane vlasnika. Čimbenici koji mogu utjecati na zdravstvenu ispravnost vode su: lokacija bunara, odnosno udaljenost od potencijalno mogućih zagađivača, materijali od kojih je bunar

izgrađen, odnosno zaštićen od procijeđenih voda, vanjska zaštićenost, dubina bunara, kao i redovita analiza i dezinfekcija bunarske vode.
U radu su prikazani rezultati analiza koji su rađeni u ovlaštenom laboratoriju Zavoda za javno zdravstvo Požega, u kojemu se rade sve analize pitke vode na području Požeško-slavonske županije.

Slika 1. Mjesta uzorkovanja vode
Fig. 1. Water sampling points

Materijali istraživanja

Bunar 1 se nalazi u mjestu Pavlovci na adresi Pavlovci 55. Bunar je udaljen od rijeke Orljave oko 150 metara, udaljenost od septičke jame 35 metara. Na udaljenosti od 250 m nalazi se susjedova farma svinja. Dubok je oko 5 metara, zidan betonom. Izvana je zaštićen okapnicom i sitnom mrežom. Dezinficiran je 2014. godine te poslije analize vode 2016. U ljetnim razdobljima bunar ne presušuje i zadržava određeni nivo vode.
Bunar 2 se nalazi u mjestu Pavlovci na adresi Pavlovci 18. Bunar je udaljen od rijeke Orljave oko 150 metara, udaljenost od staje je 50 m. Bunar je dubok oko 5 metara, zidan betonom. Izvana je zaštićen okapnicom i sitnom mrežom. Dezinficiran je 2014. godine te poslije analize vode 2016. U ljetnim razdobljima bunar ne presušuje i zadržava određeni nivo vode.
Bunar 3 se nalazi u mjestu Pavlovci na adresi Pavlovci 45. Bunar je udaljen od rijeke Orljave oko 600 metara. Bunar je dubok oko 12 metara, zidan betonskim cijevima. Izvana je poklopljen s metalnim poklopcem. Bunar duže vrijeme nije dezinficiran do 2016. godine. U ljetnim razdobljima bunar presušuje i nema vode sve do većih padalina.
Bunar 4 se nalazi u mjestu Pavlovci na adresi Pavlovci 6. Bunar je udaljen od rijeke Orljave oko 100 metara, udaljenost od septičke jame oko 25 m. Bunar je dubok oko 5 metara, zidan betonom. Izvana je zaštićen okapnicom i sitnom mrežom. Bunar se duže vrijeme ne koristi i nije dezinficiran do 2016. U ljetnim razdobljima bunar ne presušuje i zadržava određeni nivo vode.
Bunar 5 se nalazi u mjestu Pavlovci na adresi Pavlovci 25. Bunar je udaljen od rijeke Orljave oko 150 metara, a od septičke jame oko 20 m. Bunar je dubok oko 11 metara, zidan betonskim cijevima. Izvana je zaštićen i zatvoren betonskim poklopcem. Dezinficiran je 2014. godine te poslije analize vode 2016. U ljetnim razdobljima bunar ne presušuje i zadržava određeni nivo vode.
Bunar 6 se nalazi u mjestu Vilić Selo na adresi Vilić Selo 55. Bunar je udaljen od rijeke Orljave oko 100 metara. Dubok je oko 12 metara, zidan betonskim cijevima. Izvana je

zaštićen drvenim daskama. Dezinficiran je 2014. godine te poslije analize vode 2016. U ljetnim razdobljima bunar ne presušuje i zadržava određeni nivo vode.

Metode istraživanja

U vodi prisutne patogene bakterije su bakterije fekalnog porijekla i zato se provode analize na; ukupne koliformne bakterije, *Escherichia coli,* enterokoki, *Pseudomonas aeruginosa* te ukupan broj aerobnh mezofilnih bakterija. Metoda detekcije koliformnih bakterija kao i *Escherichia coli*, enterokoka te *Pseudomonas aeruginosa* vrši se metodom membranske filtracije.

Metoda detekcije i brojanja Escherichia coli i koliformnih bakterija metodom membranske filtracije

Koliformne bakterije su primarno nepatogene i normalno obitavaju u donjem intestinalnom traktu čovjeka i toplokrvnih životinja gdje osiguravaju pravilnu probavu hrane. Koliformne bakterije uključuju 15 vrsta bakterija iz porodice *Enterobacteriaceae,* fakultativno su anaerobni Gram-negativni štapići. Infekcije ovim bakterijama javljaju se u slučaju oslabljenog imuniteta (Hrenović, n.d.).
Escherichia coli je Gram-negativni, asporogeni, aerobni i fakultativno anaerobni štapić. To je jedina vrsta iz roda *Escherichia*, porodica *Enterobacteriacae,* koja je bitna u kontekstu humane patologije. Najčešće uzrokuje infekcije mokraćnih puteva. Raznolikost ove bakterijske vrste omogućuje joj kolonizaciju i opstanak u različitim uvjetima, bilo u vanjskom okolišu ili u životinjskim domaćinima. Fiziološki je pronalazimo kao dio normalne crijevne mikroflore gdje zajedno s ostalim mikroorganizmima tvori simbiotsku vezu s nositeljem, priskrbljujući hranjive tvari, signalne molekule za regulaciju razvoja i imunosnog sustava, a ujedno pruža zaštitu od vanjskih patogena. Ipak, neki serotipovi *E.coli* mogu poprimiti patogene karakteristike i uzrokovati infekciju gotovo svih tkiva ili organa (Kosalec, 2016).
U hrani se lako i brzo razmnožavaju. Određivanje prisutnosti *E. coli* služi kao metoda za dokazivanje fekalne kontaminacije. *E. coli* osjetljiva je na uobičajene dezinficijense (Duraković i Duraković, 1998).
Detekcija i brojanje koliformnih bakterija *i Escherichia coli* metodom membranske filtracije, rađena je prema normi HRN EN ISO 9308-1;2014. Metoda se temelji na membranskoj filtraciji uzorka vode volumena 100 mL, nakon čega slijedi inkubacija na selektivnoj podlozi, na temperaturi od 37 ˚C 24 sata te brojanje i procjena kolonija nakon potvrdnog testa. Pojava tamnoplave do ljubičaste boje podrazumijeva se kao prisutnost *E. coli*. Kako bi se potvrdile vjerojatne koliformne bakterije koje nisu *E. coli,* provodi se Oksidaza test. Koliformne bakterije su oksidaza negativne. Iz broja tipičnih kolonija izbrojanih na filterima i rezultata potvrdnih testova procjenjuje se broj koliformnih bakterija te *Esherichia coli*. Procijenjeni broj tipičnih kolonija izražava se kao CFU/100 mL.

Metoda detekcije i brojanja enterokoka metodom membranske filtracije

Enterokoki su Gram-pozitivne bakterije. Ovu grupu čine bakterije roda *Enterococcus* s ukupno 16 vrsta. Široko su rasprostranjeni u okolišu što upućuje na njihovu otpornost i

u nepovoljnim uvjetima rasta i razmnožavanja. Osjetljivi su na povišene temperature iznad 55 °C. Dio su normalne mikroflore probavnog sustava kralježnjaka. Fekalni enterokoki pogodna su grupa bakterija za određivanje higijenske kvalitete vode (Hrenović, n.d.). Kod ljudi mogu uzrokovati infekcije mokraćnog sustava, kao i respiratorne probleme. Enterokoki su važni i kao uzročnici bolničkih infekcija otporni na određene grupe antibiotika (Bayraktar, 2011).
Metoda detekcije i brojanje enterokoka metodom membranske filtracije provedena je prema normi HRN EN ISO 7899-2;2000. Metoda se temelji na membranskoj filtraciji uzorka vode volumena 100 mL. Filter papir s filtratom nasađuje se na selektivnu podlogu i inkubira 44±4 sata, na 36±2 °C. Nakon inkubacije pobroje se sve kolonije boje kestena, crvene ili ružičaste, kao tipične kolonije. Dokazivanje enterokoka provodi se Bile aesculin azide agarom. Nakon inkubacije za dokazivanje enterokoka pobroje se sve kolonije tamnosmeđe ili crne boje, kao kolonije enterokoka. Rezultati se određuju iz broja tipičnih kolonija izbrojanih na filterima i rezultata potvrdnih testova. Procijenjeni broj tipičnih kolonija izražava se u CFU/100 mL.

Metoda za određivanje ukupnog broja kolonija na 22 °C i 37 °C

Aerobne mezofilne bakterije su bakterije koje rastu u temperaturnom rasponu od 20 do 45 °C, uz prisutnost kisika. Većini ovih bakterija optimalna temperatura rasta je 37 °C, što predstavlja čovjekovu tjelesnu temperaturu. Dokazivanje njihove prisutnosti i ukupnog broja obavezno se radi kod mikrobiološke analize vode, kao i svih prehrambenih namirnica. Povećani broj aerobnih mezofilnih bakterija indikator je starosti ili lošije mikrobiološke kakvoće namirnice, odnosno početka procesa kvarenja namirnice. Određivanje ukupnog broja aerobnih mezofilnih bakterija provodila se prema normi HRN EN ISO 6222;2000. Metoda se temelji na miješanju određenog volumena uzorka vode (1 mL ili 0,1 mL) s hranjivim agarom u Petrijevoj zdjelici, inkubacija je 44±4 sata na temperaturi 37±2 °C i 68±44 sati na 22±2 °C te procjena broja kolonija po mL uzorka. Procijenjen broj kolonija izražava se kao CFU/1 mL.

Metoda detekcije i brojanje Pseudomonas aeruginosa metodom membranske filtracije

Bakterije iz roda *Pseudomonas* su aerobni Gram-negativni štapići, pripadaju velikom rodu bakterija široko rasprostranjenih u tlu, vodi i svježim namirnicama. Osobito ih možemo naći u higijenski neispravnoj vodi, mesu, povrću i hrani od plodova mora. Mogu se razmnožavati na temperaturi od 4-42 °C i kao psihrotrofi najčešći su uzročnik kvarenja svježe smrznutih namirnica (Duraković i Duraković, 2001).
Pseudomonas aeruginosa je poznata kao uzročnik mnogih infekcija. Većina infekcija izazvana *P. aeruginosa* zbiva se u hospitaliziranih bolesnika, osobito onih oslabljenog imuniteta. *P. aeruginosa* je drugi po redu najčešći uzrok infekcija u jedinicama intenzivne skrbi. Bolesnicima zaraženim HIV–om, osobito onima u uznapredovalim stadijima, prijeti infekcija s *P. aeruginosa* iz vanjske sredine. Infekcije *Pseudomonasom* se mogu razviti na mnogim mjestima, uključujući kožu, potkožno tkivo, kosti, uši, oči, mokraćni sustav i srčane zaliske. Mjesto infekcije ovisi

o mjestu ulaska uzročnika u organizam i osjetljivosti bolesnika (MSD, Priručnik dijagnostike i terapije).
Metoda detekcije i brojanje *Pseudomonas aeruginosa* metodom membranske filtracije provela se prema normi HRN EN ISO 16266;2008. Metoda se temelji na membranskoj filtraciji uzorka vode volumena 100 mL, te inkubaciji koncentrata na selektivnom mediju, na 37 ˚C, 48 sati. Nakon inkubacije pobroje se sve plavozelene kolonije, kao potvrđene kolonije *Pseudomonas aeruginosa*. Filter papir na selektivnoj podlozi se pogleda pod UV lampom i pobroje sve kolonije koje fluoresciraju, kao vjerojatne kolonije *Pseudomonas aeruginosa,* zatim se dokazuju s Acetamid bujonom. Također se pobroje sve crveno-smeđe pigmentirane kolonije, kao vjerojatne kolonije *Pseudomonas aeruginosa,* koje se potvrđuju Oksidaza testom, Acetamid bujonom i King´s B medijem. Iz broja vjerojatnih kolonija, izbrojenih na filtrima i rezultata potvrdnih testova, procjeni se broj kolonija *Pseudomonas aeruginosa*. Procijenjen broj kolonija izražava se kao CFU/100 mL.

REZULTATI I RASPRAVA

U Tablici 1 prikazani su rezultati analiza pitke vode bunara 1 u 2016. godini i analiza iz 2017. godine, deset mjeseci nakon što je napravljena preporučena dezinfekcija vode. Analiza iz 2016. godine, pokazuje visoke koncentracije svih analiziranih patogenih mikroorganizama. Nakon dezinfekcije 2016. godine i analiza tijekom 2017. godine rezultati pokazuju nešto bolju mikrobiološku sliku vode, ali su i dalje prisutni ukupni koliformi i *Pseudomonas aeruginosa* kojih u vodi za ljudsku upotrebu ne smije biti. Iz prikazanih rezultata je vidljivo da postoji stalna kontaminacija bunara.
Tablica 2 prikazuje rezultate analiza pitke vode bunara 2 u 2016. godini i analiza iz 2017. godine, deset mjeseci nakon što je napravljena preporučena dezinfekcija vode. Prije analize vode u 2016. godini napravljena je dezinfekcija vode 2014. godine, kao i kod bunara 1. Analiza iz 2016. godine pokazuje nešto bolju sliku analizirane vode nego bunar 1, ali ne i zadovoljavajuću, prema maksimalno dopuštenim koncentracijama za zdravstveno ispravnu vodu. Nakon dezinfekcije 2016. i analiza tijekom 2017. godine rezultati pokazuju bolju mikrobiološku sliku vode, ali i dalje su prisutni ukupni koliformi i enterokoki kojih u vodi za ljudsku upotrebu ne smije biti.
Tablica 3 prikazuje rezultate analiza pitke vode bunara 3 tijekom 2016. godine i analize iz 2017. godine, deset mjeseci nakon što je napravljena preporučena dezinfekcija vode. Prije 2016. godine bunar nije duže vrijeme dezinficiran. Analiza vode iz 2016. godine pokazuje sličnu sliku kontaminacije vode kao i bunar 1.

Tablica 1. Mikrobiološka kvaliteta vode bunara 1
Table 1. Microbiological quality of water from well 1

Uzorak (god.)	Ukupni koliformi CFU/100 mL	E. coli, CFU/100mL	Enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	900	10	48	280	420	40
2017.	250	0	0	15	30	45
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

Tablica 2. Mikrobiološka kvaliteta vode bunara 2
Table 2. Microbiological quality of water from well 2

Uzorak (god.)	Ukupni koliformi CFU/100 mL	*E. coli,* CFU/100mL	enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	100	0	0	10	240	18
2017.	16	0	7	11	20	0
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

Iz opisa bunara je vidljivo da je bunar znatno više udaljen od rijeke Orljave, oko 600 metara, i ima veću dubinu, oko 12 metara, u odnosu na ostale bunare. Nakon dezinfekcije 2016. i analiza tijekom 2017. godine rezultati pokazuju znatno bolju mikrobiološku sliku vode, odsutnost *Escherichia coli* i enterokoka, a aerobne mezofilne su ispod maksimalno dopuštene koncentracije, ali i dalje su prisutni ukupni koliformi, i *Pseudomonas aeruginosa,* kojih u vodi za ljudsku upotrebu ne smije biti, te povećani broj kolonija poraslih na 22 °C što upućuje na stalnu kontaminaciju vode.

Tablica 3. Mikrobiološka kvaliteta vode bunara 3
Table 3. Microbiological quality of water from well 3

Uzorak (god.)	Ukupni koliformi CFU/100 mL	*E. coli,* CFU/100mL	Enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	930	4	34	90	240	30
2017.	55	0	0	15	120	15
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

U Tablici 4 prikazani su rezultati analize vode bunara 4 u 2016. godini i analize iz 2017. godine, deset mjeseci nakon što je napravljena preporučena dezinfekcija vode. Analiza iz 2016. godine pokazuje visoke koncentracije svih analiziranih mikroorganizama. Bunar do 2016. godine duže vrijeme nije dezinficiran. Nakon dezinfekcije 2016. i analiza u 2017. godini, rezultati pokazuju znatno bolju mikrobiološku sliku vode, ali ne i zadovoljavajuću, vidljiva je prisutnost svih analiziranih mikroorganizama, ali u bitno nižim koncentracijama, jedino je koncentracija aerobnih mezofilnih bakterija na 37 °C ispod maksimalno dozvoljene koncentracije.
U Tablici 5 prikazani su rezultati analize vode bunara 5 tijekom 2016. godine i analize iz 2017. godine, deset mjeseci nakon preporučene dezinfekcije vode. Prije dezinfekcije 2016. godine bunar je dezinficiran 2014. godine. Analiza iz 2016. godine pokazuje visoke koncentracije svih analiziranih mikroorganizama, slično kao i voda bunara 4. Nakon dezinfekcije 2016. i analiza iz 2017. godine rezultati pokazuju nešto bolju mikrobiološku sliku vode, ali i dalje su prisutni svi analizirani mikroorganizmi u koncentracijama većim od dozvoljenih, osim ukupnih

kolonija izraslih na 22 °C. Osobito se ističe i dalje visoka koncentracija ukupnih koliforma što potvrđuje stalan izvor kontaminacije.

Tablica 4. Mikrobiološka kvaliteta vode bunara 4
Table 4. Microbiological quality of water from well 4

Uzorak (god.)	Ukupni koliformi CFU/100 mL	*E. coli,* CFU/100mL	Enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	1200	900	400	500	720	70
2017.	45	6	3	10	200	3
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

Tablica 5. Mikrobiološka kvaliteta vode bunara 5
Table 5. Microbiological quality of water from well 5

Uzorak (god.)	Ukupni koliformi CFU/100 mL	*E. coli,* CFU/100mL	Enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	1200	10	200	700	900	60
2017.	900	1	4	40	43	13
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

Tablica 6 prikazuje rezultate analiza vode bunara 6 iz 2016. godine i 2017. godine, iz Vilić Sela, koji je smješten u neposrednoj blizini rijeke Orljave, kao i većina analiziranih bunara u selu Pavlovci. Prije dezinfekcije 2016. godine bunar je dezinficiran 2014. godine. Analiza iz 2016. godine pokazuje visoke koncentracije svih analiziranih mikroorganizama, slično kao i voda bunara 4 i 5. Nakon dezinfekcije 2016. i analiza iz 2017. godine rezultati pokazuju nešto bolju mikrobiološku sliku vode, ali još uvijek izuzetno visoku koncentraciju ukupnih koliforma, prisutnost *Escherichia coli,* Enterokoka te *Pseudomonas aeruginosa,* jedino koncentracija aerobnih mezofilnih bakterija na 37 °C zadovoljava MDK vrijednosti. Voda ovakve kvalitete nije zdravstveno ispravna i nije pogodna za ljudsku upotrebu jer su i nakon provedene dezinfekcije prisutni svi patogeni koji upućuju na fekalni izvor zagađenja bunara.

Tablica 6. Mikrobiološka kvaliteta vode bunara 6
Table 6. Microbiological quality of water from well 6

Uzorak (god.)	Ukupni koliformi CFU/100 mL	*E. coli,* CFU/100mL	Enterokoki CFU/100 mL	Broj kolonija na 37 °C, CFU/1 mL	Broj kolonija na 22 °C, CFU/1 mL	*P. aeruginosa,* CFU/100 mL
2016.	1350	810	500	600	820	30
2017.	1200	16	55	20	40	18
MDK	0	0	0	20	100	0

*MDK- maksimalno dopuštena koncentracija

ZAKLJUČCI

Iz prikazanih rezultata vidljivo je da niti jedan uzorak vode, tijekom provedenih analiza u 2016. i 2017. godini, nije zdravstveno ispravan. Nakon provedenih analiza u 2016. godini vlasnici su dezinficirali vodu svojih bunara poslije čega je vjerojatno kratko vrijeme mikrobiološka slika vode bila dobra, no nakon deset mjeseci kada su napravljene ponovne analize u 2017. godini, rezultati pokazuju nešto manju kontaminaciju, ali ne i zdravstvenu ispravnost vode. Iz dobivenih rezultata vidljivo je da postoji stalan izvor kontaminacije vode.
Analizirajući opis lokacije, udaljenost od rijeke Orljave, udaljenost od septičkih jama i dubinu bunara, osobito bunara 3 i 6 koji imaju dubinu od 12 metara, a udaljenost od rijeke Orljave bitno različitu, možemo zaključiti da rijeka nije izvor onečišćenja. Prema raspoloživim podacima možemo zaključiti da su izvori kontaminacije bunara septičke jame i staje te da je bunarsku vodu u blizini ovakvih objekata jako teško održati zdravstveno ispravnom. Dezinfekcijska sredstva su slabo učinkovita kod protočnih bunara, a doza slobodnog rezidualnog klora ovisi prije svega o vrsti i količini kontaminiranog materijala, godišnjem dobu, poroznosti tla, kao i kvaliteti materijala kojim su obzidani bunari i septičke jame.
Preporuka vlasnicima je detaljno čišćenje bunara, poboljšanje njihove zaštite od utjecaja procijeđene vode iz septičkih jama i staja te češće provođenje dezinfekcije i analiza vode.

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HEALTH SAFETY CONTROL OF THE DRINKING WATER FROM PRIVATE WELLS

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Ever since the ancient times, the inhabitants of Slavonia have used water wells in their own yards as a source of water. The abundance of water at relatively low depths and suitable soil composition allowed the excavation of wells. As the technology advanced and the standard of living among the population increased, 80 % of the population became included in the public water supply system. Unfortunately, the high costs of connection to the public water supply and the low standard of living still present in some parts of the population lead to the fact that some people still use their own water wells as the only source of water. In Pavlovci village, Požega-Slavonia County, 95 % of the households still use such water wells.

In this paper, the microbiological quality of private water wells in two villages in Požega-Slavonia County, Brestovac area, has been analysed. The owners of the wells use this water for all household purposes, including drinking. Analyses have been conducted at the certified laboratory of the Public Health Institute of the Požega-Slavonia County. Samples were tested for coliform bacteria, *Escherichia coli, Enterococcus, Pseudomonas aeruginosa,* and the total number of colonies at 37 ºC and 22 ºC. All the tested samples do not meet the Croatian legislation criteria.

Keywords: microbiological water health safety, well, *Escherichia coli*, Enterococci, *Pseudomonas aeruginosa.*

Topic: Food analysis
Sekcija: Analiza hrane

CHEMICAL CHARACTERISTICS AND OXIDATIVE STABILITY OF DALMATIAN MONOVARIETAL OLIVE OILS

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ABSTRACT

Olive oils have been proven to have various beneficial health effects due to a high content of monounsaturated fatty acids, as well as a high level of biologically active lipophilic and water-soluble compounds. In the coastal region of Dalmatia (Croatia), there is a long-standing tradition of olive cultivation and the production of high quality oils. Also, great attention has been given to native olive varieties. The objective of this study was to determine the chemical characteristics and the antioxidative potential of monovarietal olive oils from the native Dalmatian olive cultivars *Levantinka*, *Buharica* and *Drobnica*. Free acidity and peroxide value, which are the primary indicators of fruit quality, as well as the handling procedures, were determined, along with total phenolic content using the Folin-Ciocalteu method. The individual phenolics and fatty acid composition were analysed using chromatographic techniques, while the oxidative stability of oil was tested using the Rancimat apparatus. According to the obtained results, there were significant differences among the investigated samples and the connection between their chemical composition and the related antioxidant properties was detected. The highest level of phenolics was detected for the *Buharica* olive oil while the longest oxidation inhibition period (11.35 h) was detected for the *Drobnica* oil.

Keywords: monovarietal olive oils, chemical composition, phenolics, Rancimat, oxidative stability

INTRODUCTION

Virgin olive oil is increasingly popular world-wide and today it is recognized as a natural functional food. The reason for this are its beneficial health effects associated with its consumption, particularly as part of the Mediterranean diet, where olive oil is the main source of fat. It is well known that olive oil contains high levels of monounsaturated fatty acids, but other biologically active components, which are present in significantly lower amounts, are also a matter of great interest. Those compounds are mostly tocopherols, phospholipids, phenolics, squalene, volatile compounds and pigments, which have a significant effect on the organoleptic characteristics, nutritive value and oxidative stability of oil (Bayram et al., 2013; Bakhouche et al., 2015; Boskou, 2015; Kelebek et al., 2016; Sanchez de Medina, et al., 2017; Bilušić et al., 2017).

Aside from a high content of monounsaturated fatty acids, health benefits of olive oils are mainly attributed to a high content of phenolic compounds. It has been reported that olive oil contains more than thirty structurally distinct phenolic compounds from the sub-groups of phenolic alcohols, phenolic acids, lignans, flavonoids and secoiridoids. The major phenolics in olive oil are oleuropein, hydroxytyrosol and tyrosol, which act as strong antioxidants. The concentration of these compounds is influenced by different factors, including the olive cultivar, growing area, pedoclimatic conditions in the growing area, irrigation management, fruit ripening stage, harvest time, as well as the oil processing technology (Stefanoudaki et al., 2000; Tuck and Haybal, 2002, Bakhouche et al., 2015; Noorali et al., 2017; Bilušić et al., 2017; Gouvinhas et al., 2017).
In Dalmatia, the coastal region of Croatia, there is a long-standing tradition of olive cultivation and the production of high quality olive oil. In the last few years, considerable efforts have been taken to increase the olive orchards and special attention was given to native olive varieties. *Buhavica* is an autochthonous olive cultivar from the Island of Brač, *Levantinka* is a cultivar most widespread on the Island of Šolta (Central Dalmatia), while *Drobnica* is a characteristic cultivar from the Island of Korčula (South Dalmatia) (Bilušić et al., 2017).
The objective of this study was to determine the effect of the cultivar on the phenolics content, fatty acid composition and oxidative stability of monovarietal olive oils from three Dalmatian olive cultivars, *Levantinka*, *Buharica* and *Drobnica.* The approach based on the comparison of the chemical composition of olive oils provided valuable information about the influence of the detected compounds (their presence and content) on the oxidative stability of oil.

MATERIALS AND METHODS

Olive oils

Three commercially available monovarietal extra virgin olive oils from the company Uje d.o.o. (Split, Croatia) were chosen for analysis in this study: *Levantinka*, *Buharica* and *Drobnica*. Their characteristics and compositions were compared with a blended olive oil which was used as the control sample. All analyses were performed immediately after opening the oil bottle, to avoid the potential oxidation processes.

Free fatty acids and peroxide value

Free fatty acids (expressed as % of oleic acid) and peroxide value (expressed as meq O_2/kg), as main chemical quality parameters of oil, were determined in accordance with the International Olive Council regulation (COI/T.20/Doc. No 34, 2015, COI/T.20/Doc. No 35, 2016).

Fatty acid composition

The analysis of fatty acid methyl esters in the investigated olive oils was carried out on a gas chromatograph (model 3900; Varian Inc., Lake Forest, CA, USA) with FID detection, using a capillary column RTX 2330, 30 m × 0.25 mm i.d., coating thickness 0.25 μm (Restek, Bellefonte, PA, USA). The method has been previously described in Bilušić et al. (2017).

100 mg of the oil in a screw top test tube was weighted and dissolved in 2 mL of heptane. Additionally, 0.2 mL of the 2 M methanolic solution of potassium hydroxide was added. The tube was caped and shaken vigorously, and then left to stratify until the upper layer (heptane solution) became clear. The injection volume was 1 μL and the split ratio was 1:40. The carrier gas was helium and the applied flow rate was 3 mL/min. The temperature of the injector and the detector was 220 °C, while the initial oven temperature was 140 °C and the final temperature was 210 °C. The temperature was ramped up at the rate of 5 °C/min for 16 minutes. The amount of detected acids is expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all peaks.

Isolation of phenolics

The method of the extraction for phenolics from oil was as follows: oil (2 g) was dissolved in *n*-hexane (1 mL) and extracted three times by 2 min of vortex and 5 min of centrifugation (3000 rpm) with the methanol:water mixture (60:40, v/v) (2 mL). The methanolic extract was then transferred to a round bottom flask and evaporated in a rotary vacuum evaporator at 35 °C. The crude extract that remained after evaporation was dissolved in methanol (2 mL) and used for further analysis.

Total phenolic content

The total phenolic content in phenolic extracts of olive oils was estimated using the Folin-Ciocalteu method (Singleton and Rossi, 1965; Katalinić et al, 2013) calibrated against gallic acid standards. Spectrophotometric measurements were performed on a SPECORD 200 Plus, Edition 2010 (Analytik Jena AG, Jena, Germany). 0.25 mL of the methanolic extract of oil phenolics in a 25 mL volumetric flask was added to 1.25 mL of the Folin-Ciocalteu reagent and 3.75 mL of sodium carbonate solution (20 %, w/v). The flask was than filled with distilled water reaching the final volume. The samples were stored for 2 hours at room temperature in the dark and the spectrophotometric analysis was performed at 725 nm. The measurements were performed in triplicate for each sample and the results are expressed as gallic acid equivalents, in miligrams per kg of oil (mg GAE/kg).

Phenolic profile

The phenolic compounds from the polar fraction of olive oil were separated by high performance liquid chromatography on the HPLC system (Perkin Elmer, Waltham, Massachusetts, USA) using the method described in the COI/T. 20/Doc. No 29 (2015). The system carries the label Series 200 and is made up of an autosampler, a vacuum degasser, a binary pump, a column oven and a UV/VIS detector. Separation was achieved on the Ultra Aqueous C18 column (150 × 4.60 mm) filled with 5 μm size particles of stationary phase (Restek, Bellefonte, Pennsylvania, USA). Solvent A was 0.2 % phosphoric acid, while solvent B was a mixture of methanol and acetonitrile in a 1:1 ratio. The gradient elution was achieved starting with 96 % of solvent A, which was then decreased to 50 % during the first 40 minutes of the pump programme. In the following

5 min, solvent A was reduced to 40 %, after which it was brought to zero during the next 15 minutes. For the next 8 min, only solvent B was passing through the column. The steady linear increase of solvent A at 96 % was used from 68 to 70 min and the obtained solvent ratio was retained for the last 10 min to achieve the stability of the column to the initial condition. The column temperature was kept at 25 °C and the flow rate of 0.8 mL/min was applied, while injecting the sample volume of 20 μL.
For the detection of phenolic compounds, a UV detector was used at 280 nm. Phenolic compounds were identified over the retention time of the standard and quantified through the calibration curve. The resultant concentrations for oleuropein derivatives: decarboxymetyl oleuropein aglycon (dialdehyde form), oleuropein aglycon (dialdehyde form), decarboxymethyl ligstroside aglycone (oxidised dialdehyde form) and decarboxymethyl ligstroside aglycone (dialdehyde form) were obtained via the calibration curve of oleuropein. The resultant concentrations of phenolic compounds are expressed as mg/kg of olive oil.
The standard and the solvents were of analytical grade, purchased from Sigma-Aldrich (Steineheim, Germany). Deionized water (Milli-Q-water purification system, Millipore, Bedford, MA, USA) was used for the preparation of all solvents.

Oxidative stability of oil

The oxidative stability of the investigated olive oils was evaluated using the apparatus Rancimat 743 (Metrohm, Herisau, Switzerland). The olive oil samples (3 g) were tested at the temperature of 120 °C (ΔT = 1.4 °C) with the constant air flow of 20 L/h (Bilušić et al., 2017). Conductivity was measured as a function of time and the results are expressed as induction time (in hours). All determinations were performed in duplicate, and the results are presented as mean value ± standard deviation.

RESULTS AND DISCUSSION

Olive oil is one of the oldest known vegetable oils, and it is also specific in relation to other plant-derived oils, as its production includes only mechanical processes (no refining) under processing conditions. The basic chemical parameters for the quality of olive oil are free fatty acids and peroxide value. These parameters are signs of proper or inadequate handling of the olives and their processing, and they are used to assess the present state of oil (Boskou, 2011; Boskou, 2014). The results for free fatty acids content and peroxide value of the investigated oils are present in Table 1. As reported in Table 1, free acidity in all the investigated olive oil samples was within the range of the virgin olive oil category (COI/T.20/Doc. No 34, 2015). The lowest value was detected for *Buharica* oil (0.18 % of oleic acid), while the highest free acidity was determined in the blended olive oil (0.64 % of oleic acid).
The peroxide values, which measure the primary oxidation of oil, were also within the limits for extra virgin olive oils (COI/T.20/Doc. No 35, 2016) and they ranged from 6.41 to7.44 meq O_2/kg. This parameter was also the highest in the blended olive oil, and the lowest for *Buharica* oil (Table 1).

Table 1. Free acidity and peroxide value of investigated olive oils

	Free acidity (% of oleic acid)	**Peroxide value (meq O_2/ kg)**
Buharica	0.18	6.41
Drobnica	0.41	6.60
Levantinka	0.22	6.96
Blended oil	0.64	7.44

The fatty acids present in olive oils are usually detected through gas-chromatographic analysis. They usually comprise between 14 and 24 carbon atoms, and the most important are the unsaturated fatty acids; such as monounsaturated oleic (18:1) and palmitoleic acid (16:1), and polyunsaturated linoleic (18:2) and linolenic acid (18:3) (Poiana et al., 2004). The results of the fatty acid composition of the investigated Dalmatian olive oils are given in Table 2. As presented and according to expectations, the dominant fatty acid in all the investigated oils was the oleic acid, with concentrations ranging from 69.89 m/m%, in *Buharica* oil to 75.98 m/m% in the blended oil. An extremely high content of palmitic (from 12.50 to 14.52 m/m%) and linoleic (from 5.54 to 11.25 m/m%) acids was also detected. The concentration of linolenic acid was significantly lower (from 0.72 to 0.82 m/m%), which is in accordance with the European Regulation, although some authors reported higher concentrations of these compounds in other monovarietal olive oils (Poiana et al., 2014). Among saturated fatty acids, stearic acid was dominant, with the concentration ranging from 2.14 to 2.48 m/m%.

Phenolic compounds are among the most widespread classes of metabolites in nature, and are also present in olives and olive oil. They are an extremely large group of polar compounds, ranging from those with simple chemical structures (like phenolic acids and stilbenes), to highly complex substances (like flavanols, flavonids, proanthocyanidins, tannins, coumarines, lignans and lignins) (Pereira et al., 2009; Tsao, 2010). Together with other biologically active phytochemicals, phenolics are found to be major contributors to the nutritive value, stability, long shelf-life and sensory characteristics of olive oil. Furthermore, beneficial health effects of olive oils are usually attributed to their presence (Stefanoudaki et al., 2000; Tuck and Haybal, 2002, Bilušić et al., 2017).

Table 2. Fatty acid composition (m/m %) of Dalmatian olive oils

Fatty acid		***Buharica***	***Drobnica***	***Levantinka***	***Blended oil***
Palmitic acid	16:0	13.49±0.12	12.50±0.41	14.52±1.04	13.08±0.38
Hypogeic acid	16:1 ω9	0.11±0.00	0.11±0.00	0.11±0.03	0.10±0.00
Palmitoleic acid	16:1 ω7	0.97±0.01	0.89±0.04	0.98±0.23	0.87±0.03
Margaric acid	17:0	0.05±0.00	0.04±0.00	0.05±0.02	0.04±0.00
Heptadecenoic acid	17:1	0.06±0.01	0.07±0.00	0.07±0.00	0.07±0.01
Stearic acid	18:0	2.14±0.00	2.48±0.04	2.43±0.18	2.46±0.05
Oleic acid	18:1	69.89±0.09	75.80±0.50	72.09±1.08	75.98±0.20
Linoleic acid	18:2	11.25±0.02	5.54±0.09	8.03±0.14	5.59±0.04
Linolenic acid	18:3	0.81±0.00	0.80±0.01	0.72±0.00	0.82±0.02
Gadoleic acid	20:1	0.35±0.00	0.32±0.01	0.24±0.09	0.33±0.01
Behenic acid	22:0	0.12±0.01	0.13±0.00	0.11±0.01	0.13±0.02
Lignoceric acid	24:0	0.40±0.00	0.93±0.02	0.32±0.01	0.14±0.04

The analytical procedure for the determination of phenolics in olive oils involves few basic steps.
The first step is the extraction of phenolics from the apolar oil fraction using a polar solvent, which is usually methanol or a methanol/water mixture. Before extraction, olive oil is usually dissolved in an apolar solvent (hexane, petroleum ether, or chloroform) (Bakhouche et al., 2015). In our study, olive oil phenolics were extracted using a methanol: water mixture (60:40, v/v), while hexane was used as the oil solvent. The obtained extract was used for the determination of total phenolics in the samples as well for the identification of individual compounds.
Currently, the two most commonly used methods to evaluate phenolics in olive oil are the Folin-Ciocalteu method for the determination of total phenolic compounds, and high-performance liquid chromatography (HPLC) for the determination of individual phenolics (Alessandri et al., 2014).
The total phenolic content differs from oil to oil, but values that are usually reported range between 100 and 300 mg/kg (Boskou, 2014). The results for total phenolic content in oils investigated in this study using the Folin-Ciocalteu method (Figure 1) are in accordance with the mentioned data. The lowest content of phenolics was detected in *Drobnica* oil and in the blended oil (237 mg GAE/kg), while *Buharica* oil contained the highest amount of these valuable minor components (384 mg GAE/kg).

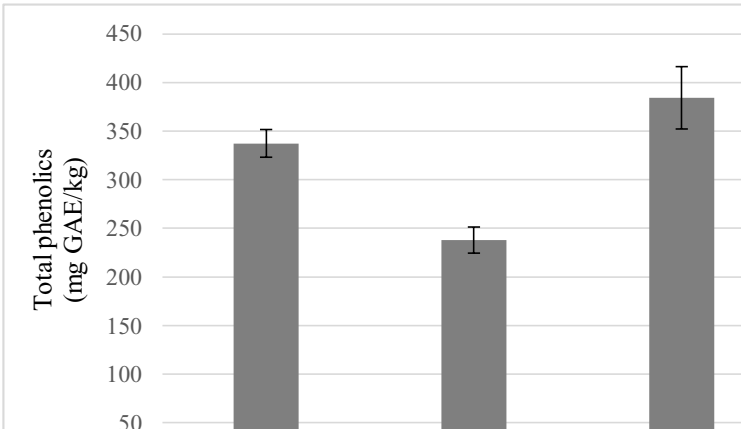

Fig. 1. Total phenolic content in investigated olive oils (GAE- gallic acid equivalents)

The second step is the chromatographic separation and characterization of individual phenolic compounds, and HPLC is the most commonly used among the separation techniques reported (Cerretani et al., 2009; Bayram et al., 2013; Bakhouche et al., 2015). The methanol-water extract of olive oil contains free phenols and phenolic acids as more polar substances, while the less polar part of the extract contains aglycones of oleuropein and ligstroside, diacetoxy and dialdehydic forms of the aglycones, flavonoids, etc. (Boskou, 2014).
The results obtained by HPLC in our study are presented in Table 2. The phenolic profile of the investigated olive oils is evidence of the notable differences between the investigated cultivars. The sum of all the detected phenolics was the

highest for *Levantinka* oil (315 mg/kg) and *Buharica* oil (287 mg/kg), while amounts that were more than two-fold lower were detected in the blended oil. There were also significant differences in the phenolic profiles among the oils.

Servili and Montedoro (2002) gave average values from the analysis of 116 commercial samples of olive oils. In their study, the dominant phenolics were oleuropein aglycones, mono- and dialdehyde forms, same in our study. The concentration of decarboxymethyl oleuropein aglycone in our study ranged from 32 to 107 mg/kg, while the amount of dialdehydic form of decarboxymethyl ligstroside aglycone ranged from 12 to 35 mg/kg.

In their study, free hydroxytyrosol and tyrosol were found in traces (concentrations less than 10 mg/kg), while in our study *Buharica* and *Drobnica* oils contained slightly higher concentrations, 12.02 and 11.13 mg/kg, respectively. Hydroxytyrosol and tyrosol are the main simple phenolics present in olive oils, with concentrations from 0 to 70 mg/kg (increased during storage).

Table 3. HPLC analysis of olive oil phenolics (mg/kg)

Phenolic compound	Olive oil			
	Buharica	***Drobnica***	***Levantinka***	**Blended oil**
Hydroxytyrosol	12.03±0.20	11.13±0.05	5.63±0.17	8.79±0.27
Tyrosol	11.45±0.01	12.32±0.09	6.23±0.09	12.11±0.35
Decarboxymethyl oleuropein aglycone	85.02±0.35	32.02±0.10	106.88±2.26	38.05±1.14
Oleuropein	25.36±0.76	9.96±0.42	28.46±0.44	5.31±0.10
Oleuropein aglycone, dialdehyde form	6.38±0.38	5.48±0.68	10.63±0.34	3.50±0.25
Decarboxymethyl ligstroside aglycone, oxidised dialdehyde form	91.33±3.38	31.95±0.21	99.85±3.19	39.59±0.83
Decarboxymethyl ligstroside aglycone, dialdehyde form	34.68±2.81	12.00±1.00	30.56±0.93	14.17±0.89
Pinoresinol	13.92±1.80	13.25±0.14	19.93±0.53	14.28±0.93
Luteolin	3.36±0.32	1.99±0.07	3.55±0.19	1.86±0.03
Apigenin	1.27±0.01	1.24±0.00	1.67±0.05	1.06±0.09
Protocatechuic acid	0.19±0.02	n.d.	n.d.	0.27±0.00
Hydroxybenzoic acid	n.d.	0.10±0.00	n.d.	n.d.
Caffeic acid	0.21±0.00	0.41±0.03	0.26±0.03	0.22±0.15
p-Coumaric acid	0.45±0.04	0.39±0.01	1.58±0.10	0.66±0.02
trans-Ferulic acid	0.72±0.06	n.d.	0.35±0.05	0.05±0.01
Cinnamic acid	0.33±0.04	0.28±0.02	0.23±0.05	0.29±0.01

n.d.- not detected

The presence of two flavones in low concentrations, apigenin and luteolin in investigated olive oils was also confirmed (Cerretani et al., 2009). Furthermore, several phenolic acids were also identified. Protocatehuic acid was detected in *Buharica* and the blended oil,

while 4-hydroxybenzoic acid was found only in *Drobnica* oil. On the other hand, *trans*-ferulic acid was lacking only in that sample. The most abundant phenolic acid was the *p*-coumaric acid, with the concentrations ranging from 0.39 mg/kg in *Drobnica* oil to 1.58 mg/kg in *Levantinka* oil. These results are in accordance with those reported in the literature (Bayram et al., 2013).

Oxidative deterioration of oils is the primary cause for its rancidity and the production of off-flavours, but also for the changes in nutritional value and the functional property changes (Hamilton et al, 1997; Jacobsen, 1999). As this degradation process occurs slowly at ambient conditions, several accelerated methods employing high temperatures and air-flow supply have been developed (Farhoosh and Moosavi, 2007). One of the most popular is the Rancimat method, which was used in this study. It is based on the conductivity changes in water caused by dissolving volatile compounds produced during the oil oxidation process (Farhoosh, 2007). The induction period (IP) is defined as the time required to reach the end-point of oxidation, which produces a sudden increase of water conductivity (Mendez et al, 1996).

According to the obtained results (Table 4), the longest induction period was obtained for *Drobnica* oil (11.35 h) and blended oil (11.33 h), while the shortest time of stability was recorded for *Buharica* oil (9.14 h). In the study by Bilušić et al. (2017), the oxidative stability of Dalmatian monovarietal olive oils extracted from *Buhavica*, *Drobnic*a, *Lastovka* and *Oblica* olives harvested in the early and late period was tested by the Rancimat assay (under the same conditions we used in our study). The induction periods ranged from 7.71 to 20.95 h and the authors concluded that the investigated oils showed a higher oxidative stability at the early stage of fruit maturity, with the exception of *Buhavica* oil. The results obtained in the study by Gharby et al. (2016) ranged from 5.9 to 16.6 h, while in the study by Mateos et al. (2006), where a lower air flow rate was used (19 L/h), the induction periods for different monovarietal olive oils ranged from 3.7 to 48.3 h.

Table 4. Oxidative stability of olive oils detected by the Rancimat method

Oil sample	Induction period (h)
Buharica	9.14±0.08
Drobnica	11.35±0.07
Levantinka	10.96±0.00
Blended oil	11.33±0.04

Different studies reported that the content of unsaturated fatty acids (the ratio of monounsaturated and polyunsaturated fatty acids) has a direct influence on the oxidative stability of oil (Velasco and Dobarganes, 2002). The highest values of this ratio were detected for Buharica (5.33) and the blended oil (5.36), while the two other oils had lower values (4.95 for *Drobnica* and 4.84 for *Levantinka*). The second ratio that has been commonlyused for the explanation of the oxidative stability of oil is the ratio of oleic and linoleic acid. In our study, this value was highest for *Drobnica* and the blended oil, 13.68 and 13.59 respectively, while it was lowest for *Buharica* oil (6.21). If we compare these data with the obtained induction periods, it can be concluded that this parameter has an effect on oil oxidation processes, which was also confirmed in the study of Bilušić et al. (2017).

Phenolics are found to be major antioxidant components in olive oil, which are also beneficial for the shelf life of the oil, and among them, hydroxytyrosol was found to be the most potent and effective antioxidant (Tsimidou et al., 1992; Boskou 1996; Boskou, 2011). Although the samples of *Levantinka* and *Buharica* contained the highest amount of total phenolic compounds (detected by the Folin-Ciocalteu method), these samples were the most susceptible to oxidation and resulted inthe shortest inhibition time. Aside from the fact that the method used for the determination of total phenolics is not specific, it is also very important to point out that the composition of phenolics (individual compounds or their mutual ratio) is a more important factor for the oxidative stability of oil. The effect of tyrosol and its derivative hydroxytyrosol on the oxidative stability of oil has been previously reported (Noorali et al., 2017), but the results obtained in this study cannot confirm this, since *Buharica* oil, with the highest content of these compounds, showed the lowest stability, and two-fold lower content was detected in *Levantinka* (11.86 mg/L). Similar conclusions could be made after analysing the results for other detected phenolics from the group of secoridoids. Although it has been established that molecules containing an orthodiphenolic group (e.g. oleuropein aglycone and derivatives) are great antioxidants, which are mainly responsible for the oxidative stability of olive oil (Cinquanta et al., 1997), the results of this study do not support this observation.

CONCLUSIONS

The fact that olive oil is a functional food with health benefits was confirmed once more by this study. The high content of unsaturated fatty acids and minor components (phenolics) with proven positive effects on human health was detected in all samples, although significant differences between different Dalmatian olive oil cultivars were established. The effect of oil composition on its oxidative stability was confirmed only for the fatty acid profile, while the effect of the phenolics (total content and individual compounds) was not confirmed. Olive oil contains some other substances that could affect its stability, e.g. squalene which could have a positive effect, or metals and pigments, which could accelerate oil oxidation. Furthermore, olive oil sterols (especially Δ5-avanasterol with an ethyldiene group on the side chain) are found to be very effective in retarding oxidation in heated oils, so the presence of these compounds in oils should also be analysed and taken into consideration.

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THE EVALUATION OF COLOUR COMPONENTS AND ANTHOCYANINS IN *Babica* AND *Crljenak kaštelanski* WINES

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ABSTRACT

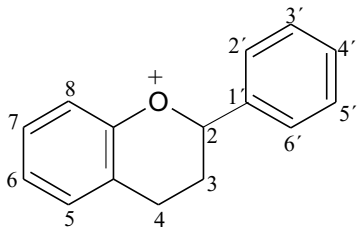
Babica and *Crljenak kaštelanski* are historic and autochthonous red grape cultivars of the Kaštela wine region (Central Dalmatia, Croatia). In the last few years, *C. kaštelanski* has been getting much moreattention, since it was discovered that it is a parent of the popular American variety *Zinfandel* and the Italian variety *Primitivo.* Therefore, *C. kaštelanski* has become economically important and it has been increasingly cultivated in Croatia, while other cultivars, like *Babica*, have almost vanished from the Dalmatian vineyards. As the colour of red wines is an important element of wine quality and it is the first feature that influences its commercial acceptance, this study was conducted with the aim of investigating the evaluation of the colour components and anthocyanins in *Babica* and *C. Kaštelanski* wines. The anthocyanin profile was determined using HPLC, while monomeric anthocyanins and basic colour characteristics of wine (density, hue, chromatic structure, and brilliance) were detected spectrophotometrically. The total monomeric anthocyanins in *Babica* were higher than in *C. kaštelanski* and the dominant anthocyanin in both wines was malvidin-3-glucoside, with over 71 % of all detected anthocyanins in *Babica* and with over 52 % in *C. kaštelanski*. The other colour parameters were mainly higher for *C. kaštelanski*, probably due to the almost six-fold higher content of cyanidin derivatives in *C. kaštelanski* wine.

Keywords: red wine, vinification, colour, anthocyanins, HPLC

INTRODUCTION

Colour is one of the most important attributes of red wine and it is well known that it depends on the phenolic composition of wine, primarily on the level and the profile of anthocyanins and other compounds that are formed during the vinification process (Bautista-Ortín et al., 2006; Jensen et al., 2008; He et al., 2012).
Anthocyanins are natural, water-soluble pigments from the group of flavonoids, responsible for the red, blue and purple colour of different fruits, vegetables, flowers, leaves, etc. (Heim et al., 2002). Anthocyanidins are the basic chemical structures of

anthocyanins (glycoside form, bonded to a sugar). They consist of an aromatic ring (A) bonded to a heterocyclic ring that contains oxygen and is bonded by a carbon-carbon bond to a second aromatic ring (B). Anthocyanins have diverse chemical structures, with differences related to the number and position of hydroxyl and methoxyl groups in the aromatic ring B, as well as sugar molecules bounded to a heterocyclic ring. The most common anthocyanins are usually conjugated to sugars (usually glucose), hydroxycinnamates and organic acids (malic or acetic acid). Almost 200 different anthocyanins have been identified in plants. (Belitz et al., 2004; Shahidi and Weerasinghl, 2004; Jackson, 2008; Jensen et al., 2008; Katalinić et al., 2010; He et al., 2012; Ma et al., 2012; Ristovski et al., 2014; Generalić Mekinić et al., 2016). The most common anthocyanidins in nature are pelargonidine, cyanidine, delfinidine, peonidine, petunidin and malvidin (Fig. 1).



Anthocyanidins	**3**	**5**	**7**	**3´**	**4´**	**5´**
Cyanidin	OH	OH	OH	OH	OH	-
Cyanin	O-Glu*	OH	OH	OH	OH	-
Peonidin	OH	OH	OH	OCH_3	OH	-
Delphinidin	-	OH	OH	OH	-	OH
Pelargonidin	OH	OH	OH	-	OH	-
Malvidin	OH	OH	OH	OCH_3	OH	OCH_3

** Glu - glucose*

Fig. 1. Structures of common anthocyanidins (Stalikas, 2007).

The anthocyanin composition of red wines depends on the anthocyanin profile of the grape variety and also on the applied winemaking technique (Bautista-Ortín et al., 2006; He et al., 2012). The red wine pigments are absorbed into the wine from red grape berry skin during its soaking in the must (maceration). In the fermentation and during the maturation, the present monomeric anthocyanins undergo a wide variety of reactions and form new compounds (anthocyanin-derived new pigments), which are crucial for the colour of wine, its intensity and stability (Bautista-Ortín et al., 2006; Jackson, 2008; Ivanova et al., 2011; He et al., 2012). In the red wines made from *Vitis vinifera* grapes the main monomeric anthocyanins are the 3-*O*-monoglucosides of the six anthocyanidins, including pelargonidin-3-*O*-glucoside (callistephin), cyanidin-3-*O*-glucoside (kuromanin), delphinidin-3-*O*-glucoside (myrtillin), peonidin-3-*O*-glucoside (peonin), petunidin-3-*O*-glucoside (petunin) and malvidin-3-*O*-glucoside (oenin) (Gao et al., 1997; Bautista-Ortín et al., 2006; Jackson, 2008; Jensen et al., 2008; He et al., 2012). Different scientific papers point out that the anthocyanins that possess more hydroxyl groups in the B ring can contribute more blueness, while the degree of methylation of the B ring in anthocyanins molecules, like in malvidin-3-*O*-glucoside, increases wine redness

(Jackson, 2008; He et al., 2012). Malvidin glycosides are the major anthocyanins in grapes, and among the most stable, due to the dimethoxylation of the molecule (Generalić Mekinić et al., 2016).
Anthocyanins also differ in their susceptibility to oxidation, so these changes could be more significant to wine-colour stability than the total anthocyanin content in wine. The initial purplish colour of young red wines is generated by the residual level of anthocyanins in copigment associations (Jackson, 2008) and it fades as the copigment complexes dissociate and the free anthocyanins react with other compounds from the group of flavonoids and non-flavonoids (such as flavonols, flavan-3-ols, oligomeric proanthocyanidins, and cinnamic acids and its derivatives) (Liao et al., 1992; Boulton, 2001; Jackson, 2008; Busse-Valverde, 2011; He et al., 2012). Among these compounds, flavan-3-ols, such as (+)-catechin or (-)-epicatechin, are recognized as powerful cofactors which can most easily and intensely form coloured complexes (Liao et al., 1992; González-Manzano, 2009; He et al., 2012). They can increase colour density, affect colour tint, and give a more purple hue to young red wines by provoking displacement of anthocyanin equilibria towards their coloured forms (He et al., 2012). Furthermore, better understanding of self-association and copigmentation can help us in predicting young wine colour attributes from the phenolic profiles of raw material - red grapes (Liao et al., 1992; Boulton, 2001; He et al., 2012).
As the colour of red wine is an important element of its quality and the first feature that influences its commercial acceptance, the objective of this study was to investigate and compare the anthocyanin profile and colour attributes of two young wines, produced using the same winemaking procedure, from two historic red grape cultivars from the Kaštela wine region; *Babica* and *C. kaštelanski*.

MATERIALS AND METHODS

All used chemicals were of adequate analytical grade. The measurements were performed on a Specord 200 spectrometer (Analytik Jena GmbH, Germany) while the individual anthocyanins were separated and identified using the Perkin Elmer HPLC-UV/Vis system (all from the 200 Series, Perkin Elmer, Walthamn, Massachusetts, USA).

Wine samples

In this study, young red wines produced from two autochthonous grape cultivars from the Kaštela wine region (Central Dalmatia, Croatia): *Babica* and *Crljenak kaštelanski* were analysed. In both vinifications 100 kg of grapes were used and thetraditional winemaking procedure was applied. The destemmed and crushed grapes were distributed into fermentation tanks and treated with potassium metabisulphite (10 g/100 L). After sulfiting, 10 g/100 L of active dry Burgundy Yeast (E. Begerrow GmbH & Co., Langenlonsheim, Germany) was added to initiate the alcoholic fermentation. The cap of grape solid was kept soaked using a mechanical barrier. The fermentation temperature was in the range from 25 to 27°C and the fermentation was accomplished within six days. After the fermentation has been completed, the must was devoted and the rest was pressed. The wine was then sealed using the tank's floating lid and paraffin oil.

Spectrophotometric analysis of monomeric anthocyanins and wine colour parameters

The amount of total anthocyanins in samples was determined using the bisulphite bleaching method (Amerine and Ough, 1980; Katalinić et al., 2010) and the monomeric anthocyanins content was calculated using the molar absorption coefficient for malvidin 3-glucoside. The results are expressed as mg of malvidin-3-glucoside equivalents per litre (mg M-3-gl/L). The measurements were performed in triplicate, and the results are expressed as means ± SD.
Wine colour intensity (CI), hue (T) and chromatic structure (optical density (OD) at 420, 520 and 620 nm) were determined by measuring the absorbance of the samples at 420, 520 and 620 nm (Glories 1984), and the colour parameters were calculated according to the equations described in Babincev et al. (2016). Colour intensity (CI) represents the amount of wine colour and it was calculated as the sum of absorbance at 620 nm, 520 nm and 420 nm, and the hue was calculated as the ratio between absorbance at 420 nm and absorbance at 520 nm (Glories, 1984; Bautista-Ortín et al., 2006; Babincev et al., 2016). Optical density represents the contribution of red (OD 520), yellow (OD 420) and blue (OD 620) colour to the colour of the red wines as described in Glories (1984).

HPLC analysis of anthocyanins

The HPLC analysis of anthocyanins was performed according to the previously published protocol with minor modifications (Fredotović et al., 2017). The separation, quantification, and identification of anthocyanins were carried out using a Kinetex C18 core-shell column (150×4.60 mm, 5 μm, Phenomenex, Torrance, CA, USA). The elution solvents were 0.3% perchloric acid in water (solvent A) and 0.3% perchloric acid in methanol (solvent B). The applied flow rate was 0.6 mL/min and the injected volume was 10 μL. The detection was carried out at 520 nm and the peaks were identified according to their retention times. The quantification was determined using a standard curve for malvidin 3-*O*-glucoside. The analysis was performed in duplicate, and the results are expressed as means ± SD.

RESULTS AND DISCUSSION

Red wine is characterized by deep purple to pale red colour, though most young red wines initially have a purplish-red hue (Jackson, 2008). The red pigment of wine increases with aging, while the share of the yellow pigment decreases. The colour intensity also decreases during wine aging, while the colour hue increases (Poiana et al., 2007; Babincev et al., 2016). All of these changes in colour parameters can be explained by the transition of monomeric anthocyanins (which contribute to the red colour of wine) into polymeric forms (Pasku, 2005; Badicev et al, 2016).
The data presented in Fig. 2 show the colour parameters of investigated wines: colour intensity, hue and the contribution of yellow (OD 420), red (OD 520), and blue (OD 620) pigments to the overall wine colour.
The highest absorption of the samples was detected at the wavelength of 520 nm, as expected, since red wines have a maximum spectrum at this wavelength due to the presence of anthocyanins. The contribution of the red pigment was slightly higher in *C. kaštelanski* wine (49.59 %), in relation to *Babica* (45.38 %). In contrast, the share of the yellow pigment was

higher in *Babica* wine than in *C. kaštelanski* (43.78 % vs 37.64 %). The participation of the blue pigment in wine colour was 10.84 % for *Babica* and 12.78 % for *C. kaštelanski*. This parameter is one of the major characteristics of young red wines (Poiana et al., 2007; Babincev et al., 2016).

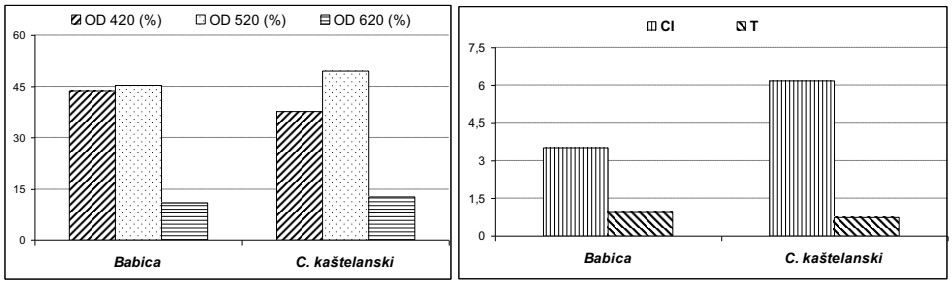


Fig. 2. Colour parameters (CI- colour intensity; T- hue; OD- optical density) of *Babica* and *C. kaštelanski* young wines

Colour intensity is defined as the amount of colour, determined by the content and structure of the anthocyanins present in wine (Glories, 1984; Ivanova et al., 2011). This parameter is usually defined as the sum of the absorbances recorded at 420, 520 and 620 nm (Glories, 1984) and in our case it was considerably higher for *C. kaštelanski* wine. Since the effect of red and blue colour in *Babica* wine was lower, it was expected that the colour intensity will be lower, since the ratio between the yellow and red colour in the wine *Babica* was lower. The wine hue is defined as the ratio of A_{420}/A_{520}, and gives a measure of the wine's redness (Glories, 1984). Wine hue indicates the development of a colour towards orange and it increases through wine aging. In this study, the detected hue was higher for *Babica* (0.97) in relation to the wine *C. kaštelanski* (0.76). While young wines have the value of 0.5-0.7, the upper limit is around 1.2-1.3 (Fig.2) (Poiana et al., 2007; Babincev et al., 2016).
The determination of total monomeric anthocyanins was performed using the spectrophotometric bisulphite bleaching method and the results are shown in Fig 3. The sum of anthocyanidin derivatives detected by HPLC is presented in Table 1 and the content of the individual compounds detected by HPLC in Table 2.

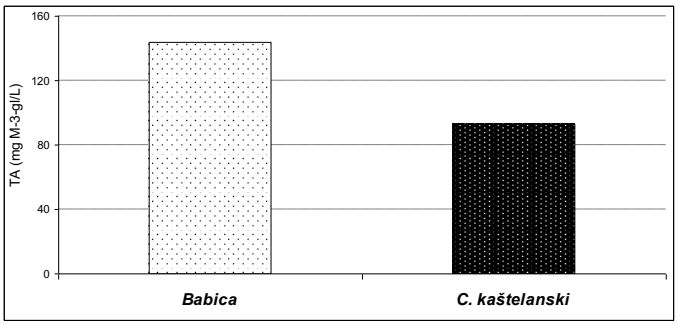


Fig. 3. The content of total anthocyanins (TA) in *Babica* and *C. kaštelanski* wines

Table 1. The content of anthocyanidin derivatives in *Babica* and *C. kaštelanski* wines

	Babica	***C. kaštelanski***
Total delphynidins (mg/L)	4.10	10.41
Total cyanidins (mg/L)	0.45	2.69
Total petunidins (mg/L)	6.43	10.39
Total peonidins (mg/L)	4.87	13.68
Total malvidins (mg/L)	67.95	58.43

The content of total monomeric anthocyanins in *Babica* was higher (143.73 mg M-3-gl/L) than in the *C. kaštelanski* (92.91 mg M-3-gl/L) wine. The dominant anthocyanin in both wines was malvidin-3-glucoside, with over 71 % of all detected anthocyanins in *Babica* and with over 52 % in *C. kaštelanski*. In our previous study on red grape anthocyanidins, we also investigated the profile of anthocyanidins in *Babica* and *C. kaštelanski* grape skin extracts. The results of that study also confirmed the higher content of malvidin derivatives in Babica (60.1 %) than in C. kaštelanski (52.1 %), which is in accordance with these results (Generalić Mekinić et al., 2016). Aside fromthe content of total malvidins, the *C. kaštelanski* wine contained higher proportions of delphynidins, cyanidins, petunidins and peonidins (Table 2).

The results for individual compounds are presented in Table 2. Generally, the dominant anthocyanin in wines is malvidin-3-O-glucoside (Liao et al., 1992; Gao et al., 1997; He et al., 2012), which was also confirmed by our results as expected. The anthocyanins identified in *Vitis vinifera* L. varieties are 3-O-monoglucosides and 3-O-acylated monoglucosides of the main anthocyanidins, and the acylated forms are the esters of the coumaric and caffeic acid (Dimitrovska et al., 2011).

In *Babica*, among other detected anthocyanins, there were petunidin-3-*O*-glucoside (7.33 %), malvidin-3-(6-*O*-coumaroyl) glucoside (5.13 %), peonidin-3-*O*-glucoside (4.83 %), delphinidin-3-O-glucoside (3.90 %), malvidin-3-*O*-acetylglucoside (3.09 %) and malvidin-(6-*O*-caffeoyl) glucoside (1.39 %). Furthermore, in *C. kaštelanski* wine the presence of peonidin-3-*O*-glucoside (12.86 %), petunidin-3-*O*-glucoside (8.95 %), delphinidin-3-O-glucoside (7.84 %), malvidin-3-(6-*O*-coumaroyl) glucoside (4.16 %), malvidin-3-*O*-acetylglucoside (2.65 %) and malvidin-(6-*O*-caffeoyl) glucoside (1.50 %) was confirmed. In comparison to *Babica*, slightly higher amounts of delfinidin-3-O-acetylglucoside (3-fold higher) and cyanidin-3-O-glucoside (10-fold higher) were detected in *C. kaštelanski*.

Studies showed that acylated anthocyanins contribute to the red colour of young red wine and that their concentrations vary during aging, while monomeric forms disappear within a few months after fermentation (He et al., 2012). Therefore, small amounts of acylated anthocyanins present in the investigated samples are not surprising. Most of these free anthocyanins will react, combine or condense with other present phenolics and form more complex and stable pigments, while a relatively small fraction will disappear by degradation, oxidation, precipitation, or formation of other colourless compounds (Jackson, 2008; He et al., 2012).

Table 2. Anthocyanin composition (%) of *Babica* and *C. kaštelanski* young

Anthocyanins	**Babica**	**Crljenak**
Delphinidin-3-*O*-glucoside	3.90 ± 0.02	7.84 ± 0.33
Cyanidin-3-*O*-glucoside	0.17 ± 0.01	1.82 ± 0.37
Petunidin-3-*O*-glucoside	7.33 ± 0.14	8.95 ± 0.48
Peonidin-3-*O*-glucoside	4.83 ± 0.42	12.86 ± 0.58
Malvidin-3-*O*-glucoside	71.49 ± 0.52	52.81 ±0.24
Delphinidin-3-*O*-acetylglucoside	0.98 ± 0.05	3.05 ± 0.16
Cyanidin-3-*O*-acetylglucoside	0.04 ± 0.00	0.49 ± 0.02
Petunidin-3-*O*-acetylglucoside	0.15 ± 0.00	0.95 ± 0.06
Peonidin-3-*O*-acetylglucoside	0.30 ± 0.01	0.60 ± 0.05
Petunidin-(6-*O*-caffeoyl)glucoside	0.13 ± 0.00	0.60 ± 0.01
Malvidin-3-*O*-acetylglucoside	3.09 ± 0.02	2.65 ± 0.03
Malvidin-(6-*O*-caffeoyl)glucoside	1.39 ± 0.02	1.50 ± 0.01
Cyanidin-(6-*O*-coumaryoyl)glucoside	0.32 ± 0.00	0.50 ± 0.01
Petunidin-(6-*O*-coumaryoyl)glucoside	0.05 ± 0.00	0.37 ± 0.01
Peonidin-3-(6-*O*-coumaroyl)glucoside	0.69 ± 0.00	0.85 ± 0.03
Malvidin-3-(6-*O*-coumaroyl)glucoside	5.13 ± 0.02	4.16 ± 0.04

CONCLUSION

According to the obtained results, notable differences in content of total anthocyanins, their chemical profile, and ratios of individual compounds in young wine from *Babica* and *C. kaštelanski* grapes were detected. Since the winemaking procedure was the same, those differences could be attributed to the grape variety and the chemical potential of the used raw material. In addition, anthocyanins in these red grapes/wines can be used as chemical markers for the differentiation of the grape cultivars. Although the content of total monomeric anthocyanins in *Babica* was significantly higher, other investigated parameters imply that the wine obtained from *C. kaštelanski* is highly coloured. Therefore, it can be concluded that the presence and the amount of individual anthocyanins have a considerable effect on the colour parameters of wine.

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PARTICLE SIZE DISTRIBUTION AND COLOUR OF WHITE CHOCOLATE WITH THE ADDITION OF ENCAPSULATED BLUEBERRY JUICE

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ABSTRACT

The cocoa bean is one of the richest known natural sources of antioxidants thatcontains more phenolic compounds and has higher antioxidant activity than tea or red wine. Many epidemiological studies show that dark chocolate may lower blood pressure and the incidence of cardiovascular diseases. On the other hand, white chocolate is a confectionery product consisting of sugar and milk powder dispersed in cocoa butter as a continuous fat phase. Unlike dark and milk chocolate, white chocolate does not contain dark cocoa particles that are rich in polyphenols. Polyphenol compounds from berry fruits have also shown beneficial effects on human health and the reduction of risk of cardiovascular disease, type II diabetes, and cancer.
Since the application of polyphenols in the creation of functional foods has recently attracted great interest in various research studies, our goal was to produce white chocolate enriched with 1%, 3% and 5% of polyphenol compounds extracted from blueberries.
Blueberry encapsulate material changed the values of lightness (L*), a* (red tone), and b* (yellow tone) measured on the surface of the enriched chocolate in accordance with the applied concentrations, while concentration of the added encapsulate material did not have any influence on the particle size distribution of the final product.

Keywords: white chocolate, blueberry juice encapsulate material, particle size distribution, colour

INTRODUCTION

The application of polyphenols in the creation of functional food has recently attracted great interest in various researchstudies in the field of food technology, due to their potential health benefits to humans (Fang & Bhandari, 2010). Several studies on rodents have revealed an attenuation of brain ageing when strawberries, blueberries, or blackberries are ingested, where the authors have proposed that the benefits are due to the presence of polyphenol compounds. Also, numerous investigations have shown that these phytochemicals have beneficial effects for human health and for the reduction of risk of

cardiovascular disease, type II diabetes, and cancer (Tavares et al., 2012). Blueberries contain plenty of phenolic compounds, including anthocyanins, quercetin, kaempferol, myricetin, chlorogenic acid and procyanidins (Howard et al., 2003).
However, the effectiveness of polyphenols depends on preserving their stability, bioactivity and bioavailability, since these natural compounds are very sensitive to light and heat and lack in long-term stability. Also, the taste of most phenolic components isunpleasant, which also limits their application in food. These deficiencies can be effectively alleviated by using encapsulated polyphenols, instead of free compounds (Fang and Bhandari, 2010; Munin and Edwards-Levy, 2011). The encapsulation technique encapsulates bioactive compounds in a biopolymer, protecting it from water, oxygen, light and other conditions in order to improve their stability and to turn liquid solutions into powders for easier handling. Freeze drying has been proved to be the most suitable method for drying thermo sensitive substances, because it minimizes thermal degradation reactions (Tumbas Šaponjac et al., 2016).
Chocolate is a complex rheological system that can be defined as a suspension consisting of non-fat particles (sugar, cocoa solids and milk powder) dispersed in cocoa butter as a continuous fat phase (Zarić et al., 2016). Dark cocoa particles are rich in polyphenols, particularly in flavan-3-ols (Mursu et al., 2004). Cacao products have more flavonoids and greater antioxidant capacity per serving than tea, red wine, fruit and vegetables, which are renowned for their high content of flavonoids (Shiina et al., 2009). Recent epidemiological studies suggest that chocolate may lower blood pressure and the incidence of cardiovascular diseases. However, white chocolate does not contain dark cocoa particles, only cocoa butter, sugar and milk. Thus, white chocolate lacks cocoa polyphenols (Rimbach, Egert and de Pascual-Teresa, 2011).
Since the market is increasingly searching for a functional food that can improve our life style and contribute to the reduction of disease risk, today's investigations are focused on finding new methods of creating nutritionally enriched functional confectionery products. Accordingly, this study was designed to examine the impact of encapsulated blueberry juice on the colour and particle size distribution of functional white chocolate.

MATERIALS AND METHODS

The materials used in this paper included white chocolate (denoted as control) (Eugen chocolate, Serbia) and blueberry juice encapsulated in whey (denoted as B) in order to protect the bioactive components from processing and storage (Frutarom Etol, Slovenia).

Pre-crystallization of white chocolate mass

The pre-crystallization of chocolate mass was carried out in a modified Brabender farinograph where the original kneader is connected with two thermostats by means of two-way taps. This enables an immediate temperature change in the kneader and a temperature change in the treated chocolate mass within half a minute (Pajin et al., 2012). White chocolate was placed in a farinograph fryer at 42 ºC for 30 minutes in order to melt without the risk of protein denaturation. Then the stirrer of the farinograph was switched on and the chocolate mass was gently stirred at 42 ºC for 1h, followed by another 1h at 29.5 ºC. The pre-crystallized

chocolate mass was then poured in a plastic mould, and cooled in a refrigerator at 5 ºC for 1.5 h.

Pre-crystallization of enriched chocolate mass

The same procedure was carried out in order to produce the control sample of white chocolate and chocolate samples enriched with 3 concentrations of encapsulated blueberry juice (1, 3 and 5%), where the certain concentration of encapsulate material was added to the melted chocolate after 30 minutes at 42 ºC. The following samples were obtained: 1%B, 3%B, and 5%B.

Determination of particle size distribution

Particle size distribution of encapsulated blueberry juice and chocolate samples was determined using the Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, England). The Scirocco dispersion unit was used for dispersing encapsulated blueberry juice in the air, while the Hydro 2000 μP dispersion unit was used for dispersing chocolate in sunflower oil. The samples were added at ambient temperature until an adequate obscuration was obtained (5-10% for dry samples and 10-20% for liquid samples). The results were quantified as the volume-based particle size distribution by means of the Mastersizer 2000 software.

Determination of surface colour

The surface colour of encapsulated blueberry juice and chocolate samples was measured in triplicate. The CIELab colour coordinates (L* – lightness, a* – redness to greenness, and b* – yellowness to blueness) were determined using the MINOLTA Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan) using D 65 lighting, a 2º standard observer angle and an 8-mm aperture in the measuring head. The Chroma Meter was calibrated using a Minolta calibration plate (No. 11333090; Y = 92.9, x = 0.3159; y = 0.3322) (CIE, 1976).

RESULTS AND DISCUSSION

Particle size distribution

Consumer acceptance depends primarily on the taste of chocolate, but also very much on mouth-feel, which mainly depends on the particle size and the viscosity of the molten chocolate mass. During production, the particles have to be milled to a specific particle size of 15– 30 μm. If particles are bigger, the final product will have a gritty mouth-feel andsmaller particles also increase the specific surface area, more liquid phase is needed to cover it, and viscosity increases (Bolenz, Holm & Langkrär, 2014). Figure 1 represents the particle size distribution of encapsulated blueberry juice, compared to the particle size distribution of white chocolate and enriched chocolate with the maximum concentration of added encapsulate material.

White chocolate has multimodal particle size distribution, since it was milled using a five roll mill. It can be also noticed from the figure that the largest volume of the blueberry juice encapsulate material consists of particles with diameters in the interval of 10-20 μm. This is very important in chocolate production, since consumers dislike sandy mouth-feel, and prefer quick melting without sticking (Bolenz & Manske, 2013). Sample volume of encapsulated blueberry juice, white chocolate, and enriched chocolate with blueberry juice encapsulate material, with the corresponding interval of particle sizes, is shown in Table 1.

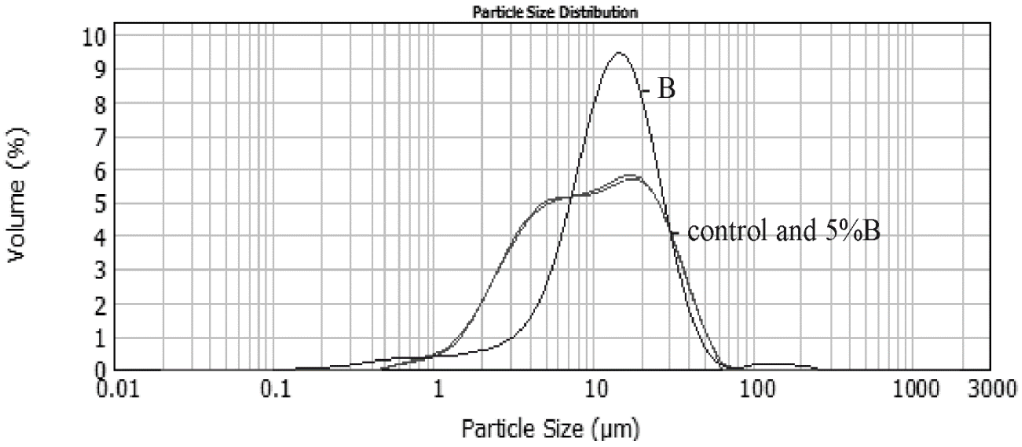

Fig. 1. Particle size distribution of encapsulated blueberry juice, white chocolate and enriched chocolate (5% of polyphenols)

Table 1. Sample volume of encapsulated blueberry juice and chocolate samples with the corresponding interval of the particle size distribution

Particle size (μm)	Sample volume (%)				
	B	control	1% B	3% B	5% B
0.1	2.41	0.89	0.78	0.98	1.07
1	31.53	50.29	50.80	50.54	50.28
10	40.72	25.06	25.33	25.48	25.55
20	16.68	13.49	13.20	13.24	13.29
30	5.52	6.39	6.34	6.22	6.25
40	2.40	3.89	3.55	3.54	3.56
100	0.74	0	0	0	0
500					

The addition of blueberry juice encapsulate material to white chocolate slightly increased the particle size distribution of enriched chocolates in the interval of 0.1-1 μm, in accordance with the added concentrations, since the encapsulate material contains a large volume of particles in that range (2.41%), compared to the control white chocolate (0.89%). More than half of the volume (50.29%) of white chocolate consists of particles in the range of 1-10 μm, where 31.53% of the volume of the

blueberry juice encapsulate material consists of particle sizes in that range. It has a minimal impact on the reduction of volume with particles in that range of samples with the encapsulate material added. One-quarter of the sample of white chocolate (25.06%) has the particles in the range of 10-20 μm, and 13.49% in the range of 20-30 μm. Only 10.28% of the white chocolate sample has the particles in the range of 30-100 μm. Considering the samples of encapsulated blueberry juice, it can be confirmed that the largest volume consists of particles with diameters in the interval of 10-20 μm, as much as40.72%, which increased the sample volume with particles in the range of 10-20% of the enriched chocolates. The encapsulate material volume consisting of particles in the range of 20-30 μm amounts to 16.68%, which is higher than in the control white chocolate. On the other hand, blueberry juice encapsulate material has a lower volume consisting of particles in the range of 30-100 μm compared to white chocolate, and also it contains particles with a diameter of 100-500 μm, unlike the control and the enriched chocolate, which do not contain them.

Surface colour

Colour is one of the most important characteristics of foods, since it is considered as a quality indicator that determines their acceptance (Chranioti et al., 2015). The values of lightness (L*), a* (red tone), and b* (yellow tone) measured on the surface of white chocolate and the encapsulated blueberry juice are shown in Figure 2.
The sample of white chocolate has a very high L* value (75.05), indicating a bright surface of white chocolate, as well as a high share of the yellow tone. The blueberry encapsulate material has the lowest L* value (40.42) or the darkest surface colour compared to white chocolate and the enriched chocolates. The addition of the encapsulated blueberry juice to white chocolate led to the creation of a new type of coloured chocolate, in whichthe increase in the concentration of the encapsulate material affects its surface colour. The chocolate with 5% of the encapsulate material has the lowest L* value or the darkest surface area compared to the control sample and other enriched chocolates.
The control white chocolate has a negative a* value (-1) on its surface, which includes green tones. On the other hand, the blueberry juice encapsulate material has an a* value, which amounts to 11.56 and increases the red tone in the enriched chocolates in accordance with the added concentrations. The chocolate sample with the maximum concentration of blueberry compounds has an even higher a* value than the pure blueberry juice encapsulate material.
As expected, the white chocolate has a high b* value (20.32) or a high intensity of the yellow tone, while the blueberry juice encapsulate has a negative b* value and a high intensity of the blue tones. The addition of 1% of the encapsulate material changed the chocolate colour to a greyish with a positive b* value. On the other hand, the addition of 3% and 5% of the encapsulate material influenced the final products with an increase in blue colour, where the sample containing 5% has a higher intensity of the blue colour compared to the sample with 3% of blueberry juice encapsulate material.

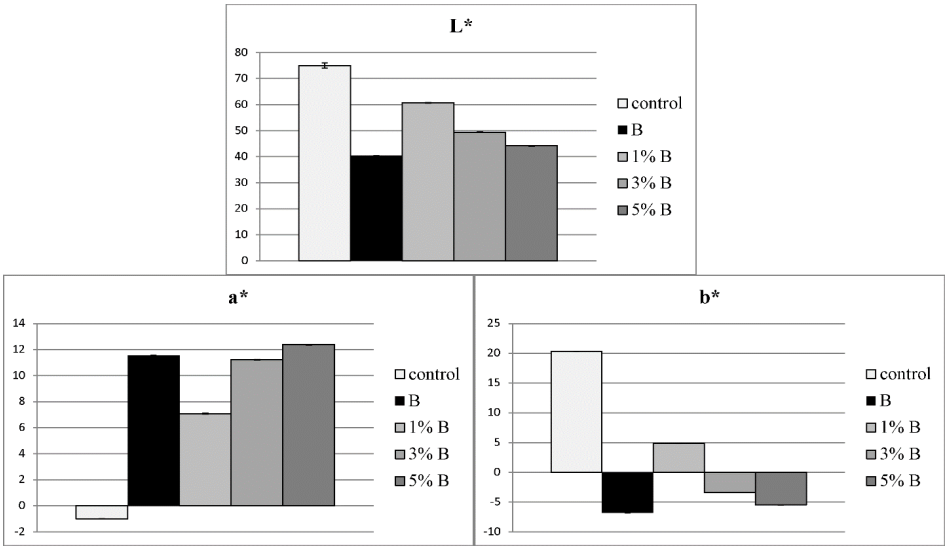

Fig. 2. Colour on the surface of the encapsulated blueberry juice, white chocolate and enriched chocolates (CIEL*a*b* system)

CONCLUSIONS

The blueberry encapsulate material has particle a size distribution in a suitable interval for chocolate production, since 88.93% of the sample volume has particle sizes in the range of 1-30 μm. Accordingly, the addition of blueberry juice encapsulate material to white chocolate did not increase the particle size distribution in the enriched chocolate samples with 1, 3 and 5% of the encapsulate material.
Since white chocolate has a very bright surface with green and yellow tones, the addition of blueberry compounds led to the creation of a new type of coloured enriched chocolate. The chocolate with 1% of the encapsulate material has a greyish colour, while the increase in the concentration of blueberry juice encapsulate material to 3% significantly increased the intensity of the red and blue tones. As expected, chocolate with 5% of the encapsulate material has the darkest surface, with the highest value of the red and the blue tones, compared to the other chocolate samples.

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Topic: Production of safe food and food with added nutritional value

Sekcija: Proizvodnja zdravstveno sigurne i nutritivno vrijedne hrane

PROIZVODNJA I STABILIZACIJA HLADNO PREŠANOG ULJA KOŠTICE ŠLJIVE

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SAŽETAK

Postupkom hladnog prešanja iz koštice šljive (*Prunus domestica* L.) dobije se kvalitetno jestivo ulje. Cilj ovog rada bio je ispitati utjecaj procesnih parametara prešanja koštice šljive na efikasnost proizvodnje hladno prešanog ulja te njegovu kvalitetu. Prilikom prešanja mijenjani su sljedeći procesni parametri: frekvencija elektromotora (brzina pužnice), temperatura zagrijavanja glave preše i nastavak za izlaz pogače. Prešanje je provedeno s kontinuiranom pužnom prešom. Primjenom standardnih metoda određeni su osnovni parametri kvalitete proizvedenog hladno prešanog ulja. Ispitan je i utjecaj dodatka antioksidanasa na oksidacijsku stabilnost ulja primjenom Testa održivosti na 98 °C. Korišteni su sljedeći prirodni antioksidansi: ekstrakt zelenog čaja, ekstrakt ružmarina i eterično ulje primorskog vriska te sintetski antioksidans oktil galat. Rezultati ispitivanja pokazuju da procesni parametri hladnog prešanja značajno utječu na iskorištenje ulja koštice šljive. Veće iskorištenje sirovog ulja i hladno prešanog ulja postignuto je kod temperature zagrijavanja glave preše 100 °C, frekvencije elektromotora 35 Hz i nastavka za izlaz pogače 10 mm. Dodatkom prirodnih antioksidanasa postignuta je bolja zaštita ulja od oksidacijskog kvarenja nego dodatkom sintetskog antioksidansa. Eterično ulje primorskog vriska pokazalo je najbolji antioksidacijski utjecaj.

Ključne riječi: koštice šljive, hladno prešanje, procesni parametri, oksidacijska stabilnost, antioksidansi

UVOD

Šljiva (*Prunus domestica* L.) je listopadno stablo iz porodice ruža (*Rosaceae*) kojem pripadaju i marelice, breskve, nektarine, višnje i trešnje. Suhe šljive su poznate po antioksidacijskim i antikancerogenim svojstvima, imaju umjereno prirodno laksativno djelovanje te se mogu koristiti u slučaju zatvora posebno kod male djece ili starijih osoba (Mahmood i sur., 2009). Provedena istraživanja Swain-a i Hillis-a (1959) pokazuju da su šljive bogate polifenolima, antocijanima i flavanolima za koje je poznato i utvrđeno da imaju antioksidacijska svojstva. Imaju i svojstva koja usporavaju starenje (tzv. anti-age svojstva) tako da je sve češća primjena komponenata iz šljiva u kozmetičke svrhe (Mahmood i sur., 2009; Özcan i sur., 2015). Izuzev samog ploda šljive, značajna je i koštica šljive koja se u zadnje vrijeme sve više istražuje š jer je

mnogim istraživanjima utvrđeno da sadrži 40-50 % ulja. Koštica šljive je nusproizvod prehrambene industrije iz koje se prešanjem može dobiti jestivo hladno prešano ulje, a njegova konzumacija može smanjiti rizik obolijevanja od kroničnih bolesti. Danas je poznato ulje koštice šljive koje se koristi u kozmetičke svrhe zbog svojih regenerirajućih i hidratizirajućih svojstava te povoljnog utjecaja na kožu. Istraživanjem koje su proveli Özcan i sur. (2015) utvrđeno je da bi koštice šljive mogle biti dobre sirovine za proizvodnju jestivog ulja jer imaju mali udio slobodnih masnih kiselina i nisku vrijednost peroksidnog broja. Također, koštice šljive bogate su nezasićenim masnim kiselinama od kojih je najzastupljenija oleinska kiselina (74,19 %) i linolna (19,14 %) tako da bi ulje koštice šljive bilo izuzetno stabilno pri obradi i proizvodnji proizvoda poput margarina, krema, majoneze ili salatnih preljeva. Poznato je kako koštica marelice ima značajna antioksidacijska svojstva te da se od nje proizvodi jestivo ulje koje se preporučuje konzumirati zbog povoljnog utjecaja na naše zdravlje. Međutim, koštica šljive ima visok udio kampesterola, čak i veći udio nego koštica marelice. Također, utvrđeno je da je bogata γ-tokoferolom (85,5 %), a u manjim udjelima tu su α-tokoferol (11,0 %) i δ-tokoferol (3,5 %). Navedene komponente imaju antioksidacijska svojstva i može se pretpostaviti kako bi ulje koštice šljive imalo čak i veće antioksidacijsko djelovanje u našem organizmu nego ulje koštice marelice (Hassanein, 1999). Utvrđeno je postojanje amigdalina u košticama šljive. Prema kemijskom sastavu je cijanogeni glikozid i poznat je po svom toksičnom djelovanju. Međutim, koncentracije koje se nalaze u samim košticama šljive su vrlo male i nemaju toksičan utjecaj na naše zdravlje (Ghiulai i sur., 2006). Iako prekomjerna konzumacija koštica koje sadrže veliku količinu amigdalina može uzrokovati akutno ili kronično trovanje ljudi i životinja (Silem i sur., 2006), dokazano je kako je u ulju dobivenom iz koštice šljive amigdalin prisutan u tragovima ili ga uopće nema tako da ne može imati štetan utjecaj na naš organizam (Ghiulai i sur., 2006). Danas se preferira hladno prešanje za izdvajanje ulja iz biljnog sjemena, umjesto konvencionalnog postupka u kojem se ekstrakcija ulja provodi organskim otapalom. Kod proizvodnje hladno prešanih i nerafiniranih ulja ne postoji faza koja bi omogućila uklanjanje nepoželjnih kontaminanata iz ulja i znatno su strožiji uvjeti kvalitete sirovine (Dimić, 2005). U proizvedenom hladno prešanom ulju zadržana je u potpunosti nutritivna vrijednost te se može koristiti izravno kao jestivo ulje (Belitz, Grosch i Schieberle, 2004). Također, postupkom hladnog prešanja osigurava se maksimalno zadržavanje bioaktivnih spojeva kao što su esencijalne masne kiseline, fenolne i flavonoidne tvari, tokoferoli i dr. (Teh i Birch, 2013; Krist i sur., 2005) i senzorska svojstva ulja jer ovdje nema termičke pripreme sirovine (kondicioniranje) prije provedbe prešanja. Tokoferoli i tokotrienoli su prirodna skupina spojeva u biljnim uljima koji imaju antioksidacijska svojstva. Postupkom hladnog prešanja koštica dobiva se sirovo ulje koje ide na pročišćavanje (sedimentacija, filtriranje, centrifugiranje) radi dobivanja hladno prešanog ulja (Dimić i Turkulov, 2000; Shahidi, 2005). Kao nusprodukt prešanja uljarica dobiva se uljna pogača u kojoj zaostane određena količina ulja, proteini, minerali, vlakna i drugi sastojci (Zubr, 1997; Quezada i Cherian, 2012). Razni istraživači ukazuju na to da procesni parametri mogu utjecati na iskorištenje ulja tijekom prešanja raznih uljarica. U prijašnjim istraživanjima (Jokić i sur., 2014) provedena je optimizacija postupka proizvodnje hladno prešanog orahovog ulja primjenom pužne preše te je utvrđeno da procesni parametri prešanja utječu na iskorištenje

ulja. Također je utvrđeno (Moslavac i sur., 2014) da parametri hladnog prešanja sjemenki podlanka *Camelina sativa* L. utječu na iskorištenje sirovog ulja i hladno prešanog ulja. Dodatkom prirodnih antioksidanasa utječe se na porast stabilnosti ulja prema oksidacijskom kvarenju.
Predmet istraživanja ovog rada bio je ispitati utjecaj procesnih parametara prešanja (frekvencija elektromotora, temperatura zagrijavanja glave preše, nastavak za izlaz pogače) koštice šljive na efikasnost proizvodnje sirovog ulja i hladno prešanog ulja. Na proizvedenom hladno prešanom ulju ispitani su osnovni parametri kvalitete: peroksidni broj, slobodne masne kiseline, udio netopljivih nečistoća te udio vlage i hlapljivih tvari. Kako bi se odredila efikasnost proizvodnje sirovog ulja prešanjem, provedeno je određivanje količine ulja u košticama šljive i pogači metodom po Soxhlet-u. Primjenom Testa održivosti ulja na 98 °C ispitan je utjecaj dodatka antioksidanasa na oksidacijsku stabilnost ulja koštice šljive tijekom 20 sati testa.

MATERIJALI I METODE

Materijali

Za ispitivanje utjecaja procesnih parametara hladnog prešanja na iskorištenje ulja korištene su koštice šljive prikupljene na području Slavonije i Baranje, očišćene od mezokarpa i osušene u prirodnim uvjetima. Čuvane su neoljuštene u vrećama na tamnom i suhom mjestu pri sobnoj temperaturi. Koštice su drobljene neposredno prije prešanja kako bi se koštica oslobodila i mogla koristiti za proizvodnju hladno prešanog ulja.

Antioksidansi:
Ekstrakt ružmarina tip OxyLess®CS prirodni je ekstrakt listova ružmarina (*Romarinus officinalis* L.) proizveden u praškastom obliku u Francuskoj (tvrtka Naturex). Udio karnosolne kiseline je od 18 % do 22 %, zaštitni faktor (PF) je veći od 12 %, suha tvar ekstrakta je 92 - 98 %. U ispitivanju je upotrijebljen u udjelu 0,2 % računato na masu ulja.
Ekstrakt zelenog čaja prirodni je ekstrakt dobiven iz listova zelenog čaja (*Camellia sintensis* L.), proizveden je u praškastom obliku u Francuskoj (tvrtka Naturex). Udio epigalokatehin galata (EGCG) veći je od 45 %, udio ukupnih polifenola veći od 98 %, udio kofeina manji je od 2 %, a udio katehina veći od 80 %. U ispitivanju je upotrijebljen u udjelu 0,2 % računato na masu ulja.
*Eterično ulje primorskog vriska d*obiveno je parnom destilacijom cvjetnih vrhova primorskog vriska (*Satureja montana* L.). Eterično ulje korišteno u ovom ispitivanju proizvedeno je na Institutu za ratarstvo i povrtlarstvo iz Novog Sada u Srbiji. U ispitivanju je upotrijebljeno u udjelu 0,1 % računato na masu ulja.
*Oktil galat*je sintetski antioksidans, a u istraživanju dodan je u ulje u udjelu 0,01 %.

Proizvodnja hladno prešanog ulja

Hladno prešanje koštica šljive provedeno je primjenom kontinuirane pužne preše modela SPU 20 (Elektromotor-Šimon, Srbija), kapaciteta prerade uljarica 20 kg/h, snage elektromotora 1,5 kW. Za provedbu eksperimenta hladnog prešanja korišteno je 0,5 kg sirovine.

Određivanje parametara kvalitete ulja

Određivanje udjela ulja u košticama šljive i pogači kao nusproduktu prešanja provedeno je primjenom metode ISO 734-1:1998 (Određivanje masti po Soxhletu).
Kiselost jestivih biljnih ulja nastaje kao rezultat hidrolize triacilglicerola u prisustvu vode i lipolitičkih enzima, a izražena je kao udjel slobodnih masnih kiselina. Određivanje udjela slobodnih masnih kiselina u ispitivanom svježe proizvedenom hladno prešanom ulju koštice šljive provedeno je standardnom metodom HRN EN ISO 660:1996 koja se temelji na principu titracije s otopinom natrij-hidroksida. Rezultat se izražava kao udjel (%) slobodnih masnih kiselina (SMK) prema izrazu (1).

$$SMK\ (\%\ oleinske\ kiseline) = \frac{V \cdot c \cdot M}{10 \cdot m} \quad (1)$$

Gdje je:
V = utrošak otopine natrij-hidroksida za titraciju uzorka (mL)
c = koncentracija otopine natrij-hidroksida za titraciju, c(NaOH) = 0,1 mol/L
M = molekulska masa oleinske kiseline, M = 282 g/mol
m = masa uzorka ulja za ispitivanje (g)

Određivanje peroksidnog broja ispitivanog hladno prešanog ulja provedeno je standardnom metodom HRN EN ISO 3960:2007. Rezultat je izražen kao mmol aktivnog kisika koji potječe iz nastalih peroksida prisutnih u 1 kg ulja (2).

$$Pbr = \frac{(V_1 - V_0) \cdot 5}{m}\ (mmol\ O_2/kg) \quad (2)$$

Gdje je:
V_1 = volumen otopine natrij-tiosulfata, c ($Na_2S_2O_3$) = 0,01 mol/L utrošen za titraciju uzorka ulja (mL)
V_0 = volumen otopine natrij-tiosulfata utrošen za titraciju slijepe probe (mL)
m = masa uzorka ulja (g)
Određivanje udjela vode i hlapljivih tvari u ulju koštice šljive provedeno je prema metodi HRN EN ISO 662:1998 i izračunato iz izraza (3):

$$\%\ vode = \frac{m_1 - m_2}{m_1 - m_0} \times 100 \quad (3)$$

Gdje je:
m_0 = masa staklene posudice (g)
m_1 = masa staklene posudice i uzorka prije sušenja (g)
m_2 = masa staklene posudice i uzorka nakon sušenja (g)

Određivanje količine netopljivih nečistoća u ulju koštice šljive provedeno je prema metodi HRN EN ISO 663:1992 i izračunato prema izrazu (4):

$$\%\ netopljive\ nečistoće = \frac{m_2 - m_1}{m_0} \times 100 \quad (4)$$

Gdje je:
m_0 = masa uzorka (g)
m_1 = masa osušenog filter lijevka (g)
m_2 = masa filter lijevka s nečistoćama nakon sušenja (g)

Izračunavanje stupnja djelovanja prešanja

Na temelju udjela ulja u sirovini i dobivenoj pogači, može se izračunati prinos prešanog ulja, odnosno stupanj djelovanja prešanja (Dimić i Turkulov, 2000).
Količina sirovog ulja dobivenog prešanjem izračunata je prema jednadžbi (5) (Dimić, 2005):

$$U = U_0 - U_p \times \left(\frac{a}{b}\right)(\%) \quad (5)$$

Gdje je:
U –količina prešanog ulja (%);
Uo – udio ulja u sirovini (%);
Up – udio ulja u pogači (%);
a – suha tvar u sirovini (%);
b – suha tvar u pogači (%).
Za izračunavanje Stupnja djelovanja prešanja (P) korištena je jednadžba (6):

$$P = \left(\frac{U}{U_0}\right) \times 100\ (\%) \quad (6)$$

Gdje je:
U - količina prešanog ulja (%)
Uo - udio ulja u sirovini (%)

Određivanje oksidacijske stabilnosti ulja

Priprema uzoraka ulja

U staklene čaše ulilo se 30 g ulja te određeni udio ispitivanih antioksidanasa i promiješalo se staklenim štapićem. Zatim su se tako pripremljeni uzorci zagrijali na temperaturu od 70 °C i održavali uz miješanje 30 minuta kako bi se antioksidansi otopili i jednoliko rasporedili u ulju. Uzorci su se ohladili na sobnu temperaturu, prekrili satnim stakalcem i stavili u sušionik na

konstantnu temperaturu od 98 °C čime započinje ispitivanje oksidacijske stabilnosti ulja koštice šljive, sa i bez dodanih antioksidanasa.

Test održivosti na 98 °C

Oksidacijska stabilnost ulja određena je prema metodi ubrzane oksidacije ulja i to primjenom Testa održivosti na 98 °C. Tijekom ovog testa, svakih 60 min određena je vrijednost peroksidnog broja u prvih 6 sati testa. Nakon toga, vremenski intervali za određivanje stupnja oksidacije ulja produženi su i mjerena je vrijednost peroksidnog broja (Pbr) u osmom, desetom, petnaestom i dvadesetom satu. Uzorci ulja su držani ukupno 20 sati na konstantnoj temperaturi u sušionku.

REZULTATI I RASPRAVA

Prije provedbe hladnog prešanja koštica šljive, određen je udio ulja u košticama te je srednja vrijednost iznosila 36,28 %. Standardnom metodom određen je i izračunat udio vlage u košticama pri čemu je dobivena vrijednost 5,42 %.

Utjecaj procesnih parametara prešanja na iskorištenje ulja

Rezultati ispitivanja utjecaja procesnih parametara prešanja koštica šljive (frekvencija elektromotora, temperatura zagrijavanja glave preše, veličina otvora za izlaz pogače) na iskorištenje sirovog ulja i hladno prešanog ulja prikazani su u Tablicama 1-3. Na proizvedenom hladno prešanom ulju ispitani su osnovni parametri kvalitete prema Pravilniku o jestivim uljima i mastima NN 41/12 (Tablica 4).
Utjecaj frekvencije elektromotora, odnosno brzine pužnice, tijekom prešanja koštica šljive na efikasnost proizvodnje sirovog i hladno prešanog ulja prikazan je u Tablici 1. Ispitan je utjecaj tri frekvencije elektromotora: 25, 30 i 35 Hz, uz konstantnu temperaturu zagrijavanja glave preše (100 °C) i veličinu otvora za izlaz pogače (8 mm). Prešanjem koštice šljive kod frekvencije elektromotora 25 Hz dobiveno je 186 mL sirovog ulja temperature 44 °C, a nakon 28 dana sedimentacije (taloženja) i vakuum filtracije, volumen hladno prešanog ulja (finalnog ulja) iznosio je 140 mL. Udio zaostalog ulja u pogači iznosio je 19,96 %, a stupanj djelovanja preše 50,26 %. Povećanjem frekvencije elektromotora tijekom prešanja na 30 Hz, zapaženo je smanjenje proizvedenog sirovog i hladno prešanog ulja, stupanj djelovanja preše se smanjio što rezultira i nešto većim udjelom zaostalog ulja u pogači. Daljnjim porastom frekvencije elektromotora na 35 Hz došlo je do porasta proizvodnje sirovog ulja koštice šljive te nakon 28 dana taloženja i vakuum filtracije i najveća količina finalnog ulja (158 mL). Prikazani rezultati ukazuju na pojavu da se kod ovih uvjeta prešanja dobilo više ulja koštica šljive kod veće frekvencije elektromotora (35 Hz) u odnosu na 25 i 30 Hz. Ova pojava je u suprotnosti s istraživanjima drugih autora (Kartika i sur., 2010) koji ističu da frekvencija elektromotora također ima utjecaj na iskorištenje ulja, tj. da se pri manjoj vrijednosti ovog parametra dobiva više ulja jer se stvaraju veći tlakovi pa se više ulja iscijedi iz uljarice. Kod prešanja koštice šljive razloge treba tražiti u sastavu koštice šljive i utjecaju relativno visoke temperature grijača glave preše na iskorištenje ulja.

Tablica 1. Utjecaj frekvencije elektromotora (brzine pužnice) tijekom prešanja koštice šljive kod temperature grijača glave preše od 100 °C i otvorom nastavka 8 mm na iskorištenje hladno prešanog ulja, s polaznom masom sirovine 0,5 kg
Table 1. Effect of frequency electric motor (speed screw) during pressing plum kernel at press head temperature 100 °C and nozzle size 8 mm on the yield of cold pressed oil, with initian raw-material mass of 0.5 kg

Parametri prešanja	V sirovog ulja (mL)	V ulja nakon 28 dana taloženja i vakuum filtriranja (mL)	T sirovog ulja (°C)	m pogače (g)	w ulja u pogači (%)	Stupanj djelovanja preše (%)
F = **25 Hz**	186	140	44	332,24	19,96	50,26
F = **30 Hz**	172	132	46	330,88	20,31	49,39
F = **35 Hz**	190	158	43	323,90	19,64	51,06

Udio ulja u košticama šljive je 36,28 %, a udio vode 5,42 %.

Utjecaj temperature zagrijavanja glave preše (80, 90, 100 °C) kod konstantnih parametara otvora za izlaz pogače (12 mm) i frekvencije elektromotora (25 Hz) na iskorištenje ulja prikazan je u Tablici 2. Rezultati dobiveni tijekom ovog ispitivanja pokazuju da se porastom temperature glave preše povećavaju volumen i temperatura sirovog ulja te količina proizvedenog hladno prešanog ulja šljive, uz postepeno smanjenje udjela zaostalog ulja u pogači. Tijekom zagrijavanja glave preše na 100 °C proizveden je veći volumen sirovog ulja i hladno prešanog ulja nakon 28 dana sedimentacije i vakuum filtracije uz najniži udio zaostalog ulja u pogači (14,67 %). Rezultat porasta količine proizvedenog ulja s porastom temperature zagrijavanja glave preše može se objasniti tako što se prešanjem povećava i procesni tlak, a to rezultira i većim cijeđenjem ulja iz koštice. Martinez i sur. (2013) također ukazuju da temperatura prešanja značajno utječe na iskorištenje sirovog i hladno prešanog ulja. Također se porastom temperature snižava i viskoznost ulja što dovodi do većeg iskorištenja ulja tijekom prešanja.

Tablica 2. Utjecaj temperature zagrijavanja glave preše tijekom prešanja koštice šljive pri 25 Hz i s nastavkom otvora 12 mm na iskorištenje hladno prešanog ulja, s polaznom masom sirovina 0,3 kg
Table 2. Effect of temperature heating head presses during pressing plum kernel at 25 Hz and nozzle size 12 mm on yield of cold pressed oil, with initial raw-material mass of 0.3 kg

Parametri prešanja	V sirovog ulja (mL)	V ulja nakon 28 dana taloženja i vakuum filtriranja (mL)	T sirovog ulja (°C)	m dobivene pogače (g)	w ulja u pogači (%)	Stupanj djelovanja preše (%)
T = **80 °C**	70	44	37	224,72	18,51	48,98
T = **90 °C**	80	49	37	215,50	15,53	57,19
T = **100 °C**	90	71	44	206,99	14,67	59,56

Utjecaj nastavka na glavi preše, tj. veličini otvora za izlaz pogače (5, 8, 10 mm) tijekom prešanja koji utječe na vrijednost procesnog tlaka stvorenog u preši i djeluje na iskorištenje sirovog i finalnog hladno prešanog ulja prikazan je u Tablici 3. Korištenjem nastavka za izlaz pogače promjera 5 mm i prešanjem kod konstantnih uvjeta frekvencije elektromotora 25 Hz i temperature zagrijavanja glave preše na 100 °C, dobiveno je 224 mL sirovog ulja temperature 44 °C. Nakon 28 dana sedimentacije (taloženja) i završne vakuum filtracije volumen proizvedenog hladno prešanog ulja šljive iznosio je 178 mL, a udio zaostalog ulja u pogači 15,53 %, te stupanj djelovanja preše 61,30 %. Upotrebom nastavka većeg promjera otvora 8 mm te prešanjem uz navedene vrijednosti temperature glave preše i frekvencije, dobiven je manji volumen sirovog ulja (186 mL) i finalnog ulja (140 mL). Analizom zaostalog ulja u pogači utvrđena je vrijednost 19,96 % te izračunat manji stupanj djelovanja preše 50,26 %. Kod sljedećeg ispitivanja utjecaja veličine otvora glave preše korišten je nastavak promjera 10 mm, a dobiveni rezultati su: volumen sirovog ulja (232 mL), volumen finalnog ulja nakon taloženja i vakuum filtracije (200 mL), udio zaostalog ulja u pogači je najniži (14,97 %) i najviši stupanj djelovanja preše (62,70 %). Iz navedenih podataka se može zaključiti da se primjenom nastavka veličine otvora 10 mm dobiju veće vrijednosti volumena proizvedenog sirovog ulja i hladno prešanog ulja te manji udio zaostalog ulja u pogači u odnosu na primjenu nastavka veličine 5 i 8 mm. Razlog tome treba tražiti u sastavu koštice šljive i visokoj temperaturi zagrijavanja glave preše tijekom provedbe ovog prešanja.

Tablica 3. Utjecaj veličine otvora glave preše za izlaz pogače tijekom prešanja koštice šljive kod temperature grijača glave preše od 100 °C i 25 Hz na iskorištenje hladno prešanog ulja, s masom polazne sirovine 0,5 kg
Table 3. Effect of nozzle size head presses during pressing plum kernel at press head temperature 100 °C and 25 Hz on yield of cold pressed oil, with initial raw-material mass 0.5 kg

Parametri prešanja	**V sirovog ulja (mL)**	**V ulja nakon 28 dana taloženja i vakuum filtriranja (mL)**	**T sirovog ulja (°C)**	**m dobivene pogače (g)**	**w ulja u pogači (%)**	**Stupanj djelovanja preše (%)**
N = **5 mm**	224	178	44	307,77	15,53	61,30
N = **8 mm**	186	140	44	332,24	19,96	50,26
N = **10 mm**	232	200	52	325,30	14,97	62,70

Kvaliteta proizvedenog ulja

Na svježe proizvedenom hladno prešanom ulju koštice šljive provedeno je određivanje osnovnih parametara kvalitete prema Pravilniku o jestivim uljima i mastima (NN 41/12). Rezultati analiza prikazani su u Tablici 4 gdje se može uočiti da su svi ispitivani parametri zadovoljili navedeni Pravilnik, osim udjela netopljivih nečistoća koji je iznosio 0,455 %, a maksimalna dopuštena vrijednost prema Pravilniku je 0,1 %.

Udio netopljivih nečistoća može se smanjiti dodatnom filtracijom ili centrifugiranjem tako da se može reći da je proizvedeno ulje dobre kvalitete. Parametri kvalitete ulja Pbr i SMK pokazuju da u sirovini nije došlo do procesa kvarenja ulja.

Tablica 4. Početne kemijske karakteristike ispitivanog ulja koštice šljive
Table 4. Initial chemical characteristics of the tested plum kernel oil

Parametar kvalitete	**Ulje koštice šljive (hladno prešano)**
Pbr (mmol O_2/kg)	0,0
SMK (% oleinske kiseline)	0,315
Voda (%)	0,085
Netopljive nečistoće (%)	0,455

SMK – slobodne masne kiseline, izražene kao % oleinske kiseline;
Pbr – peroksidni broj, mmol O_2/kg.

Tablica 5 prikazuje oksidacijsku stabilnost ili održivost proizvedenog hladno prešanog ulja koštice šljive, sa i bez dodanih antioksidanasa, koja je određena Testom održivosti na 98 °C. Rezultati u Tablici 5 pokazuju da je tijekom 20 sati provođenja ovog testa održivosti došlo do porasta vrijednosti Pbr kod svih ispitivanih uzoraka ulja. Nakon 20 sati testa ulje koštice šljive bez dodanog antioksidansa (kontrolni uzorak) ima peroksidni broj (Pbr) 6,16 mmol O_2/kg što je vrijednost unutar granica propisanih Pravilnikom o jestivim uljima i mastima, a to znači da je ulje izuzetno dobre kvalitete i otporno prema oksidacijskom kvarenju. Dodatkom eteričnog ulja primorskog vriska u udjelu 0,1% postignuta je najbolja zaštita ulja od oksidacijskog kvarenja, vrijednost Pbr nakon 20 sati testa je iznosio 1,26 mmol O_2/kg. Također, jako dobru zaštitu ostvario je i ekstrakt ružmarina (tip Oxy Less CS) dodan u udjelu 0,2 %, gdje je vrijednost Pbr nakon 20 sati testa iznosila 1,97 mmol O_2/kg. Ekstrakt zelenog čaja dodan u udjelu 0,2 % pružio je slabiju zaštitu ulja, ali i dalje se može smatrati dobrim antioksidansom za stabilizaciju ovog ulja. Vrijednost Pbr nakon 20 sati provođenja testa bila je 2,22 mmol O_2/kg. Najslabiju zaštitu pružio je sintetski antioksidans oktil galat dodan u udjelu 0,01 %, gdje je vrijednost Pbr bila 2,46 mmol O_2/kg na kraju testa. Na Slici 1 vidljiv je utjecaj dodatka navedenih antioksidanasa na oksidacijsku stabilnost ulja nakon 20 sati provedbe testa. Ulje koštice šljive, samo po sebi, ima znatnu oksidacijsku stabilnost, primjećuje se da su dodani prirodni antioksidansi imali bolju funkciju u zaštiti od oksidacijskog kvarenja nego sintetski antioksidans oktil galat. Dodatak antioksidanasa ne bi trebao utjecati na senzorska svojstva ulja (boja, miris, okus). Međutim, ekstrakt zelenog čaja promijenio je boju ulja u crvenkastosmeđu, a ekstrakt ružmarina u svjetlonarančastu boju. Eterično ulje primorskog vriska dalo je ulju blagi miris karakterističan za navedeno eterično ulje. Potrebno je naglasiti kako su se ove promjene dogodile tek nakon provođenja Testa održivosti na 98 °C u kojem je ulje tijekom 20 sati zagrijavano, ali ne i prilikom samog dodatka antioksidanasa u ulje.

Tablica 5. Oksidacijska stabilnost hladno prešanog ulja koštice šljive, sa i bez dodanog antioksidansa, određena testom održivosti na 98 °C
Table 5. The oxidative stability of cold pressed plum kernel oil, with or without added antioxidants, determined by the test of sustainability at 98 °C

Uzorak	Udio antioksidansa (%)	Pbr (mmol O_2/kg)										
		0. sat	1.	2.	3.	4.	5.	6.	8.	10.	15.	20.
Ulje koštice šljive	-	0	0	0,52	1,31	1,95	2,35	2,44	2,93	3,05	3,75	6,16
Ekstrakt zelenog čaja	0,2			0,25	0,58	0,56	0,96	1,02	1,49	1,5	2,03	2,22
Ekstrakt ružmarina (Oxy Less CS)	0,2			0	0,24	0,49	0,54	0,47	0,76	1,01	1,02	1,97
Eterično ulje primorskog vriska	0,1			0	0,45	0,47	0,51	0,49	0,49	0,76	1,01	1,26
Oktil galat	0,01			0	0,56	0,77	0,74	1,14	1,19	1,38	1,59	2,46

ZAKLJUČCI

Temeljem dobivenih rezultata ispitivanja procesnih parametara prešanja koštica šljive, može se zaključiti da frekvencija elektromotora, temperatura zagrijavanja glave preše i veličina otvora glave preše za izlaz pogače utječu na iskorištenje sirovog i hladno prešanog ulja. Frekvencija elektromotora regulira brzinu pužnice i time utječe na vrijeme trajanja prešanja koštice kod određenog tlaka. Prešanjem koštica kod frekvencije elektromotora 35 Hz proizvedena je veća količina sirovog ulja i hladno prešanog ulja nego kod 25 i 30 Hz. Korištenjem nastavka za izlaz pogače većeg promjera (10 mm) proizvedena je veća količina sirovog ulja i hladno prešanog ulja uz manji udio zaostalog ulja u pogači te veći stupanj djelovanja preše. Zagrijavanjem glave preše tijekom prešanja dolazi do kondicioniranja koštice i omekšavanja pogače što rezultira većim iskorištenjem ulja. Porastom temperature glave preše dobivena je veća količina sirovog ulja i hladno prešanog ulja uz manji udio zaostalog ulja u pogači. Proizvedeno hladno prešano ulje koštice šljive je odlične kvalitete, a osnovni parametri kvalitete su u skladu s Pravilnikom o jestivim uljima i mastima.
Dodatak ispitivanih antioksidanasa u ulje koštice šljive dodatno poboljšava stabilnost ulja budući da je vrijednost peroksidnog broja niža od vrijednosti peroksidnog broja uzorka ulja bez dodatka antioksidansa. Primjena prirodnih antioksidanasa (ekstrakt zelenog čaja, ekstrakt ružmarina, eterično ulje primorskog vriska) bolje je zaštitila ulje koštice šljive od oksidacijskog kvarenja za razliku od sintetskog antioksidansa oktil galata. Eterično ulje

primorskog vriska pokazalo je najbolju zaštitu ulja od oksidacijskog kvarenja, a zatim ekstrakt ružmarina i ekstrakt zelenog čaja.

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THE PRODUCTION AND STABILIZATION OF COLD-PRESSED PLUM KERNEL OIL

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The cold pressing of the plum kernel (*Prunus domestica* L.) is used to obtain high-quality edible oil. The aim of this study was to investigate the influence of process parameters of pressing plum kernel on the efficiency of production of cold-pressed oil and its quality.
Following process parameters were investigated: electromotor frequency, temperature of head presses, and nozzle size. Pressing was carried out with a continuous screw press. Using standard methods, the basic quality parameters of produced cold pressed oil were determined. Oxidative stability of oil with added antioxidants was determined by the test of sustainability at 98°C. Natural antioxidants such as green tea extract, rosemary extract, winter savory essential oil and synthetic antioxidant octyl gallate were used. The results showed that the process parameters of cold pressing had a significant impact on the yield of plum kernel oil. Higher yield of crude oil and cold-pressed oil was obtained at a temperature of heating head presses 100 °C, electromotor frequency of 35 Hz and using nozzle size of 10 mm. The results showed that natural antioxidants have greater protection of oil in comparison to synthetic antioxidants. Winter savory essential oil had the best antioxidant effect.

Keywords: plum kernel, cold pressing, process parameters, oxidative stability, antioxidants

UTJECAJ HOMOGENIZACIJE I SASTOJAKA NA REOLOŠKA SVOJSTVA SALATNE MAJONEZE S DODATKOM PULPE MANGA

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SAŽETAK

Reološka svojstva majoneze su vrlo bitna kod izbora recepture, procesa kondicioniranja i kontrole kvalitete. U ovom radu ispitivan je utjecaj procesa homogenizacije i sastojaka na reološka svojstva salatne majoneze s dodatkom pulpe manga. Ispitivani sastojci su ugljikohidrati (glukoza, saharoza, maltodekstrin), žumanjak jajeta (svježi, pasterizirani, cijelo jaje u prahu) i mliječna komponenta (punomasno mlijeko u prahu, obrano mlijeko u prahu, sirutka u prahu). Proces homogenizacije majoneze proveden je sustavom rotor-stator Tip 1 i Tip 2 pri brzini rotora 10 000 °/min u vremenu od tri minute. Mjerenje reoloških svojstava svježe proizvedene majoneze provedeno je pri temperaturi od 25 °C na rotacijskom viskozimetru s koncentričnim cilindrima. Iz dobivenih eksperimentalnih podataka izračunati su reološki parametri: koeficijent konzistencije, prividna viskoznost i indeks tečenja. Rezultati istraživanja pokazuju da proces homogenizacije i sastav (mliječna komponenta, vrsta ugljikohidrata i žumanjka) utječu na reološka svojstva salatne majoneze s dodatkom pulpe manga. Veća prividna viskoznost i konzistencija majoneze postiže se dodatkom maltodekstrina, punomasnog mlijeka u prahu, cijelog jajeta u prahu i homogenizacijom kod brzine rotora 15 000 °/min tijekom 3 minute.

Ključne riječi: reološka svojstva, salatna majoneza, pulpa manga, sastojci, proces homogenizacije

UVOD

Majoneza kao emulzija ulje-voda predstavlja jedan od najčešće korištenih umaka u svijetu. Dobro je prihvaćena kod potrošača te je odličan dodatak kod prehrane djece i odraslih. U posljednje vrijeme preferira se razvoj majoneze u pravcu novih okusa koje pristaju zahtjevnim individualnim prehrambenim navikama pojedinih skupina potrošača. Majoneza predstavlja sustav emulzije ulje/voda s visokim udjelom jestivog biljnog ulja. To je proizvod ograničenog vremena trajanja, a njezini sastojci veoma brzo podliježu nepoželjnim promjenama, kao što su enzimski i mikrobiološki procesi te kemijske reakcije oksidacije koje mogu dovesti do kvarenja i nepoželjnih organoleptičkih promjena (Dimić i Turkulov, 2000.). Žumanjak jajeta je vrlo važan za

stabilnost ovog proizvoda, ali utječe i na boju majoneze (Hasenhuettl i Hartel, 2008). Salatna majoneza mora sadržavati minimalno 50 % jestivog biljnog ulja koji čini uljnu fazu (Narodne novine 39/99). Jestivo biljno ulje ima vrlo važnu funkciju u stvaranju emulzije ovih proizvoda, doprinosi okusu, izgledu, teksturi i oksidacijskoj stabilnosti na vrlo specifičan način (McClements i Demetriades, 1998). Reološka svojstva hrane važan su čimbenik kvalitete (Mezger, 2002), a naročito proizvoda koji predstavljaju emulziju tipa ulje/voda (majoneze, umaci i preljevi). Poznavanje reoloških svojstava značajno je kod kreiranja željene viskoznosti i konzistencije majoneze (Štern i sur., 2001), u kontroli kvalitete tijekom proizvodnje, skladištenja i transporta (Juszczak i sur., 2003; Munizaga i Barbosa, 2005). Reološka svojstva majoneze uglavnom su određena udjelom uljne faze, prisutnošću emulgatora, stabilizatora i zgušnjivača (Wendin i Hall, 2001; Mancini i sur., 2002). Kvaliteta i stabilnost ovih proizvoda tipa emulzije ulje/voda, kao i njihova viskoznost, ovisi o procesu homogenizacije (Wendin i sur.,1999), dispergiranosti kapljica biljnog ulja u kontinuiranoj vodenoj fazi majoneze, žumanjku jajeta (Guilmineau i Kulozik, 2007; Xiong i sur., 2000; Laca i sur., 2010), vrsti ugljikohidrata (Ruiling i sur., 2011) te vrsti i udjelu mliječne komponente (Dybowska, 2008). Kod ovih proizvoda sitne kapljice jestivog ulja su mehaničkim postupkom raspršene i dispergirane u kontinuiranoj vodenoj fazi octa te se djelovanjem prirodnog emulgatora iz žumanjka jajeta postiže veća stabilnost cijelog sustava (Kiosseoglou, 2003; Castellani i sur., 2006). Danas se reološko ponašanje majoneze kontinuirano proučava s obzirom da utječe na stav potrošača sastavom, konzistencijom, okusom, ali i primjenom na salate, pomfrit ili druga jela (Franco i sur., 1995; Akhtar i sur., 2005; Abu-Jdayil, 2003).
U ovom radu istraživan je utjecaj parametara procesa homogenizacije (brzina rotora, vrijeme trajanja, tip sustava rotor-stator) te sastojaka (mliječna komponenta, vrsta ugljikohidrata, žumanjka jajeta) na reološka svojstva salatne majoneze s dodatkom voćne pulpe manga pri temperaturi od 25 °C.

MATERIJALI I METODE

Materijali

Za ispitivanje utjecaja sastojaka i procesa homogenizacije na reološka svojstva salatne majoneze s dodatkom pulpe manga korišteni su sljedeći sastojci:

- Rafinirano suncokretovo ulje (linolni tip), Villa di Olio
- Ugljikohidrati (glukoza, saharoza, maltodekstrin), Claro-prom d.o.o., Zagreb
- Žumanjak kokošjeg jajeta (svježi, pasterizirani, cijelo jaje u prahu)
- Alkoholni ocat, Badel, Zagreb
- Morska sol
- Senf
- Destilirana voda
- Mliječna komponenta (punomasno mlijeko u prahu, obrano mlijeko u prahu, sirutka u prahu)
- Vinska kiselina, Alkaloid, Skoplje
- Voćna komponenta (pulpa manga), Prehrambeno-tehnološki fakultet Osijek.

Salatna majoneza proizvedena je sa 65 % uljnom fazom koju čini rafinirano suncokretovo ulje (linolni tip). Alkoholni ocat, morska sol i senf za proizvodnju salatne majoneze nabavljeni su u lokalnoj trgovini. Žumanjak jajeta nabavljen je od privatnog dobavljača te je priređen kao svježi i pasterizirani (68 °C, 3 minute), a cijelo jaje u prahu od proizvođača Elcon, Zagreb. Od cijelog jajeta u prahu dobivamo jedno tekuće jaje tako da u 12 g jaja u prahu dodamo 48 g tople vode i dobro promiješamo. Od mliječne komponente punomasno mlijeko u prahu (proteini 26,3 %, šećeri 39,8 %, masti 26 %) i obrano mlijeko u prahu (proteini 32,3 %, šećeri 51,3 %, masti 1,2 %) nabavljeno je iz firme Dukat d.d., a sirutka u prahu iz firme Zdenka.

Metode

Priprema salatne majoneze

Svi ispitivani uzorci salatne majoneze s dodatkom pulpe manga korišteni za ispitivanje reoloških svojstava pripremljeni su na tradicionalan način, bez upotrebe konzervansa, pri sobnoj temperaturi u količini 200 g za pojedini uzorak. Korištenjem voćne pulpe manga (5 %) kod izrade salatne majoneze željela se postići blaga voćna aroma, okus i boja proizvoda koji bi bio zanimljiv potrošačima. Kontrolni uzorak salatne majoneze pripremljen je sa 65 % uljnom fazom koju čini rafinirano suncokretovo ulje linolnog tipa. U suncokretovom ulju dominira esencijalna linolna masna kiselina (do 75 %) koja daje biološku aktivnost majonezi te alfa tokoferol kao prirodni antioksidans koji štiti ovo ulje od oksidacijskog kvarenja (Dimić, 2005.). U Tablici 1. Prikazana je osnovna receptura za pripremu salatne majoneze s dodatkom pulpe manga. Ostali uzorci majoneze rađeni su s različitim sastojcima čiji je utjecaj ispitivan na promjenu reoloških svojstava.
Za proizvodnju salatne majoneze korišten je laboratorijski homogenizator s rotor/stator sustavom, model D-500 (Wiggenhauser, Njemačka-Malezija) s područjem brzine rotacije rotora (10000 – 30000 °/min.). Kod izrade salatne majoneze primijenjen je sustav rotor/stator Tip 2, a čine ga rotor oznake ER30 i stator oznake S30F. Uzorci su pripremljeni na isti način tako da se u izvagane sastojke dodaje 1/2 suncokretovog ulja, zatim žumanjak jajeta, ocat, voda i ostali sastojci uz voćnu komponentu pulpe manga, uključi se homogenizator i polagano dodaju preostali dio suncokretovog ulja, a zatim homogenizira do 3 min kod brzine rotora 10000 °/min.
Uzorci su pripremljeni na isti način tako da se u izvagane sastojke dodaje 1/2 suncokretovog ulja, zatim žumanjak jajeta, ocat, voda i ostali sastojci uz voćnu komponentu pulpu manga, uključi se homogenizator i polagano dodaju preostali dio suncokretovog ulja, a zatim homogenizira do 3 min kod brzine rotora 10000 °/min. Priprema uzoraka salatne majoneze napravljena je pri sobnoj temperaturi svih sastojaka, a nakon izrade provedeno je mjerenje reoloških svojstava. Svi uzorci su pripremljeni na isti način, samo su se mijenjali pojedini sastojci ovisno o recepturi uzorka salatne majoneze te uvjetima procesa homogenizacije.

Tablica 1. Osnovna receptura za pripremu salatne majoneze (kontrolni uzorak)
Table 1. The basic recipe for the preparation of salad mayonnaise (control sample)

Sastojci	**Udio (%)**	**Masa (g)**
Rafinirano suncokretovo ulje (linolni tip)	65	130
Svježi žumanjak jajeta	6	12
Voćna pulpa manga	5	10
Maltodekstrin	4	8
Alkoholni ocat	4	8
Morska sol	1	2
Senf	1	2
Vinska kiselina	0,1	0,2
Sirutka u prahu	3	6
Destilirana voda	10,9	21,8
Ukupno:	100	200

Reološka svojstva

Mjerenje reoloških svojstava uzoraka salatne majoneze s dodatkom pulpe manga provedeno je rotacijskim viskozimetrom, model Rheomat 15T (Švicarska), primjenom koncentričnih cilindara. Ispitivanje reoloških svojstava svježe pripremljenih uzoraka provedeno je pri sobnoj temperaturi od 25 ^{o}C. Održavanje konstantne temperature uzorka majoneze tijekom mjerenja viskozimetrom postignuto je primjenom termostata modela TC-501P, firme Brookfield. Mjerenjem reoloških svojstava salatne majoneze praćena je ovisnost smičnog naprezanja (τ) o brzini smicanja (D) pri brzini smicanja od 2,18 s^{-1} do 137,1 s^{-1} kod uzlaznog mjerenja i od 137,1 s^{-1} do 2,18 s^{-1} kod povratnog mjerenja. Na osnovi ove ovisnosti određen je tip tekućine te je utvrđeno da su svi ispitivani uzorci majoneze imali nenewtonovska svojstva te pripadaju pseudoplastičnom tipu tekućina. Izračunate vrijednosti reoloških parametra koeficijenta konzistencije (k) i indeksa tečenja (n) dobivene su pomoću programa Microsoft Exel, uz primjenu metode linearne regresije.
Za izračun reoloških parametara koeficijenta konzistencije (k) i indeksa tečenja (n) primijenjen je Ostwald-Reinerov „stupnjeviti zakon“:

$$\tau = k \cdot D^{n} \qquad (1)$$

Gdje je:
τ - smično naprezanje (Pa)
D - brzina smicanja (s^{-1})
k - koeficijent konzistencije ($Pa \cdot s^{n}$)
n - indeks tečenja

Izračunavanje prividne viskoznosti (μ) uzoraka salatne majoneze s dodatkom pulpe manga provedeno je primjenom izraza:

$$\mu = k \cdot D^{n-1} \qquad (2)$$

REZULTATI I RASPRAVA

Reološko ponašanje majoneze u kombinaciji s voćnom pulpom danas je predmet istraživanja raznih autora. Tako Izidoro i sur. (2008) prikazuju utjecaj dodatka pulpe zelene banane na reološko ponašanje i kemijske karakteristike majoneze te utvrđuju da ona značajno utječe na porast prividne viskoznosti. Rezultati ispitivanja utjecaja sastojaka (ugljikohidrata, mliječna komponenta, žumanjak jajeta) na reološka svojstva salatne majoneze sa 65 %-tnom uljnom fazom s dodatkom pulpe manga prikazani su u Tablicama 2-4. U Tablici 2 vidljivi su rezultati ispitivanja utjecaja vrste ugljikohidrata (glukoza, saharoza, maltodekstrin) na promjenu reoloških parametara salatne majoneze s dodatkom pulpe manga. Pojedini istraživači (Mun, 2009; James, 1998) izvještavaju da ugljikohidrati kao što su modificirani škrob, inulin i dr. doprinose stabilizaciji emulzije ulje-voda te porastu prividne viskoznosti i konzistencije majoneze. Salatna majoneza proizvedena s glukozom (monosaharid) ima vrijednost prividne viskoznosti (μ) 1,0842 (Pas) kod brzine smicanja 137,1 (s^{-1}), konzistenciju prikazanu preko koeficijenta konzistencije (k) 36,21 (Pas^n) te indeks tečenja (n) 0,287. Primjenom saharoze (disaharid) kod izrade salatne majoneze proizvedena je emulzija s najmanjom prividnom viskoznošću (1,0381 Pas) i koeficijentom konzistencije (32,36 Pas^n) te indeksom tečenja (0,301). Korištenjem maltodekstrina napravljena je salatna majoneza s većom viskoznošću (1,2566 Pas) i konzistencijom (37,11 Pas^n) u odnosu na glukozu i saharozu.

Rezultati ispitivanja utjecaja mliječne komponente (sirutka u prahu, punomasno mlijeko u prahu, obrano mlijeko u prahu) na reološka svojstva izražena reološkim parametrima salatne majoneze s dodatkom pulpe manga vidljivi su u Tablici 3. Primjenom punomasnog mlijeka u prahu izrađena je salatna majoneza s većom prividnom viskoznošću (1,3791 Pas) i većim koeficijentom konzistencije (41,74 $Pa.s^n$), a najmanjim indeksom tečenja (0,307) u odnosu na primjenu sirutke u prahu i obranog mlijeka u prahu. Dybowska (2008) utvrđuje da proteini mlijeka stabiliziraju emulziju ulje-voda što se odražava i na stabilnost ovih proizvoda tipa emulzije ulje-voda.

Tablica 2. Utjecaj vrste ugljikohidrata na reološke parametre salatne majoneze s dodatkom pulpe manga (sustav homogenizacije: rotor ER 30, stator S 30F), mjerene pri 25 °C

Table 2. The influence of carbohydrate types on the rheological parameters of salad mayonnaise with addition of mango pulp (homogenization system: rotor ER 30, stator S 30F), measured at 25 °C

Uzorak	**τ pri 137,1 s^{-1} (Pas)***	**k (Pas^n)**	**n**	**R^2**
Glukoza	1,0842	36,21	0,287	0,99231
Saharoza	1,0381	32,36	0,301	0,97913
Maltodekstrin	1,2566	37,11	0,312	0,99659

τ - prividna viskoznost pri brzini smicanja 137,1 s^{-1} (Pas)
k – koeficijent konzistencije (Pas^n)
n – indeks tečenja
R^2 – koeficijent determinacije

Salatna majoneza izrađena sa sirutkom u prahu imala je najniže vrijednosti prividne viskoznosti (1,2566 Pas). Liu i sur. (2007) utvrđuju da proteini sirutke koji se mogu dodavati kao zamjena za jedan dio uljne faze emulzije utječu na reološka svojstva, teksturu i senzorska svojstva lagane majoneze. Primjenom obranog mlijeka u prahu dobivena je salatna majoneza s većom viskoznošću u odnosu na korištenje sirutke u prahu, a manju od punomasnog mlijeka u prahu.

Tablica 3. Utjecaj mliječne komponente na reološke parametre salatne majoneze s dodatkom pulpe manga (sustav homogenizacije: rotor ER 30, stator S 30F), mjerene pri 25 °C
Table 3. The influence of milk components on the rheological parameters of salad mayonnaise with addition of mango pulp (homogenization system: rotor ER 30, stator S 30F), measured at 25 °C

Uzorak	τ pri 137,1 s^{-1} (Pas)	k (Pas^n)	n	R^2
Sirutka u prahu	1,2566	37,11	0,312	0,99659
Punomasno mlijeko u prahu	1,3791	41,74	0,307	0,98602
Obrano mlijeko u prahu	1,3531	35,86	0,334	0,99300

U Tablici 4 vidljivi su rezultati ispitivanja utjecaja žumanjka kokošjeg jajeta (svježi, pasterizirani, cijelo jaje u prahu) na reološka svojstva salatne majoneze s dodatkom pulpe manga. U industriji kod proizvodnje salatne majoneze koristi se pasterizirani žumanjak kako bi se izbjegla moguća mikrobiološka kontaminacija. Ova salatna majoneza izrađena sa svježim žumanjkom jajeta imala je veću prividnu viskoznost (2,6462 Pa.s) i koeficijent konzistencije (38,48 $Pa.s^n$) te manji indeks tečenja (0,293) u odnosu na primjenu pasteriziranog žumanjka. Guilmineau i Kulozik (2007) ukazuju na pojavu da termičko tretiranje utječe na funkcionalna svojstva žumanjka kokošjeg jajeta kod izrade majoneze. Ibanoglu i Ercelebi (2007) te Kiosseoglou (2003) izvješćuju da termička denaturacija proteina žumanjka jajeta utječe na emulgirajuća svojstva kod izrade emulzija tipa ulje-voda. Proizvodnjom salatne majoneze s cijelim jajetom u prahu dobivena je znatno veća viskoznost i konzistencija emulzije što se objašnjava pojavom koaguliranja proteina iz žumanjka i bjelanjka tijekom dehidratacije jajeta.

Tablica 4. Utjecaj žumanjka jajeta na reološke parametre salatne majoneze s dodatkom pulpe manga (sustav homogenizacije: rotor ER 30, stator S 30F), mjerene pri 25 °C
Table 4. The influence of egg yolk on the rheological parameters of salad mayonnaise with addition of mango pulp (homogenization system: rotor ER 30, stator S 30F), measured at 25 °C

Uzorak	τ pri 44,1 s^{-1} (Pas)	k (Pas^n)	N	R^2
Svježi žumanjak jajeta	2,6462	38,48	0,293	0,99761
Pasterizirani žumanjak	1,5706	22,84	0,313	0,97888
Cijelo jaje u prahu	5,9121	121,34	0,202	0,98813

U Tablicama 5-7 prikazani su rezultati ispitivanja utjecaja procesnih parametara homogenizacije (brzina rotora, vrijeme trajanja homogenizacije) te vrsta sustava rotor/stator (Tip 1, Tip 2) na reološka svojstva salatne majoneze s dodatkom pulpe manga. U Tablici 5. prikazan je utjecaj brzine rotacije rotora tijekom 3 min homogenizacije (sustav rotor/stator Tip 2) na promjenu reoloških parametara salatne majoneze, mjereno pri sobnoj temperaturi 25 °C.

Tablica 5. Utjecaj brzine rotora tijekom 3 min homogenizacije na reološke parametre salatne majoneze s dodatkom pulpe manga (sustav rotor-stator: Tip 2), mjerene pri 25 °C
Table 5. The influence of rotor speed during 3 min homogenization on rheological parameters of salad mayonnaise with addition of mango pulp (rotor-stator system: Type 2), measured at 25 °C

Uzorak	τ pri 77,92 s^{-1} (Pa.s)	k (Pa.s^n)	n	R^2
10 000 °/min	2,6013	66,55	0,2557	0,99797
12 000 °/min	2,6752	76,78	0,2293	0,99537
15 000 °/min	3,4826	87,29	0,2604	0,98526

Dobiveni rezultati pokazuju da se izradom majoneze s dodatkom pulpe manga, kod brzine rotora homogenizatora 10 000 °/min, proizvela majoneza s prividnom viskoznošću (μ) 2,6013 (Pa.s) i konzistencija izražena koeficijentom konzistencije (k) 66,55 (Pa.s^n) te indeks tečenja (n) 0,2557. Primjenom veće brzine rotacije rotora 12 000 °/min proizvedena je majoneza s većom prividnom viskoznosti 2,6752 (Pa.s) i koeficijentom konzistencije 76,78 (Pa.sn) te manjim indeksom tečenja 0,2293. Daljnjim porastom brzine rotora homogenizatora na 15 000 °/min tijekom proizvodnje ove majoneze, došlo je do stvaranja takve emulzije ulje/voda koja ima još veću prividnu viskoznost (3,4826 Pa.s) i veći koeficijent konzistencije (87,29 Pa.s^n) u odnosu na primjenu 12 000 °/min. Ova pojava se događa jer je primjenom veće brzine rotora došlo do stvaranja većeg broja kapljica ulja manjeg promjera, a to rezultira porastom viskoznosti i konzistencije ove emulzije.
U Tablici 6 prikazan je utjecaj vremena trajanja homogenizacije (1, 2, 3 min), kod brzine rotora 10 000 °/min, primjenom sustava rotor/stator Tip 2, na reološka svojstva izražena reološkim parametrima salatne majoneze s dodatkom pulpe manga. Dobiveni rezultati pokazuju da se homogenizacijom tijekom 1 min dobiju parametri: prividna viskoznost (μ) 1,2756 (Pa.s) kod brzine smicanja 137,1 s^{-1}, koeficijent konzistencije (k) 51,64 (Pa.s^n) te indeks tečenja (n) 0,2479. Produženjem vremena trajanja homogenizacije tijekom izrade salatne majoneze s 1 min na 2 min, dobiva se emulzija veće prividne viskoznosti 1,6148 (Pa.s) i većeg koeficijenta konzistencije 59,39 (Pa.s^n). Daljnjim porastom vremena izrade majoneze na 3 min, došlo je do ponovnog porasta prividne viskoznosti 1,7082 (Pa.s) i koeficijenta konzistencije 66,55 (Pa.sn) te smanjenja indeksa tečenja 0,2557.
Rezultati ispitivanja primjene različitog sustava rotor/stator (Tip 1, Tip 2) na reološka svojstva salatne majoneze s dodatkom pulpe manga, kod brzine rotora 10 000 °/min i vremenu trajanja homogenizacije 3 min, prikazani su u Tablici 7. Dobiveni rezultati ukazuju na pojavu da se primjenom sustava rotor/stator Tip 2 dobije stabilnija emulzija ulje/voda s većom prividnom viskoznošću 1,7082 (Pa.s) i koeficijentom konzistencije

66,55 (Pa.s^n), a manjim indeksom tečenja 0,2557 u odnosu na primjenu sustava rotor/stator Tip 1 (rotor oznake SR20 i stator oznake S20C).

Tablica 6. Utjecaj vremena homogenizacije, kod brzine rotora 10 000 ^{o}/min, na reološke parametre salatne majoneze s dodatkom pulpe manga (sustav rotor-stator Tip 2), mjerene pri 25 °C
Table 6. The influence of homogenization time at rotor speed 10.000 rpm on rheological parameters of salad mayonnaise with addition of mango pulp (rotor-stator system: Type 2), measured at 25 °C

Uzorak	τ pri 137,1 s^{-1} (Pa.s)	k (Pas^n)	n	R^2
1 min	1,2756	51,64	0,2479	0,97541
2 min	1,6148	59,39	0,2674	0,99719
3 min	1,7082	66,55	0,2557	0,99797

Razlog tome je taj što je konstrukcijska izvedba sustava rotor/stator kod Tipa 2 takva da se pri toj brzini rotora postiže bolja disperzija kapljica ulja u vodenoj fazi pri čemu nastaje veći broj kapljica ulja manjeg promjera što rezultira porastom prividne viskoznosti i konzistencije salatne majoneze s dodatkom pulpe manga.

Tablica 7. Utjecaj tipa sustava homogenizacije rotor-stator, kod brzine rotora 10 000 ^{o}/min i vremena 3 min, na reološke parametre salatne majoneze s dodatkom pulpe manga, mjerene pri 25 °C
Table 7. The influence of rotor-stator homogenization system type at rotor speed 10.000 rpm and time 3 min, on rheological parameters of salad mayonnaise with addition of mango pulp, measured at 25 °C

Uzorak	τ pri 137,1 s^{-1} (Pas)	k (Pa.s^n)	n	R^2
Tip 1	1,0196	36,07	0,2753	0,98396
Tip 2	1,7082	66,55	0,2557	0,99797

Tip 1 (rotor SR 20, stator S 20C), Tip 2 (rotor ER 30, stator S 30F)

ZAKLJUČCI

Ispitivanjem utjecaja procesa homogenizacije i sastojaka na reološka svojstva salatne majoneze s dodatkom pulpe manga može se zaključiti da salatna majoneza pripada nenewtonskim tekućinama pseudoplastičnog tipa. Korištenjem voćne komponente pulpe manga postigao se blago voćni okus salatne majoneze. Rafinirano suncokretovo ulje (linolni tip) u uljnoj fazi osigurava oksidacijsku stabilnost i povećava biološku aktivnost salatne majoneze. Najprikladnija receptura majoneze u pogledu veće viskoznosti i konzistencije, a manjeg indeksa tečenja, ostvarena je primjenom

punomasnog mlijeka u prahu, maltodekstrina i cijelog jajeta u prahu. Također, veća viskoznost i konzistencija salatne majoneze ostvarena je izradom kod brzine rotora 15 000 o/min tijekom tri minute homogenizacije i primjenom sustava rotor-stator Tip 2.

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THE INFLUENCE OF HOMOGENISATION AND INGREDIENTS ON THE RHEOLOGICAL PROPERTIES OF SALAD MAYONNAISE WITH THE ADDITION OF MANGO PULP

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The rheological properties of mayonnaise are very important for the choice of formula, process conditions and quality control. In this paper, the influence of homogenization process and ingredients on the rheological properties of salad mayonnaise with the addition of mango pulp was examined. The tested ingredients were carbohydrate (glucose, sucrose, maltodextrin), egg yolk (fresh, pasteurized, whole egg powder) and milk component (whole milk powder, skimmed milk powder, whey powder). The homogenization process of mayonnaise was carried out using a rotor-stator system Type 1 and Type 2 at a rotor speed 10000 rpm for a period of three minutes. The rheological measurements were performed on a rotating viscometer with concentric cylinders at 25 °C, and the rheological parameters, apparent viscosity, consistency coefficient and flow behaviour index have been calculated. The results show that the homogenization process and ingredients (milk component, types of carbohydrates and egg yolk) influence the rheological properties of salad mayonnaise with the addition of mango pulp. The higher apparent viscosity and consistency of mayonnaise was achieved with the addition of maltodextrin, whole milk powder and whole egg powder and homogenization at a rotor speed of 15.000 rpm for 3 minutes.

Keywords: rheological properties, salad mayonnaise, mango pulp, ingredients, homogenization process

Topic: Production of safe food and food with added nutritional value/
Sekcija: Proizvodnja zdravstveno sigurne i nutritivno vrijedne hrane

THE INFLUENCE OF GUM ACACIA ON MILK FERMENTATION PROCESS AND CHARACTERISTICS OF FERMENTED MILKS DURING STORAGE

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ABSTRACT

Acacia gum was evaluated for its effect on the durability, sensory properties and health benefits of fermented milk with probiotics. For this purpose, viable cells and pH development were evaluated during fermentation and storage of three fermented milks enriched with 1.5 % fructooligosaccharides (FOS), 0.75% FOS + 0.75% acacia gum, and 1.5 % acacia gum. The carbohydrate profile, dietary fibre content, texture and sensory analyses of the final product were also examined at specific intervals during storage. The addition of acacia gum to fermented milk will enrich the product with prebiotic, which prolongsfermentation in the distal colon, without significantly modifying the production protocols. Except for the prebiotic effect, several other advantages of acacia gum were noticed: lower lactose levels and absence of fructose in the final product, which benefitsintolerant consumers, as well as lowers blood glucose levels, a potential benefit to diabetics. . Texture evaluation demonstrated small and non-significant differences in most attributes. The overall preference of the consumer panel was in favour of the FOS enriched fermented milk, which might be explained by the sweetening effect of FOS and consumers, who are accustomed to the existing sensory profile of the Ddary, i.e. fermented milk, on the market. These differences in taste could be overcome by flavouring and/or sweetening the fermented milk.

Keywords: fermented milk, prebiotics, fructooligosaccharides, gum acacia

INTRODUCTION

Probiotic foods and beverages are gaining popularity as more and more consumers become aware of their health benefits. Therefore, the development of such products is one of the research priorities for the food industry. However, the survival of the health-conferring microorganisms in these media remains a main challenge for the manufacturers. As reported, prebiotics can improve the probiotics' ability to survive. Furthermore, when probiotics and prebiotics werebeen used together, a biggeradvantage to the host was

noticed due to their synergistic action (de Vrese and Schrezenmeir, 2008; Vitali et al., 2010). Like probiotics, prebiotics are also involved in the treatment of irritable bowel syndrome and inflammatory bowel diseases (Balakrishnan and Floch, 2012; Ghouri et al., 2014).

Gum acacia, also known as gum arabic (E-Number 414), is an edible, dried, gummy exudate from the stems and branches of Acacia senegal and A. seyal that is rich in non-viscous soluble fibre (Ali et al., 2009). It has been studied for its health-conferring properties for a long time. Calame and Weseler (2008) found in their research on healthy human volunteers that gum acacia produced a greater increase in bifidobacteria and lactobacilli than an equal dose of inulin, and resulted in fewer gastrointestinal side-effects, such as gas and bloating. Gum acacia was also found to have proabsorptive properties during gastrointestinal malfunction (Codipilly et al., 2006), to lower blood lipids in humans (Jensen et al., 1993) and to ameliorate oxidative stress and inflammation during chronic renal failure in rats (Ali et al., 2013). Taking into account its high intestinal tolerance (Cherbut et al., 2003), its technological advantages like colour stability in beverages (Chung et al., 2016) and non-viscous consistency and its proven prebiotic role (Michel et al., 1998), it can be concluded that gum acacia is an excellent choice for “functionalization” of food products. Enrichment of fermented milk with gum acacia as a source of dietary fibre as well as a prebiotic conferring health benefits has already been done, and some procedures are even patented (Hoyda et al., 1990).

Acacia gum is an all-natural dietary fibre based on soluble dietary fibre content of over 90%, produced by the company Nexira, France. Recent in vitro studies found that acacia gum increases the transepithelial electrical resistance and modulates interleukin secretion, hence exerting potential positive effect on a gut barrier and inflammation (Daguet et al., 2016). In simulated gastrointestinal tract,Terpend and Possemiers (2013) have noticed several advantages of acacia gum in comparison to FOS. Unlikethe fast fermentation of FOS in the ascending colon, acacia gum undergoes prolonged and selective fermentation in the distal colon, thus providing higher digestive tolerance. This was also postulated by Goetze and Fruehauf (2008) in their clinical trials. Acacia gum has a specific bifidogenic effect, it stimulates butyrogenic bacteria like *Faecalibacterium prausnitzii,* and it is free of fructose, so, unlike FOS, it can be consumed by fructose-intolerant people. Namely, food intolerance is nowadays a huge and increasing problem for a growing number of consumers. There is a new dietary concern that is rising very quickly: the FODMAPs intolerance. FODMAPs (Fermentable, Oligo-, Di-, Mono-saccharides and Polyols) are poorly absorbed short-chain carbohydrates, including fructose (in excess of glucose), lactose, polyols, fructans, and galacto-oligosaccharides (Halmos et al., 2014). New nutritional drinks are emerging with the claim of low FODMAPs, declaring on the packaging that they contain “no inulin or fructooligosacharides” and highlighting the use of “low FODMAP fibre to support digestive health”, which includes acacia gum as soluble fibre. This new trend, together with gluten-free or lactose-free products, is targeting the increasing number of intolerant people to make their lives easier.

The research was done in cooperation with adairy company, interested in addingacacia gum to in their symbiotic fermented milk product.The acacia gum was evaluated for its effect on the durability, sensory properties and health benefits of the product. Two

concentrations of acacia gum were examined in the final product during this research, yielding three samples withtheir current standard as negative control:

- Sample 1 - Fermented milk product supplemented with 1.5% fructooligosacharides (FOS), their current standard (hereinafter referred toas FOS)
- Sample 2 - Fermented milk product supplemented with 0.75 % FOS + 0.75% - acacia gum (AG) (hereinafter referred to as FOS+ AG)
- Sample 3 - Fermented milk product supplemented with 1.5% - acacia gum (hereinafter referred to as AG)

MATERIALS AND METHODS

The production of fermented milk samples was done in a dairy company using regular production procedures. Namely, raw milk (20 tons), once pasteurized on reception, was homogenized and split into two containers. FOS was added (1.5%) to the first, and AG (1.5 %) was to the second container. The milk in both containers was homogenized and pasteurized again. Equal amounts of milk from both containers were aseptically transferred into a third container, thus the sample became FOS+AG. The inoculation of the milk (fat content: 3.2%, lactose content: 4.7%) was done by Chr. Hansen direct vat culture containing single strain culture blend of *Streptococcus thermophilus*, *Lactobacillus acidophilus* LA-5, and *Bifidobacterium lactis* BB-12. The temperature and the pH were monitored and the end of the fermentation was determined when the pH value reached 4.5. After that, the procedure of aseptic packaging and cooling of the fermented milk commenced. The next day, samples (1L commercial package, i.e. Tetra Pak carton) were ambiguously marked (only the researchers knew the samples' identity) and refrigerated at low temperature (4-7 ^{o}C) for further measurements at regular intervals of 5 days during 45 days.
The absence of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the inoculation set is the reason that the product is marketed as fermented milk rather than "yogurt", which under the Macedonian regulation (Sl. vesnik br. 96/2011) is defined as milk fermented by both *S. thermophilus* and *L. delbrueckii subsp. bulgaricus*. In this case, *S. thermophilus* is the main carrier of the milk fermentation.
Taking into account the purpose of the acacia gum addition, the following protocols were used to assess the influence of this component on the fermentation process and the finished product.

Counting of viable cells

The viable count of lactic acid bacteria in the samples was measured using the Milles and Misra surface viable count method (Miles et al., 1938; Hedges, 2002). After a series of appropriate dilutions, the samples were planted on MRS agar plates and incubated on 37 ^{o}C for 48 h in hermetically closed jar using anaerobic atmosphere generation bags (Merck, Darmstadt, Germany). The grown colonies were manually counted and this number was multiplied by the plate dilution resulting into bacterial count, colony forming units per gram of fermented milk (cfu/g). pH was measured during the fermentation as well as during storage usinga pH-meter (Sartorius PB-11, Göttingen, Germany).

Growth curves were generated using averaged data of triplicate measurement of the samples. The standard deviations are shown as error bars. For description of microbial kinetics, growth model was fitted to the colony count data.

Determination of carbohydrates

Preparation of carbohydrate extracts from fermented milk samples was done according to Richmondand and Harte (1987). Ten grams of each sample were accurately weighted into large conical centrifuge tubes and absolute ethanol was added to bring the final ethanol concentration to 80 % (vol/vol). Slurries were mixed and allowed to stand at room temperature to precipitate proteins. Ethanol (80% vol/vol) was then added to make a total volume of 50 ml. Samples were centrifuged at 5000 rpm for 5 min, the supernatant decanted, and the precipitate washed with ca. 25 ml additional 80% (vol/vol) ethanol. The combined extract and washings were then concentrated (absence of alcohol odor) using a rotary vacuum evaporator (25 to 27 °C). Sample extracts were made up to 25.0 ml with water and filtered through Whatman no. 42 paper. The samples were filtered through a 0.45 μm syringe membrane (Waters Corp., Milford, United States) placed in vials, sealed, and frozen (-10 °C) for subsequent HPLC analysis.
Agilent 1200 high performance liquid chromatography system (Agilent Technologies, Inc, Santa Clara, United States) was used for quantification of sugar concentration in the samples. Supelcosil LC-NH_2 column, 250×4.6 mm, 5μm particle size, (Supelco analytical, Sigma Aldrich Group, Taufkirchen, Germany) was used to separate the containing sugars in 15 min using isocratic mobile phase acetonitrile/water = 75/25 % (v/v) at 50 ^{o}C (Muntean and Muntean, 2010). Refractive index detector, also thermostated at 50 ^{o}C, was used for detection of the analytes and the data was processed by the Agilent ChemStation software. External standards were used to quantify fructose, glucose, galactose, sucrose, maltose and lactose in the samples while fructooligosaccharides present in the FOS and FOS+AG samples as well as eventual - acacia gum derivates in the AG sample, were quantified as lactose equivalents.
The data from the HPLC analysis are presented as average values of two independent analyses along with the standard deviation.

Dietary fibre analysis

High-molar-weight dietary fibre (DF), including water soluble fractions (gum acacia), was determined by AOAC 985.29 method. However, low-molar-weight DF including inulin (degree of polymerization (DP) from 10 to 60) and fructooligosaccharides (FOS, DP from 2 to 10) cannot be determined by this method. Hence, dietary fibre content was analyzed only in theFOS+ AG and AG samples.

Texture analysis

The texture of the samples was studied using a texture analyzer - XT2 (Stable Micro Systems, Godalming, England) and the method of back extrusion. Firmness, consistency, cohesiveness and index of viscosity were measured inall samples placed in a standardized container (75% full). The force during compression of the sample

was measured with 35 mm probe and extension bar using 5 kg load. Data were averaged from 5 replicates.
Fluidity, which is correlated with the viscosity of the fermented milks, was obtained with an in-house method using a burette. The time needed to pass 10 ml of fermented milk through the burette was measured. The reciprocal value of the time multiplied by 10 (ml), results in ml/min which is the volume of the sample flowing throw in 1 min (fluidity). The higher this value, the sample has higher fluidity and lower viscosity. Data were averaged from 3 replicates.

Sensory evaluation

The samples of fermented milk were evaluated for their sensory quality by two separate panels. An expert panel, which took place in the dairy company, was performed by their laboratory staff members that are usuallyresponsible for the sensory evaluation of all produce of the company. It consisted of 5 judges who were given a specific questionnaire for descriptive analysis of the samples and instructions on how to evaluate the intensities of the attributes. The other panel consisted of consumers with some knowledge of the technology of dairy production and took place at standardized test room (Faculty of Technology and Metallurgy). Each of the samples was served in a randomized order in a transparent plastic cup labeled with three digit random number. The panel consisted of at least 15 random panelists selected from a larger group of possible testers due to their declaration as frequent yogurt drinkers, non-smokers and persons sensitive to taste and smell. Before the tasting, the samples were introduced to them and they were briefed on the attributes that should be evaluated. The acceptance of color, smell, taste, and consistency, as well as overall acceptance was graded from 1 (not acceptable) to 5 (very good).

Statistical analysis

The experimental data were statistically analyzed using Anova with the Tukey post-hoc test (SPSS software, IBM Corporation, Armonk, New York, USA) for the determination of statistically significant difference between the two populations of values at 95% confidence interval. The equality of variances was checked by the Levine's test. Principal component analysis was used to study the correlation between different parameters.

Influence of fermented milk supplemented with acacia gum on glucose level in healthy individuals and people with diabetes

The influence of FOS+ AG and AG samples on blood glucose levels was examined on 9 people divided into three groups:

- Healthy people (3),
- People with type 1 diabetes (3)
- People with type 2 diabetes (3)

Volume of 200 ml fermented milk was consumed byeach of the test subjects and glucose levels in their blood were measured using rapid test (One Touch Select, LifeScan Inc.) in regular time intervals from 0 to 120 min. The test was conducted over three consecutive days, examining one sample each day. The FOS sample was used as negative control. The data were averaged for each subject group and presented.

RESULTS AND DISCUSSION

Milk fermentation

Cell concentration and pH

The viable count and pH development during the three fermentations (FOS, FOS+AG and AG) is presented in Figures 1 and 2. Linear growth can be noticed in all three fermentations starting from around 4.0 - 4.5 log cfu/g and reaching 6.5 - 7.0 log cfu/g after 4.5 hours. No lag phase was noticed at the beginning, which means that the culture was well adapted to the environment. The specific growth rate (μ) of the culture in the FOS sample was 0.51 h-1, while in the samples with acacia gum added (FOS+ AG and AG), it was slightly higher, 0.55 h-1. Minor change of the pH value, from 6.5 to 6.3, was noticed in the first hour of the fermentation after which, the decrease was most intensive, and in the third hour, the pH values fell below 5.0. It seems that the decrease of pH in this period is faster in the FOS sample than in the other two samples. However, all samples reached pH 4.5 at approximately the same time.
In this case, the culture *Streptococcus thermophilus* in this case was the main fermenting bacteria, since *Lactobacillus delbrueckii* subsp. *bulgaricus* was absent. *Lactobacillus acidophilus* LA-5, and *Bifidobacterium lactis* BB-12 are added as probiotic bacteria exerting health benefits to the consumer, and probably did not influence the fermentation (production of lactic acid) much. In contrast to *Lactobacillus*, *S. thermophilus* is also able to produce energy through aerobic respiration. Through fermentation, it converts lactose to lactic acid at the optimal pH of 4.6 (Radke-Mitchell and Sandine, 1986). In yogurt, *L. bulgaricus* provides *S. thermophilus* with formic acid, which provides better growth, while *S. thermophilus* releases amino acids, mainly valine, to accelerate *L. bulgaricus* growth. This protocoorperative association is capable of producing lactic acid at a greater rate. However, in this case, the selection of the cultures was done to lessen the production of lactic and acetic acid, hence prolonging the shelf life of the fermented milk. Namely, when pH value decreased below the optimal value of 4.6, *S. thermophilus* lowered its metabolism, so it can maintain the pH homeostasis, hence the conversion of lactose into lactic acid significantly slows down (Hutkins and Nannen, 1993). Also, *Bifidobacterium lactis* when co-cultivated with *S. thermophilus* produces less acetic acid due to the inhibition of its heterofermentative features (Rodrigues et al., 2011).

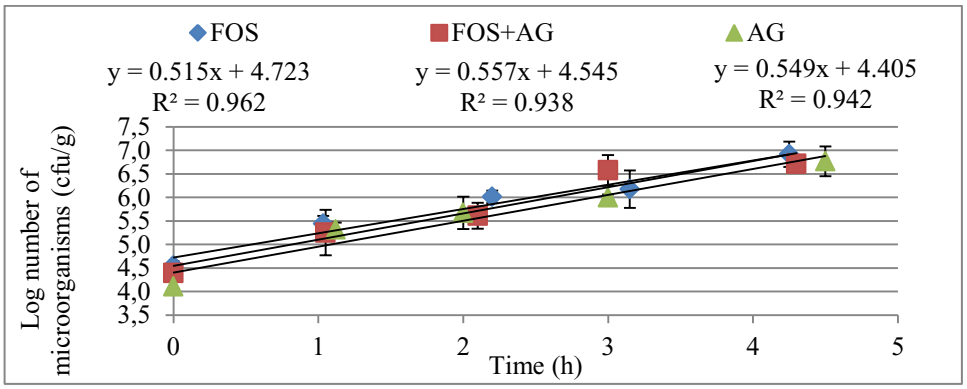


Fig. 1. Lactic acid bacteria viable count during fermentation of milk

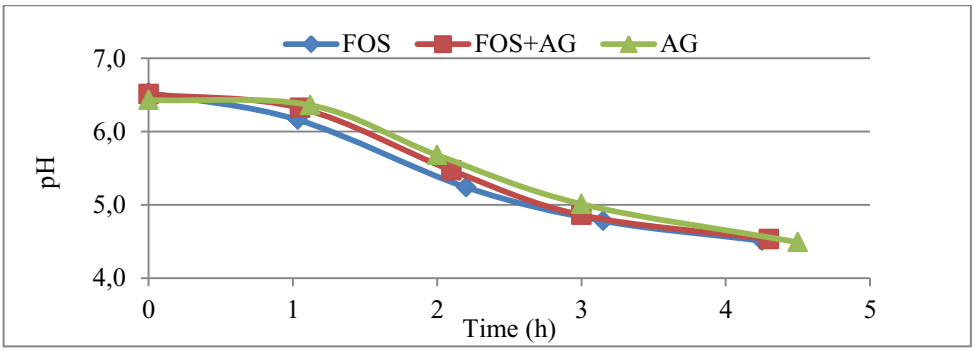


Fig. 2. pH during fermentation of milk

Carbohydrate profile of milk before and after fermentation

Carbohydrate analysis was done onmilk and onthe finished product. Table 1 presents the concentrations of the two main carbohydrate constituents in milk: galactose and lactose. As can be seen, the concentration of galactose in the milk was minor, whereas it was significantly increased during the fermentation. The cultures were utilizing the glucose as the main carbon source. Therefore, after hydrolyzing the lactose into glucose and galactose, the galactose was accumulating. The concentration of lactose decreased from the initial 4.73 % in milk to 4.01 %, 3.40 % and 3.77 % for the FOS, FOS+AG and AG samples, respectively. Accordingly, higher amounts of lactose were consumed by the starter cultures in theFOS+ AG and AG samples, in comparisonto theFOS sample, which explains the slightly higher specific growth rate in these samples. However, pH change was not affected, so most probably lactose was utilized through the pyruvate-formate lyase-mediated pathway with production of formic acid and ethanol to yield extra ATP, contrary to the conventional catabolic route leading only to acetic and lactic acids (Van der Meulen et al., 2006).
Figure 3. presents the carbohydrate profile (except galactose and lactose) of the milk with added FOS before and after fermentation. The identification of the components was not

possible due to a lack of external standards. However, it was tentatively concluded that the chromatogram peaks with different retention times (presented on x-axis) correspond to different fructooligosaccharide. As can be seen, some FOSs were diminishing during the fermentation and some were accumulating/appearing.

Table 1. Concentration of galactose and lactose before and after fermentation

Sample	Identification	Concentration (%)	
		Before fermentation	After fermentation
FOS	Galactose	0.04	0.69
	Lactose	4.72	4.01
FOS+ AG	Galactose	0.04	0.54
	Lactose	4.72	3.40
AG	Galactose	0.04	0.72
	Lactose	4.72	3.77

These results suggest that the starter cultures were able to hydrolyze the fructooligosaccharides. On the other hand, no fructose or glucose were qualified in the milk after fermentation, which indicates that the bacteria utilized these carbohydrates very fast. This finding is in agreement with the behaviour of other lactic acid bacteria which could ferment complex matrices without acidic or enzymatic hydrolysis prior to fermentation (Choi et al., 2012). It was found that oligosaccharides are hydrolyzed extracellularly with the cell wall associated enzyme β-fructosidase, induced by the presence of fructooligosaccharides, sucrose and fructose in the medium (Goh et al., 2007). Recently, several studies were published about different *Lactobacillus* species that utilize fructooligosaccharides as an energy source (Zubaidah, 2013; Endo et al., 2012). The preference fordifferent fructooligosaccharides such as 1-kestose (GF_2) and nystose (GF_3) was studied for different *Lactobacillus* species by Endoand Tamura (2015).
The metabolism of fructooligosaccharides as energy and carbon sources can be undertaken by two major pathways: glycolysis (Embden-Meyerhof pathway), which is mostly used by the homofermentative lactic acid bacteria, and 6-phosphogluconate / phosphoketolase pathway mostly used by the heterofermentative bacteria (Axelsson, 1998).

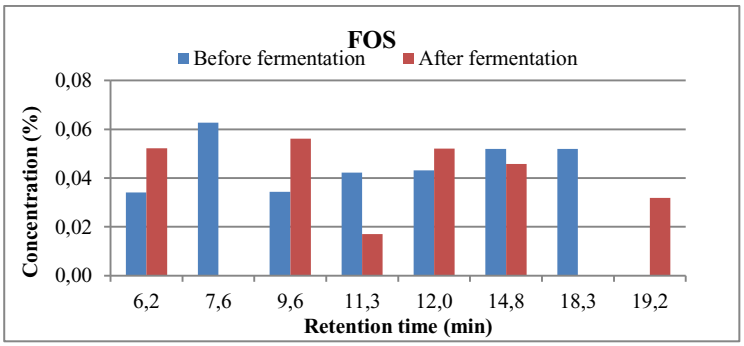


Fig. 3. Carbohydrate profile of the milk with added FOS before and after fermentation-different retention time presents various fructooligosaccharides

Fermented milk during storage

Cell concentration and pH

Packages containing 1 L of each sample of fermented milk were stored in a refrigerator at 4-7 °C and a new package was opened for each set of analysis during the 45 days. The viable count and pH changes during the storage are given in Figure 4 and 5. The viable count and pH data were fitted with fifth and third order polynomial equation (solid line), respectively. In the first 7 days of storage, lactic acid bacteria continued growing reaching log 10, log 9.7 and log 8.4 cfu/g in the samples FOS, FOS+AG and AG. By this time, the pH was below 4.5 in all three samples so the environment was unfavourable for such a high number of bacteria. Thus after this day, a decrease of viable count was noticed. The lowest count was measured on the 14th day (between log 6 and 7 cfu/g) after which the viable count started growing again. The reason for reactivation of the cells is explained by their adaptation to the stress that is sublethal (Jin et al., 2015). Low pH triggers different regulation of the genes in the cells so they can survive. However, after a while, the new generation of cells is already accustomed to the new environment and can reactivate their genes for cell division. Pan et al. (2009) have shown that the presence of oligosaccharides (fructooligosaccharides and xylooligosaccharides) can enhance the survival of probiotic cultures to simulated stress conditions (gastrointestinal juice, heat treatment and phenol solution). The presence of gum acacia was also shown to improve the survivability of spray-dried *Lactobacillus paracasei* strain to gastric juices (Desmond et al., 2002).
It is important to observe that, even when the viable count in the samples was the lowest, it was not below log 6 cfu/g, which is the limit for probiotic products. Namely, according to Codex Alimentarius (2003) and also some of the national regulations of EU Member States, to materialize most of the putative health benefits associated with probiotics, an adequate amount of viable cells must be delivered at the time of consumption. Therefore, the minimal number of probiotics in products islog 6 cfu/g. Although in the first days of storage the increase of colony count in FOS was the highest, during the period between 20 and 35 days of storage, AG had maintained higher viable cells compared to both other samples (log 9.5 cfu/g, Figure 4).

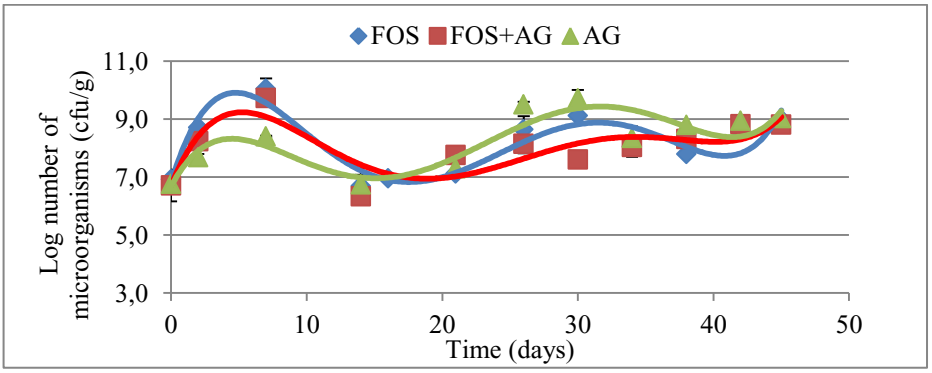


Fig. 4. Lactic acid bacteria viable count during cold storage of fermented milk

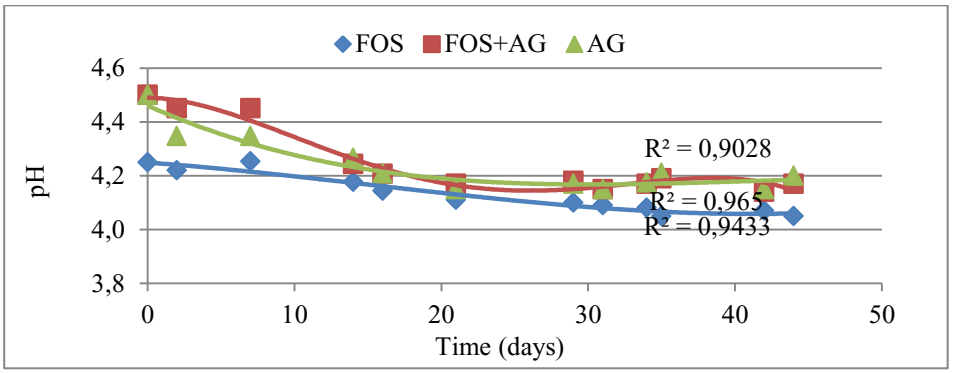


Fig. 5. pH during cold storage of fermented milk

It can be noticed that the pH was changing relatively slow in all samples. During the storage period of 45 days, the pH decreased by 0.2 units for the FOS sample and 0.3 units for the FOS+AG and AG samples. The initial pH for FOS was lower than in the other two samples (4.2), so the final pH (after 45 days) of this sample reached the lowest valueof 4.05. After day 21, the pH change was relatively stable in all three samples contributing to long shelf life of the samples.

Mono-, di- and oligo- saccharide profile of fermented milk during storage

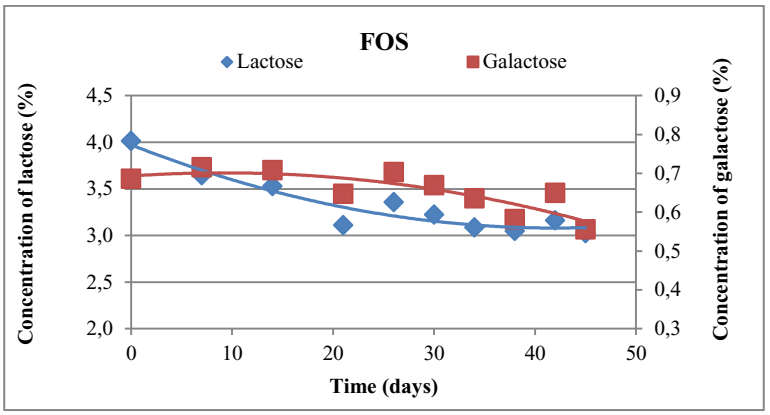
The concentrations of galactose and lactose in the samples FOS, FOS+ AG and AG samples during storage are presented in Figure 6 a-c. Usually, as stated before, the fermentation is mainly due to metabolization of the glucose moiety of lactose, while a relevant portion of the galactose is excreted in the medium. Since fructooligosaccharides are influencing the culture metabolisms, slightly different pattern of carbohydrate utilization was seen during storage of the three samples. As can be noticed in Figure 6a, the concentration of galactose in FOS was not changing significantly in the first 20 days, while in the other two samples a significant increase was observed. The higher concentration of galactose during storage of FOS+ AG and AG can be explained by partial fermentation of the acacia gum by the culture. Namely, galactose is one of the monomers in the molecule of gum acacia, hence its utilization by the culture strains can result in liberation of galactose. Not all lactic acid bacteria are able to ferment galactose, but S. thermophilus and L. acidophilus are species that are known to utilize it to some extent (Abrahamson, 2015). After day 20, decrease of galactose was observed in all three samples, which means that the cultures activated the galactose utilization genes. The final concentration of galactose was lower in FOS (0.56 %) than in FOS+AG (0.63 %) and AG (0.62 %).

Lactose was consumed in all three samples, reaching 3.0 % in FOS and FOS+ AG and 2.6 % in AG. These values are in agreement with the results of other authors determining the lactose levels in fermented milk (Alm, 1982; Sieber et al., 1997; Galvao et al., 1995). The same authors commented on the consumption of yogurt and

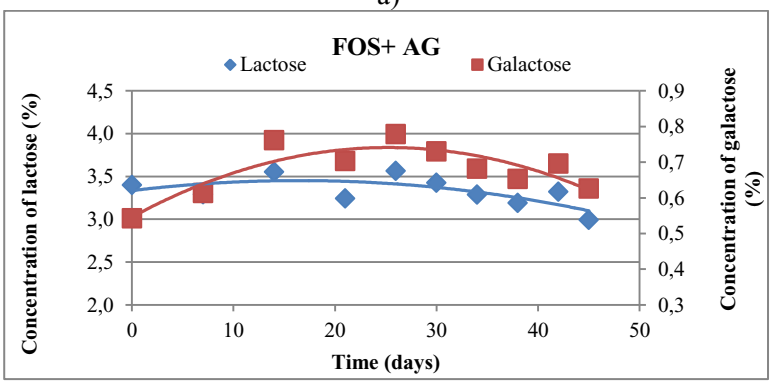
fermented milks by lactose intolerant people. With the exception of the Caucasian race, the lactase activity decreases in most people at the age of 4 to 6. Lactose intake can cause symptoms of bloating, flatulence, abdominal pain, and diarrhea due to the lactose reaching the large intestine. However, most lactose intolerant people are able to digest small amounts of milk or milk products, which are very important sources of calcium. The studies show that despite the presence of lactose in yogurts and fermented milks, they are very well tolerated by lactose intolerant people (Sieber eet al., 1997). Alm (1982) has tested fermented milk on eight individuals that showed symptoms of abdominal distress and diarrhea afterconsuming low fat milk, whereas ingestion of the same quantity of yogurt or acidophilus milk did not result in any symptoms. This advantage of fermented dairy products is ascribed to the presence of living lactic acid bacteria which survive passage through the stomach and also to the lactase present in these products.
Galvaoand Fernandes (1995) measured the beta-galactosidase (lactase) activity in different fermented milks and concluded that these products would probably be tolerated by most hipolactasic persons.
Figure 7 a-c presents the carbohydrate profile (except galactose and lactose) of the samples during storage. The HPLC column can separate and elute only low molecular compounds, so fructooligosaccharide with a higher degree of polymerization (DP) as well as the acacia gum molecules with high molecular weight were not qualified in the chromatogram. Representative chromatogram of a sample containing FOS is given in Appendix 1. Fructose and glucose, which are main monomers in FOS, were not detected, most probably because they were immediately consumed by the present bacteria. The FOS sample has shown to have the larger concentration of low molecular carbohydrates, which correlates with the addition of 1.5 % fructooligosaccharides in the milk (Figure 7a). As seen before, some compounds, like the one eluted on ret. time 9.6 min and 12.0 min are being consumed, while other compounds, like the one eluted on ret. time 11.3 min and 14.8 min are accumulated. The components that are accumulating are probably derivatives of fructooligosaccharides with larger DP. Except galactose and lactose, the AG sample contains only couple of low molecular components as shown in Figure 7c. The one with ret. time 6.1 min, found in each sample, is a constituent of the milk, which is probably lactulose. Lactulose is produced from lactose during the heat processing and the storage of dairy products. This sugar seems to have unique growth-promoting properties for certain desirable types of Lactobacilli in the intestinal tract of infants (Adachi and Patton, 1961). The other qualified carbohydrates are most likely derivatives from the added acacia gum, which was partially fermented by the culture. The AG + FOS samples show a carbohydrate profile in between the other two.

High-molar-weight dietary fibre in fermented milk during storage

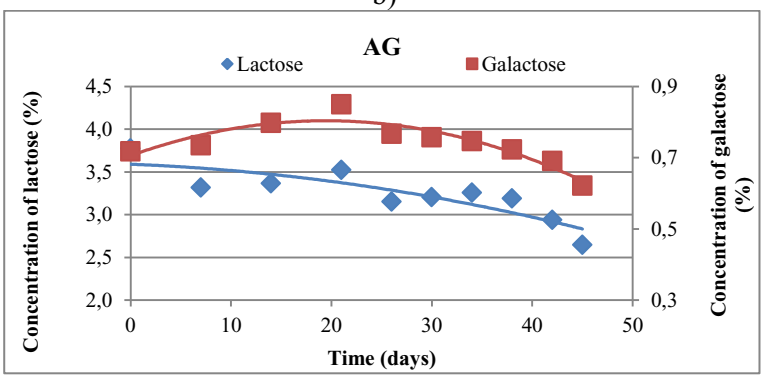
High-molar-weight DF (gum acacia included) of the FOS+FIB and FIB samples is presented in Figure 8. As one can conclude, the concentration of dietary fibre during storage decreased in both samples. In FOS+AG and AG the fibre reduction rate was 0.02 day-1 and 0.03 day-1 reaching 55 % and 56 % total decrease at the end of the storage (45 days), respectively.



a)



b)

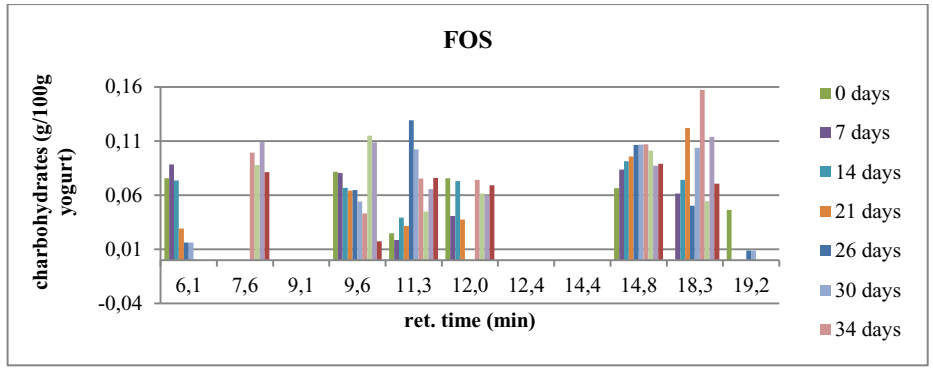


c)

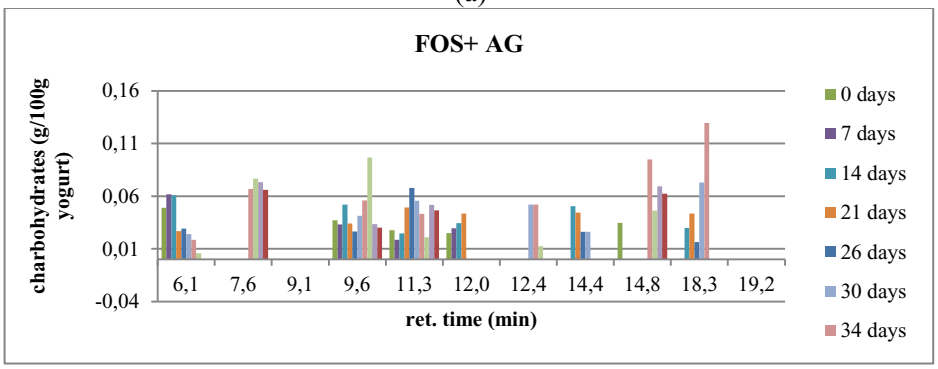
Fig. 6. Galactose and lactose concentrations in samples: FOS (a), FOS+AG (b) and AG (c) during storage

Gum acacia's stability in acidic environments has been mentioned in several articles (Paquin, 2009; Montenegro et al., 2012). Acacia gum was also successfully applied in orange juice (pH 3.2) without any degradation during 10 min at 100 °C. Most possible explanation of the DF decrease during storage is the utilization of the gum acacia by the culture. Taking into account its properties as prebiotic (stimulating the growth of

advantageous bacteria in the colon), fermentation of gum acacia by the live bacteria present in the fermented milk during storage is to be expected.



(a)



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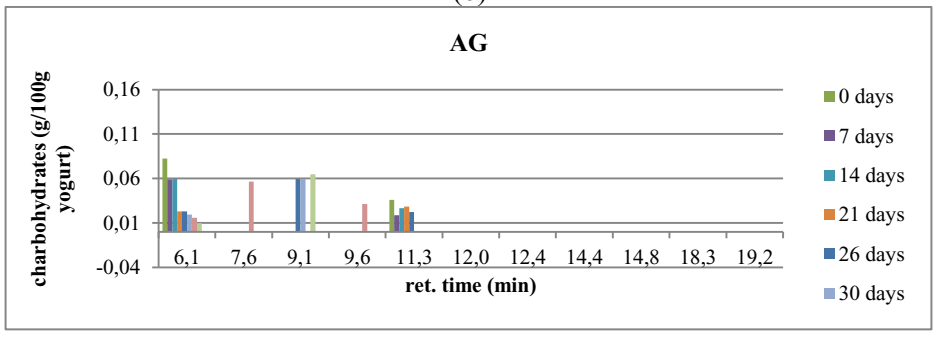

(c)

Fig. 7. Carbohydrate profile of samples: FOS (a), FOS+ AG (b) and AG (c) during storage

Texture of fermented milk during storage

Table 2 presents the data from the texture analysis of fermented milk during storage. Different letters in a row, under each textural parameter, designate significantly

different means by the Tukey test ($p<0.05$). The values that are statistically different between the three samples (FOS, FOS+AG and AG) are highlighted in grey. As perceived, the parameters' firmness and consistency are not significantly different between the samples, except at day 21 of storage, when FOS had the highest value ($p<0.05$). FOS has also significantly higher index of viscosity at day 21 and 42, while for cohesiveness, the same sample had higher values in days 21, 30, 38 and 42. In the literature, the construct of cohesiveness is defined as a mechanical textural attribute relating to degree to which a substance can be deformed before it breaks, or how well the product withstands a second deformation relative to its resistance under the first deformation. Adjectives that are listed as descriptors of cohesiveness include: fracturable, crumbly, crunchy, brittle, crispy, crusty, chewy, tender, tough, short, mealy, pasty, and gummy (Steele et al., 2015).
The change during time of all textural parameters was very similar, hence only the cohesiveness will be discussed. As seen, in the first 14 days, the texture has not changed much for any of the samples. The highly branched structure of the gum acacia molecules leads to compact, relatively small hydrodynamic volume. Thus, solutions containing less than 10% of gum acacia have a low viscosity and respond to Newtonian behavior (Williams et al., 1990). However, starting from day 21, a variation of the texture parameters during time was noticed. The texture of gum acacia solutions can be modified by the presence of acids or bases, as these change the electrostatic charge on the macromolecule. In very acidic solutions, acid groups are neutralized, thus inducing a more compact conformation of the polymer, which leads to a decreased viscosity (Montenegro et al., 2012).

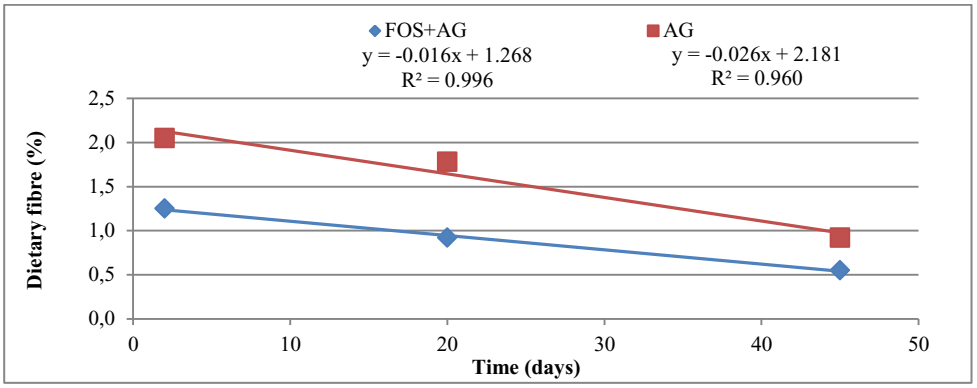

Fig. 8. Concentration of dietary fibre in FOS+AG and AG samples during storage

During this experiment, we observed a sinusoidal change of the textural parameters, so it can be concluded that other factors influence the texture of the samples as well. Production of exopolisacharides by the present cultures can be one of the reasons. Namely, several LABs, such as *Streptococcus thermophilus*, lactobacilli and bifidobacteria, are able to direct some part of the sugar pool towards biosynthesis of

exopolysaccharides (EPSs), which can improve technological characteristics like stability and texture, of fermented dairy products (Baltasar et al., 2010).

Fluidity of fermented milk during storage

Fluidity of the three samples during storage is presented in Figure 9. It was also noticed that the fluidity, which is correlated (negatively) with the viscosity of the samples, was changing during time. Whereas the fluidity of FOS was continuously decreasing during 34 days (down by 13 %), the FOS+AG and AG samples showed a significant increase of the fluidity on day 22 and again a decrease on day 34. On the second day of storage, FOS had statistically higher fluidity from the other two samples ($p<0.05$). On the day 22 it was the other way around; AG had the highest fluidity, while on day 34, all samples had similar fluidity.
The decrease of the fluidity in FOS can be explained with EPS's production mentioned before. The significant fluidity increase of the FOS+AG and AG samples, on the other hand, could be explained by the different conformation of the gum acacia molecule in acidic environment and its fermentation.

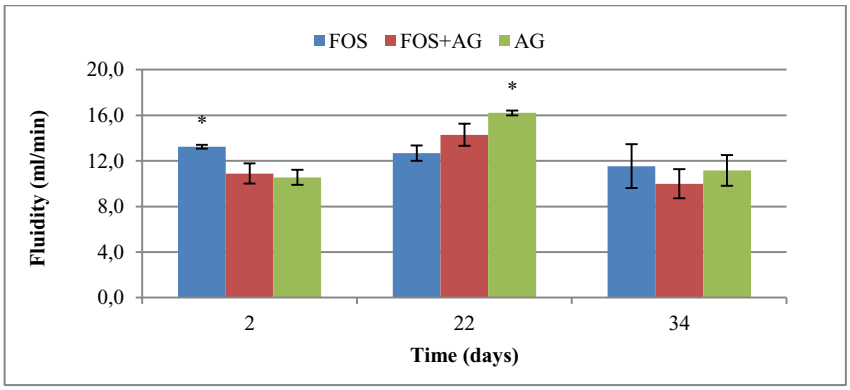

Fig. 9. Fluidity of the FOS, FOS+AG and AG samples during storage. The bars marked with an asterisk are statistically different from the other bars ($p<0.05$).

Sensory evaluation of fermented milk during storage

Consumer panel

The data of the consumers' sensory evaluation of the three samples during storage are presented in Figure 10. Grades that are statistically different between the samples are marked with an asterisk. As observed, due to high variation, most of the data are with no statistical significance. In the case of consistency, no difference between the samples was significant at any time during storage. However, the taste, the sourness and the overall acceptance of FOS received statistically higher grades than the other two samples at some points during storage.

Table 2. Textural parameters of the FOS, FOS+AG and AG samples during storage.

Texture parameter	Firmness (g)			Consistency (g sec)			Cohesiveness (g)			Index of viscosity (g sec)		
Time (days)	FOS	FOS+AG	AG	FOS	FOS+AG	AG	FOS	FOS+ AG	AG	FOS	FOS+AG	AG
0	34.7±0.5^{a}	34.7±0.51^{a}	34.9±0.54^{a}	295.0±3.77^{a}	295±3.47^{a}	296.2±4.12^{a}	6.2±0.14^{a}	6.3±0.08^{a}	6.4±0.08^{a}	1.4±0.16^{a}	1.5±0.07^{a}	1.5±0.17^{a}
7	34.3±0.37^{a}	34.2±0.26^{a}	34.1±0.56^{a}	292.3±4.13^{a}	291.7±2.62^{a}	290.6±5.47^{a}	6.3±0.04^{a}	6.2±0.19^{a}	6.1±0.30^{a}	1.5±0.1^{a}	1.4±0.11^{a}	1.4±0.15^{a}
14	34.3±0.45^{a}	33.7±0.68^{a}	34.2±0.37^{a}	292.4±4.23^{a}	285.4±5.71^{a}	290.9±2.49^{a}	6.3±0.24^{a}	6±0.21^{a}	6.0±0.08^{a}	1.4±0.14^{a}	1.5±0.17^{a}	1.4±0.09^{a}
21	35.6±0.42^{a}	34.6±0.39^{b}	33.6±0.42^{c}	302.9±3.99^{a}	293.1±3.52^{b}	285.2±3.2^{c}	6.8±0.14^{a}	6.2±0.29^{b}	5.7±0.12^{c}	1.7±0.14^{a}	1.5±0.16^{b}	1.2±0.1^{b}
26	34.9±0.82^{a}	34.8±0.69^{a}	35.0±1.5^{a}	295.9±7.05^{a}	294.5±5.65^{a}	296.3±13.2^{a}	6.5±0.3^{a}	6.4±0.57^{a}	6.5±0.58^{a}	1.6±0.24^{a}	1.6±0.32^{a}	1.6±0.32^{a}
30	35.3±0.44^{a}	35.6±0.44^{a}	35.2±0.48^{a}	298.2±4.14^{a}	301.2±3.7^{a}	296.8±4.03^{a}	6.7±0.05^{a}	6.6±0.09^{a}	6.5±0.08^{b}	1.7±0.13^{a}	1.7±0.13^{a}	1.6±0.17^{a}
34	33.7±0.78^{a}	33.8±2.92^{a}	34.2±0.88^{a}	286.4±5.27^{a}	286.7±21.6^{a}	289.9±8.31^{a}	5.7±0.38^{a}	5.8±1.12^{a}	6.0±0.40^{a}	1.2±0.11^{a}	1.2±0.56^{a}	1.1±0.61^{a}
38	35.1±0.41^{a}	35.1±0.37^{a}	34.7±0.41^{a}	295.7±3.94^{a}	296.5±2.36^{a}	293.6±4.28^{a}	6.8±0.09^{a}	6.7±0.1^{a}	6.4±0.19^{b}	1.8±0.12^{a}	1.7±0.18^{a}	1.6±0.16^{a}
42	34.7±0.37^{a}	34.5±0.44^{a}	33.8±0.85^{a}	293.0±3.53^{a}	290.9±3.16^{a}	286.5±7.39^{a}	6.4±0.1^{a}	6.1±0.17^{a,b}	6.0±0.27^{b}	1.5±0.04^{a}	1.3±0.11^{b}	1.3±0.16^{b}

* Different letters in a row, under each textural parameter, designate significantly different means by the Tukey test ($p<0.05$).

Table 3. Intensity grades of sensory attributes from the expert panel

Time (day)		14			19			26			33			43		
Sensory attributes		FOS	FOS+AG	AG	FOS	FOS+ AG	AG	FOS	FOS+ AG	AG	FOS	FOS+AG	AG	FOS	FOS+AG	AG
Appearance	Syneresis	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	3.2±0.45^{a}	2.0±0.71^{b}	3.8±0.84a	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}	2.0±0.00^{c}	4.3±0.50^{b}	5.0±0.00^{a}
	Homogeneity of surface	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}	2.0±0.00^{a}	1.5±0.58^{a}	2.8±0.45^{a}	2.6±0.55^{a}	2.8±0.45^{a}	3.0±0.00^{a}	2.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}
	Firmness	3.0±0.00^{a}	3.0±0.00^{a}	3.5±1.00^{a}	3.0±0.00^{a}	2.5±0.58^{b}	2.8±0.50^{b}	3.0±0.00^{a}	2.2±0.45^{b}	3.0±0.00^{a}	3.0±0.00^{a}	1.8±0.50^{b}	2.3±0.50^{a,b}	3.0±0.00^{a}	2.8±0.50^{a}	3.0±0.00^{a}
Taste	Taste-overall intensity	3,0±0,00^{a}	3.2±1.26^{a}	4.5±1.00^{a}	3.0±0.00^{a}	4.0±0.82^{b}	5.0±0.00^{c}	3.0±0.00^{b}	4.2±0.45^{a}	4.8±0.45^{a}	3.0±0.00^{a}	5.0±0.00^{a}	5.0±0.00^{a}	3.0±0.00^{c}	4.3±0.50^{b}	5.0±0.00^{a}
	Sour	2.5±1.00^{a}	3.7±1.89^{a}	3.5±1.73^{a}	3.0±0.00^{b}	3.8±0.50^{a,b}	4.5±0.58^{a}	3.0±0.00^{b}	3.6±0.55^{b}	4.4±0.55^{a}	3.0±0.00^{b}	4.0±0.00^{a}	4.5±0.58^{a}	3.8±0.50^{b}	5.0±0.00^{a}	4.5±0.58^{a,b}
	Biter	1.7±0.96^{a}	1.7±0.96^{a}	1.0±0.00^{a}	1.5±0.58^{a}	1.8±0.50^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	2.0±1.15^{a}	1.8±0.50^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}
	Creamy	5.0±0.00^{a}	2.0±0.82^{b}	2.7±1.26^{b}	3.0±0.00^{a}	2.0±1.41^{b}	2.3±1.26^{b}	2.6±0.89^{a}	2.6±0.89^{a}	2.0±1.00^{a}	3.0±0.00^{a}	2.8±0.50^{a}	2.0±0.00^{b}	2.8±0.50^{a}	1.8±0.50^{b}	1.8±0.50^{b}
	Cardboard	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.4±0.55^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.3±0.50^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}
	Oxidized	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.6±0.55^{a}	2.2±1.30^{a}	1.0±0.00^{a}	1.8±0.50^{a}	1.8±0.50^{a}	1.8±0.50^{a}	1.0±0.00^{b}	4.3±0.96^{a}	4.8±0.50^{a}
	Aftertaste	2.5±1.00^{a}	2.5±1.29^{a}	4.0±1.41^{a}	1.0±0.00^{b}	4.3±0.96^{a}	5.0±0.00^{a}	1.6±1.34^{b}	4.2±0.45^{a}	3.8±1.64^{a}	1.0±0.00^{c}	4.3±0.50^{b}	5.0±0.00^{a}	1.0±0.00^{b}	4.8±0.50^{a}	4.8±0.50^{a}
Texture	Mouthfeel	3,5±1,00^{a}	2.7±1.71^{a}	3.5±1.91^{a}	3.0±0.00^{a}	3.3±1.71^{a}	3.5±1.91^{a}	3.0±0.00^{a}	2.8±0.45^{a}	2.4±0.55^{a}	3.0±0.00^{a}	3.0±0.00^{a}	2.0±0.00^{a}	3.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}
	Homogeneity	3.5±1.00^{a}	2.5±1.00^{a}	2.5±1.00^{a}	3.0±0.00^{a}	2.5±1.29^{a}	2.8±0.96^{a}	3.0±0.00^{a}	2.6±0.55^{a}	2.4±0.89^{a}	3.0±0.00^{a}	2.0±0.00^{b}	1.8±0.50^{b}	3.0±0.00^{a}	3.0±0.00^{a}	3.0±0.00^{a}
	Smoothness	4.0±1.15^{a}	2.5±1.29^{a}	3.2±1.50^{a}	3.0±0.00^{a}	2.3±0.96^{a}	2.0±1.15^{a}	3.0±0.00^{a}	2.6±0.55^{a,b}	2.2±0.45^{b}	3.0±0.00^{a}	2.0±0.00^{b}	1.5±0.58^{b}	3.0±0.00^{a}	2.0±0.00^{a}	2.0±0.00^{a}
	Mouthcoating	3.5±1.00^{a}	2.2±1.50^{a}	3.2±1.71^{a}	3.0±0.00^{a}	3.3±0.50^{a}	3.3±0.50^{a}	3.0±0.00^{a}	1.8±0.84^{b}	2.2±0.45^{a,b}	3.0±0.00^{a}	1.8±0.50^{b}	1.8±0.50^{b}	3.0±0.00^{a}	2.0±0.00^{a}	2.0±0.00^{a}
	Slimy	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.0±0.00^{a}	1.5±0.58^{a}	1.0±0.00^{a}
	Astringent	2.0±1.15^{a}	2.0±2.00^{a}	2.3±1.53^{a}	1.0±0.00^{a}	1.3±0.50^{a}	2.5±1.73^{a}	1.2±0.45^{b}	2.4±0.55^{a}	2.6±0.55^{a}	1.0±0.00^{a}	2.0±0.00^{a}	2.0±0.00^{a}	1.0±0.00^{b}	4.3±0.50^{a}	4.3±0.96^{a}

* Different letters in a row, under each day, designate significantly different means by the Tukey test ($p<0.05$).

The data were averaged at different pointsof time yielding one mean value for each sensory parameter and are presented in Figure 11. It can also be seen that consistency was not something that the consumers found different between the samples. Although the mean value is higher for FOS, large variation of the data resulted in no statistical difference. Nevertheless, the FOS grades for the taste, sourness and the overall acceptance were highest ($p<0.05$).

From the Principal Component Analysis (Figure 12), it is evident that the gender of the panelists and their frequency of the consumption of fermented milks had no influence on the sensory parameters. The taste and the sourness are closely correlated, which is consistent with the fact that the most intensive taste sensation in this case is sourness. Overall acceptance is not so far away from both, so it is most likely that this parameter was influenced bythe sourness and the taste of the samples.

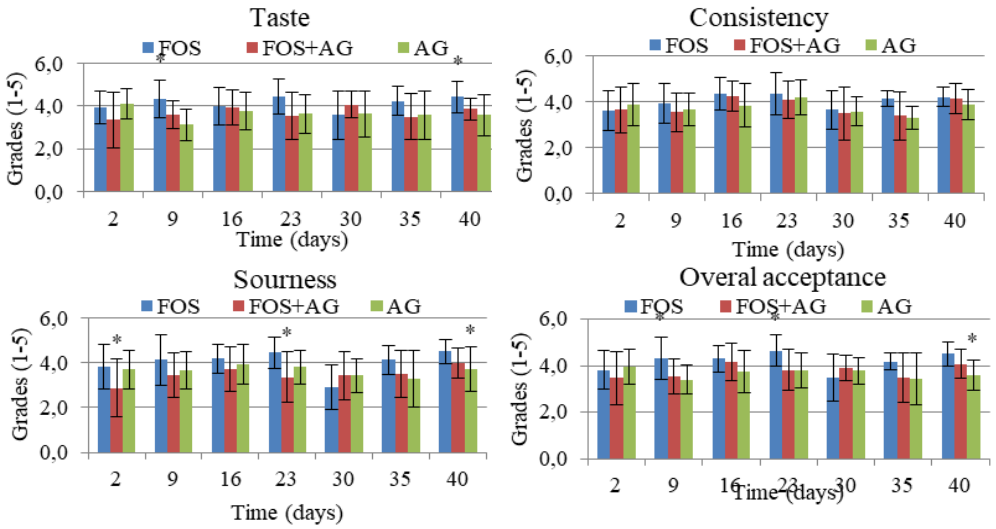

Fig. 10. Consumers' grades of sensory attributes of the three samples: FOS, FOS+ AG and AG during storage. The bars marked with an asterisk are statistically different from the other bars ($p<0.05$)

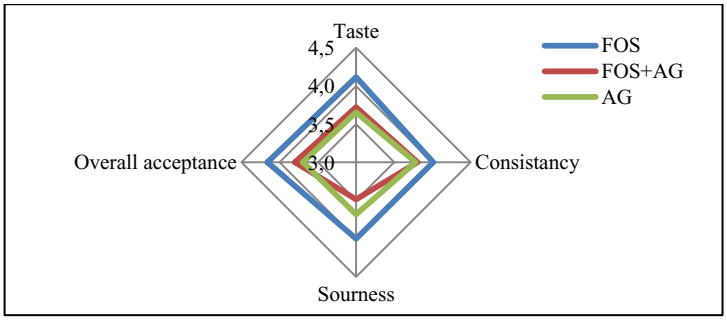

Fig. 11. Mean (for the whole time interval) consumer grades of sensory attributes for the three samples: FOS, FOS+AG and AG

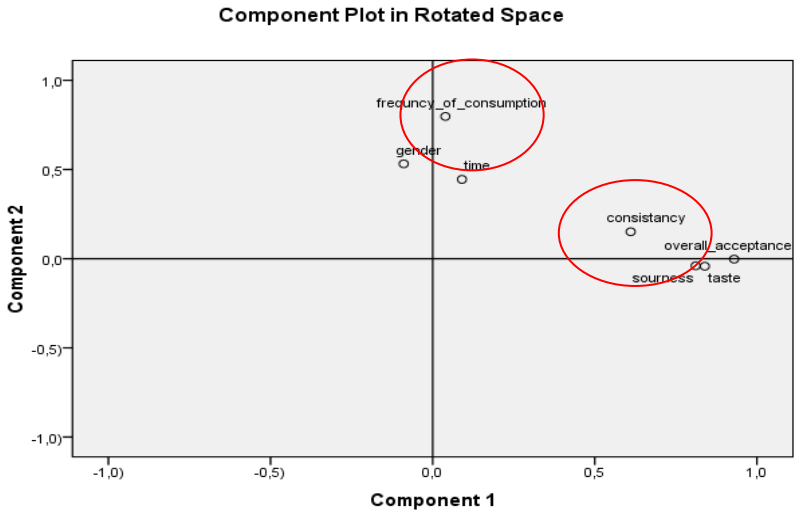

Fig. 12. Principal component analysis of the consumers' panel data

Expert panel

The expert panel was evaluating the intensities of the separate sensory sensations with grades from 1 (non existing sensation) to 5 (very intensive sensation). The obtained data are presented in Table 3. As can be concluded from these data, some sensory parameters did not change over time, like homogeneity of surface and homogeneity where all samples in most panels obtained intensity grade around 3. Bitter and cardboard tastes and slimy consistency were not sensed at all in the samples (intensity grade 1). The intensity of some sensory parameters increased during time, like syneresis, which after 19 days of storage started to increase in all samples, especially in FIB, which was graded with maximal intensity (5) at day 43 ($p<0.05$). Also the oxidized (fat oxidation) and the astringent taste were found to develop only in sample FOS+AG and AG during storage, graded with max intensity at day 43 ($p<0.05$). In some cases, the sensation of the parameter decreased during the storage of the samples like in the case of creamy taste, where, although FOS had max. intensity at day 14 it was graded in the following panels with 3. The samples FOS+AG and AG had lower intensity of creamy taste than FOS ($p<0.05$). The sample FOS also received higher intensity grades for the mouthfeel, smoothness and mouthcoating ($p<0.05$). On the other hand, FOS+AG and AG had higher intensity grades for aftertaste, sour and the overall intensity of taste ($p<0.05$). Concerning the firmness, FOS+AG had lower intensity grades than the other two samples in most of the panels (days 19, 26 and 33).

In conclusion, expert panelists had found that FOS have more pronounced texture characteristics than the samples with added acacia gum, giving higher intensity grades for the mouthfeel, smoothness and mouthcoating and lower for the astringent sensation. Conversely, the taste sensations were found to be more intensive in FOS+AG and AG although development of oxidative taste is a negative sensation and should be avoided in the finished product. Concerning the appearance, it seems as no significant difference between the samples

can be stated. The significant increase of the syneresis in FOS+AG and AG was only noticed at day 43; there was no change in the homogeneity of surface and the intensity of firmness in FOS+AG was lower compared to the other two only by approximately 1 grade.
Similar results are obtained when the data from all expert panels were averaged for each sensory parameter. The means are presented in Figure 13. The sample FOS obviously had higher intensity grades for creamy taste, mouthfeel, homogeneity, smoothness and mouthcoating, whereas AG has more intensive overall taste, sourness, aftertaste as well as astringent sensation and syneresis. Concerning FOS+ AG, it can be concluded that it has intensities in-between the other two samples or not different from AG.

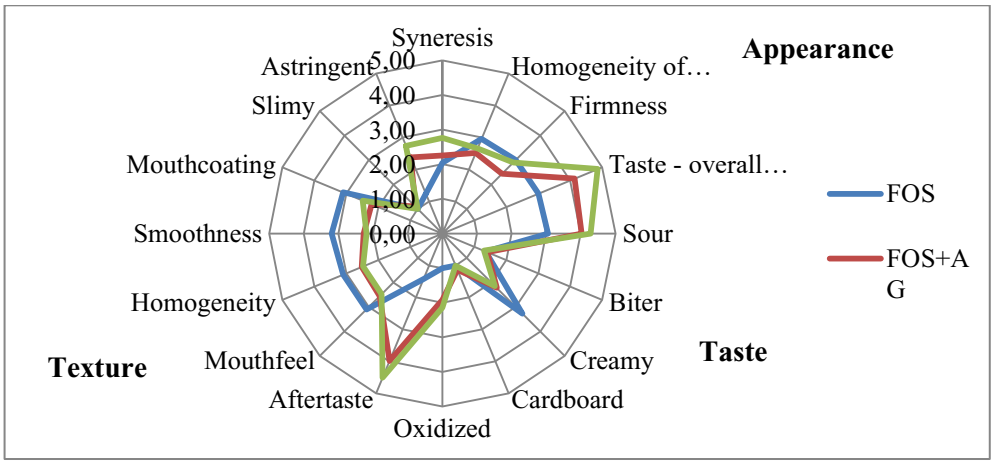

Fig. 13. Mean intensities of sensory attributes during 45 days

The relation between the sensory parameters (consumer and expert panel), the concentration of lactose, textural parameters, viable count (Log) and pH during storage were studied using Principal Component Analysis and the results are given in Figure 14. Four groups of the related parameters are distinct. It is interesting to notice that the grades obtained from the consumer panel (taste, sourness, consistency and overall acceptance) are in a separate group, i.e. not correlating with any of the intensity grades from the expert panel. The taste sensations: aftertaste, overall intensity, sour, astringent and oxidized taste, are correlated. Also, texture sensations mouthcoating, mouthfeel and smoothness are correlated with bitter and creamy taste as well as with the concentration of lactose. It can be tentatively assumed that the concentration of lactose could have some influence on these sensations. The forth group unites only Log, syneresis, slimy and time for which, except the time dependence of the viable count (Log) and syneresis, no other relation can be conceived.

The influence of fermented milk supplemented with acacia gum on glucose level in healthy individuals and people with diabetes

Glucose concentration in blood was measured in subjects consuming fermented milk and the results are presented in Table 4, and their visual presentation in Figures 15 and 16. It can be

concluded that in all three groups, the glucose level increases 30 min after consuming all samples (FOS, FOS+ AG and AG).

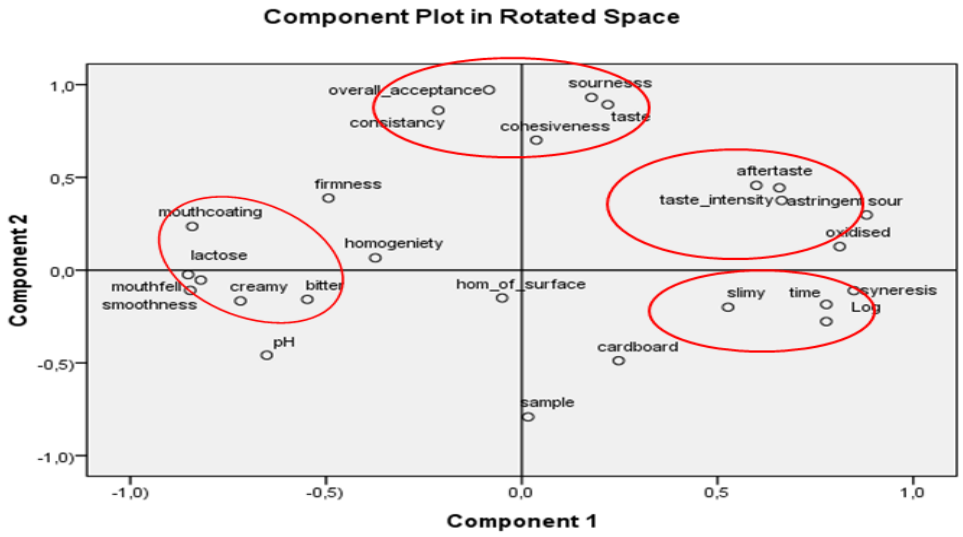

Figure 14. Principal Component Analysis of data for sensory parameters (consumer and expert panel), the concentration of lactose, textural parameters, viable count (Log) and pH

However, it can be clearly observed that the peak of blood glucose is much lower when the AG sample was consumed than the samples containing FOS, especially for the type II diabetics. Also, after 120 min, the glucose levels in subjects with diabetes (type I and type II) consuming sample AG, was significantly lower compared to the FOS sample. Similar results were obtained when acacia gum was used as a replacement for simple sugars in biscuits (Nakov et al., 2016).

Table 4. Glucose levels in subjects after consumption FOS, FOS+AG and AG

Time after consumption (min)	Glucose level (mmol/L)								
	Healthy persons			Person with Type I Diabetes			Person with Type II Diabetes		
	FOS	FOS + AG	AG	FOS	FOS + AG	AG	FOS	FOS + AG	AG
0	6.1	5.4	6.3	6.4	6.1	6.7	7.5	7.0	7.0
30	6.3	5.9	6.6	7.2	9.2	7.3	7.8	7.4	7.1
60	5.2	5.3	5.1	8.3	8.7	8.1	7.4	6.9	6.9
120	5.0	5.5	4.9	9.0	9.0	7.3	7.2	6.5	6.5

These preliminary results indicate that acacia gum could influence the glycemic index of the product. By lowering the glycemic index of fermented milk, acacia gum would make this product more acceptable to people suffering from diabetes. Further research with standardized clinical trials is needed to validate this effect and understand its mechanism.

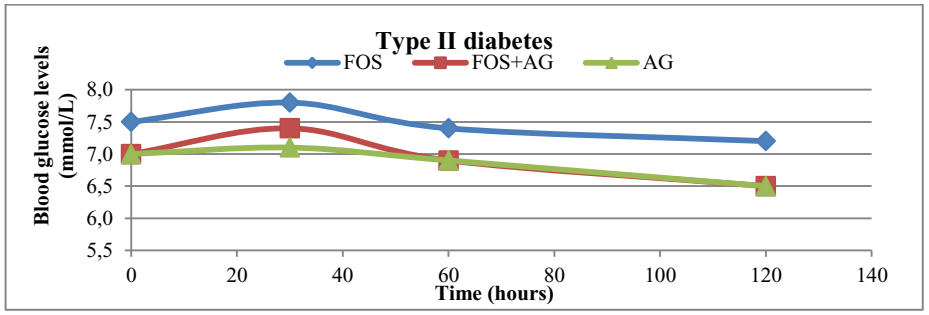

Fig. 17. Blood glucose levels in persons with type I diabetes after consumption of 200 ml fermented milk: FOS, FOS+AG and AG.

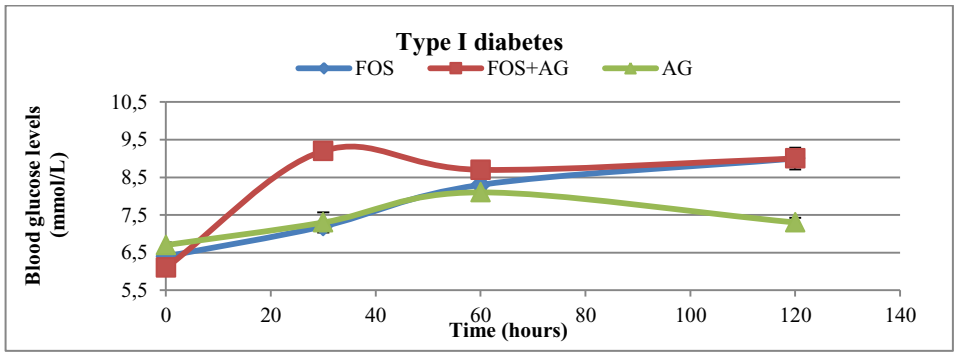

Fig. 18. Blood glucose levels in persons with type II diabetes after consumption of 200 ml fermented milk: FOS, FOS+AG and AG.

CONCLUSION

Acacia gum, fibre produced worldwide, was implemented in the production of sinbiotic fermented milk by a dairy company. Two concentrations of acacia gum in the milk were tested for their effects on the fermentation and the finished product during storage of 45 days: 0.75% (w/w) (together with 0.75% (w/w) fructooligosaccharides) and 1.5% (w/w). The current standard of the company containing 1.5% (w/w) fructooligosaccharides was used as a negative control.

Concerning the cell concentration and the pH change during the fermentation of the milk, no significant difference was noticed between the samples. All fermentations lasted for about 4.5 h reaching viable count close to log 7 cfu/g. The carbohydrate metabolism of the starter cultures, on the other hand, was affected by the presence of different types of fibre in the milk (fructooligosaccharides (FOS) and acacia gum (AG)). The lactose was consumed morein theAG sample, which was associated with the pyruvate-formate lyase-mediated pathway. Lower lactose levels would make this product more acceptable for

those who are lactose intolerant. It was also proved that fructooligosaccharides, as well as acacia gum, were consumed by the cultures, hence, detailed measurement of their concentration in the final product is needed for labelling purposes.

During the 45 day storage, the viable count in all samples varied, however, it maintained above log 6 cfu/g verifying that the product is probiotic. During the period between day 20 and 40, the AG sample had the highest cell concentration. FOS had slightly lower pH and higher total titratable acidity than the other two samples implying higher amounts of acids generated. Lactose was consumed in all three samples reaching 3.0% in FOS and FOS+AG and 2.6% in AG after 45 days. Lactulose was also found in all samples.

According to AOAC 985.29, dietary fibre was evaluated in the FOS+AG and AG samples and it was concluded that during storage the fibre was decreasing. More analyses and literature search are needed to comprehend the exact reasons for this outcome. However, the fermentation of the gum acacia by the present bacteria is the most probable explanation. Nevertheless, there is a high amount of dietary fibre still intact in the fermented milk containing acacia gum at the end of the shelf life.

In the first 14 days of storage, the texture has not changed much in any of the samples, however, sinusoidal variations werenoticed afterwards. Cohesiveness was found most different ($p<0.05$) among the samples for which FOS had higher values in days 21, 30, 38 and 42. The fluidity was also variable and found to be highest for AG at day 22. Cohesiveness is the degree to which a substance can be deformed before it break,s and in this case, can be perceived by the consumers as both good and bad, depending on their preference. Nevertheless, immense differences in the texture of a product during storage can negatively affect consumers.

From the consumer panel, it was concluded that FOS were preferred for the taste and sourness and obtained highest grades for the overall acceptance, while no statistical difference between the samples was found for consistency. The reason for these results might be the fact that the consumers are used to the existing sensory profile of fermented milk on the market. The overall preference of the consumer panel for the FOS enriched fermented milk might be explained by the sweetening effect of FOS and the lack of sweetness of acacia gum, which can also explain the aftertaste found by the expert panel in the FOS+AG and the AG. The gender of the panelists and their frequency of the consumption of fermented milks had no influence on the sensory parameters. The expert panelist pleaded that FOS have more pronounced texture characteristics, while the taste sensations were found to be more intensive in FOS+ AG and AG. The development of oxidative taste in these two is a negative sensation and should be avoided in the finished product. Concerning the appearance, it seems asno significant difference between the samples can be stated.

In conclusion, the addition of acacia gum in fermented milk will enrich the product with prebiotic with prolonged fermentation in the distal colon without significantly modifying the physical characteristics of the final product. The similar pH evolution, cell count increase and kinetics indicate that switching from one fibre to another will not be a source of processing problems. For the dairy companies, this opens a wide range of potential new developments without changes in their production process. In sweetened/flavoured fermented milks the sensory difference will be even less pronounced and the better tolerance of acacia gum, its excellent digestive health benefits and the synergistic prebiotic effects with fructooligosaccharides could be elements in favour of the use of acacia gum. Lower lactose

levels and absence of fructose are always in favour of intolerant consumers. The potential benefit to diabetics on lowering the blood glucose levels is also a great advantage of acacia gum -supplemented fermented milks that can boost the functional properties of the final product.

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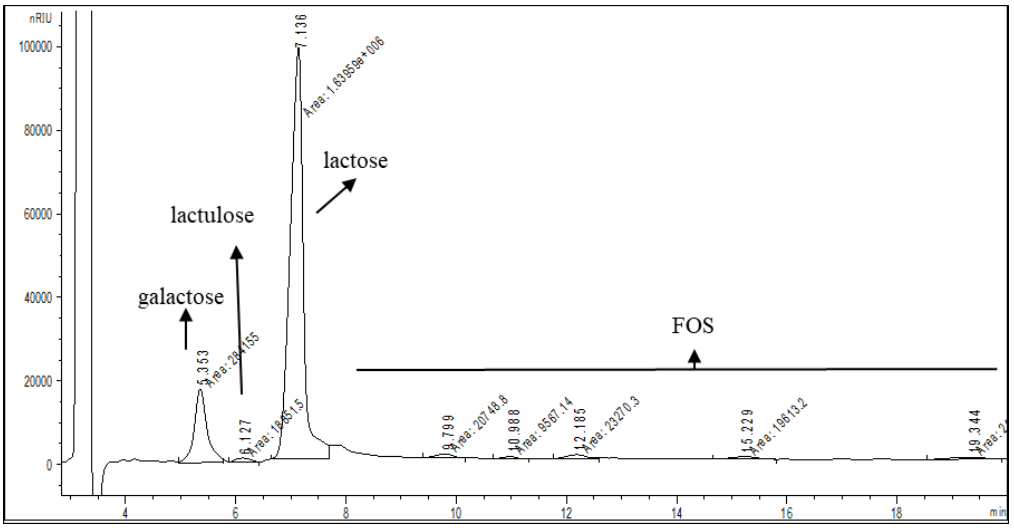
Appendix



Appendix 1. Representative chromatogram of a sample containing FOS

Topic: Production of safe food and food with added nutritional value/
Sekcija: Proizvodnja zdravstveno sigurne i nutritivno vrijedne hrane

ORIGINAL TRAPPIST CHEESE

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professional paper/stručni rad

ABSTRACT

The original Trappist cheese, the product of the monastery of Marija Zvijezda in Banja Luka, is produced for more than 135 years. The specialty of this cheese is in its secret recipe which is transferred by the word of mouthfrom a monk to a monk.
As the production started in 1882, the cheese became a synonym for semi-hard cheeses in the area of South-Eastern Europe. After the Second World War, the monks produced it only for their own needs inside the monastery of Marija Zvijezda, and in this period there was neither opportunity nor interest by the legal representatives of that time to accurately describe its organoleptic characteristics and traits. Since the production of Trappist cheese has been revived in 2008, we can try to correct this injustice.
The characteristic of the Trappist cheese is the hoop weight 1.6-2.0 kg and a natural rind which is yellowish, thin and smooth. Its consistency is soft, elastic, mild and it can be easily cut. The cut is smooth with or without very little holes, and the color is pale yellowish. Its aroma is clean, milk-specific, and it is moderately saline and easily soluble.
According to Gerber, the fat content is about 32%, the water content is about 41% while the dry matter is 56%.
Instead of a conclusion, we can only wish that the Trappist monks continue the production of Trappist cheese and that political circumstances will not influence it as it was the case up to now.

Keywords: milk, monastery of Marija Zvijezda, Trappist cheese

INTRODUCTION

This year the monastery of Marija Zvijezda will celebrate 132 years since the production of Trappist cheese has begun in Banja Luka. The cheese has been produced in all these years with more or less problems that followed the monks and their destiny in these areas. The production was interrupted only from 1996 to 2008 due to the sudden death of Father Mohor who knew the secret recipe and which he did not manage to transfer to his brothers.
The cheese production was revived again in 2008 when Father Tomislav went to France to the Monastery of Mont-des-Cats and learned the technique of cheese making and brought back the recipe for cheese production.
After the Second World War, the property of the monastery was confiscated, monks had to leave and the name “Trappist” was taken away but not the secret of cheese making.
On the territory of the former Yugoslavia, each dairy owned by the state began the production of cheese called Trappist because this name was a synonym for quality and semi-hard cheese,

but this production was never approved by the monks. At that time, the cooperation with the dairy of Banja Luka tried to be established. However, it failed because the former government had its own experts who produced "better" and "original" cheese.Now as well as before, the Trappist monks have problems regarding the protection of their intellectual property and many other producers use this name illegally for some of their products. Today in the monastery we can also find notices to consumers where Trappist monks warned about the look of the original cheese and how it differs from forgeries.
Trappist cheese is standardized and we hope that Bosnia and Herzegovina will finally protect this product under the designation of origin and thus prevent further illegal use of the Trappist name labelled on other semi-hard cheeses.

The History of Trappist Order

Trappists belong to the monastic family that follows Christ by living according to the Rule of St. Benedict of Nurisa, the father of Western monasticism, the founder of the monastery Subiaco and Monte Cassino. The name "Trappists" was received by the reform movement that began in 17th century in French Cistercian monastery of Notre Dame de La Trappe in Normandy, under the guidance of Abbot Armand Jean le Bouthillier de Rance. This reform movement was inspired by the reform movement that began 500 years ago in the monastery of Cîteaux near Dijonin France. The aim of this movement was to influence changes in a loose lifestyle of the monks in many French monasteries. Therefore, the official name of the Trappists is *Ordo Cisterciensis Strictioris Observantiae* (O.C.S.O.) which means the stricter observance of the Cistercian Order. Trappists are actually reformed Cistercians who have started their activities as insignificant local reform movement, and today in the world they serve in more than 100 monasteries. It is less known that Trappists have a female branch which has 72 monasteries, mainly in Europe. The Trappists are a contemplative order in the Catholic Church that serves to God and to people in silence, prayer and physical work. Their motto is „Ora et labora" – Pray and work. These silent monks and nuns devote their entire lives to God and their life path is governed by the cross (Ostojić, 1965.).
The monastery of Marija Zvijezda is the only Trappist monastery which produces cheese on the right side of the Rhine River that is in the former countries of the communist bloc.
Often, the religious community of Trappist monastery is misplaced with Cistercist monks who were also engaged in different productions, including the production of different cheese types. Some documents mention that Trappist cheese was made in the monasteries in Hungary, Czech Republic, Slovakia, etc. (Sanders, 1954) which is not true because in these countries there were no Trappist monasteries.

The History of Trappist Cheese

The beginning of cheese production in Marija Zvijezda monastery was in 1872, in a small dairy built by Father Franz who named that cheese a „Swiss". However, this cheese plant production did not last long due to animal diseases that caused the lack of milk.
The production of the original cheese began in 1882 when Father Ignatius from the French monastery "Port-du-Salut" arrived to the monastery in Banja Luka. He trained his brother Luka in making cheese. At the beginning, the cheese was made only for the purposes of the

monastery, and later it was made for the markets in Austria and Hungary as well as for the whole Europe. It was well known and was awarded numerous prizes at fairs all acrossEurope. At first, the monks processed the milk from their own farm and later they started to buy it from the local farmers.

There was a small sized dairy at Marija Zvijezda monastery, and the main dairy was established in Josipovac (today called Bosanski Aleksandrovac) where in 1887 a branch of Marija Zvijezda was opened.

However, the monks again encountered some problems, especially in the first year. Firstly, because of the lack of experts in cheese making, they had problems with the quality of cheese. Therefore, in 1888 abbot Bonaventure the First sent brother Dositej to France to the local monasteries, especially to Port-du-Salut, to train in cheese making for one year. When he came back he taught his brothers about cheese production and they were obliged to keep the secret of production. The secret was transmitted by the word of mouthfrom brothers to brothers or they would carry it with them into the grave. Kirin wrote about this: Even though in cheese making industry the Trappist cheese was dominantly present for the entire century, in our literature there are very little data about the technological process of making this cheese. Due to the secrecy of making cheese, there is no description of the original Trappist cheese from Banja Luka, so it can only be speculated.

This secrecy draws the following conclusion: “The quality of the cheese and the art of its production are predicated largely on the method of its preparation. Specifically, a number ofspecialists participated in the production of the cheese. Particular intervention was done by only one cheese maker. Each cheese maker knew his part of the job to perfection while the job of the others was a secret to him.” The success of the branch Josipovac prompted the Abbot Bonaventure to establish the second branch. The colonists were well developing economically in the colony Windthrst (today called Nova Topola).

The Trappists bought the land from one colonist in 1893 and onthat place they established a dairy Marienburg – Marijin Dvor (Nova Topola). The cornerstone was set on March 18, 1893. In thisdairy, besides other buildings, a cheese making plant was opened. The local people brought the milk and the monks processed it into cheese and butter.

Cheese production in both dairieswas developing successfully. Every day, 2.000-3.000 liters of milk were brought in. As the cheese plant was developing successfully, the purchase of milk rose up as much as 8.000 liters. The cheese production has reached 100 – 120 tons per year. The excess of milk was pasteurized and transported to Banja Luka where it was offered for sale. The milk was much appreciated for its quality but also because of the price.Moreover, it was cheaper than from other sellers.

The cheese was packed in packages of 4.8 kg and sent by post or railway to the clients throughout the monarchy as well as beyond its borders. The Trappist monks were also the official suppliers for the royal palace in Belgrade.

H. Renner travel writer wrote: „Now the monastery.... deals with manufacture of the „Trappist cheese“ which has a good reputation abroad as well. Since the monastery does not have enough cows, the milk for cheese making plant is taken from the close German settlements.“

Up to now, there were no reliable data on the organoleptic properties of the original Trappist cheese, and his secrecy of production does not allow us to obtain an insight into the production technology. The assumptions by different authors were the same as today's Saint-Paulin cheese that is a successor of Port-du-Salut and Port-Salut (Kirin,

2003). Mainly, the research was done to study the chemical composition, the quality and organoleptic characteristics of semi-hard cheese, which were produced in the former communist dairies.
The cheese production was revived again in 2008 when Father Tomislav went to France to the monastery of Mont-des-Cats and learned the technique of cheese making and brought back the recipe for cheese production (Budimir, 2012).

MATERIAL AND METHODS

The research is done at the Agricultural Cooperative "Livač" which is located in Aleksandrovac in Laktaši municipality, Bosnia and Herzegovina. The cooperative is engaged in the production of raw milk. Since 2008, in a newly built space for cheese production, the Trappist cheese is produced in collaboration with the monk Tomislav Topić. The cooperative provides production material and auxiliary work force and the recipe is owned by the monastery of Marija Zvijezda. Currently, about 2.5 tons of Trappist cheese are produced per month. The cheese plant is HACCP certified and has ISO 2008:2009 certificate and is under the constant supervision of a veterinary inspection.
Forty cheese samples were taken during 2012. Chemical analysis of cheese is done at the Veterinary Institute of the Republic of Srpska „Dr. Vaso Butozan“ in Banja Luka.

RESULTS AND DISCUSSION

Organoleptic properties of trappist cheese and chemical composition

The Trappist cheese belongs to the group of semi-hard cheese types and is easily cut. It has somewhat stiffer consistency but is still soft enough compared to the bad copies which are either too soft or too hard to cut. Unfortunately, due to the fact that it was not possible to forbid the use of the name „Trappist“, on the market there are different cheese plants that make cheese types based on different recipes and then they label it as „Trappist“.
The softness and ease of cutting comes from a special way of preparing and of course, due to special conditions of its ripening. It is important to note that the original Trappist cheese ripens in special conditions, where it is handled with care and it is rotated and cleaned daily. The copies of Trappist cheese which can be found on the market are produced in a way that it is „dried“ for fifteen days and then it is delivered to the stores. The ripening time of the original Trappist cheese is between 75 and 90 days as a minimum and this allows it to have a special consistency and taste.

Table 1. Organoleptic properties of Trappist cheese

Group	External appearance	Texture	Cutting	Smell and taste
semi-hard	wheel d =19 cm	soft,	smooth without or	clean milk–specific scent
	hight = 7-9 cm	elastic, mild	with very little holes	taste sweet,
	weight 1.6-2.0 kg	easily cut	pale yellow color	moderately saline
	smooth rind, dry	plastic		easily soluble
	yellowish, thin			

The cheese is produced exclusively in the form of a wheel, 19 cm in diameter and 7-9 cm size (Table 1).
The characteristic of the Trappist cheese is the wheel weight 1.6-2.0 kg and it has a natural rind which is yellowish, thin and smooth. Its consistency is soft, elastic, mild and can be easily cut. The cut is smooth with or without very little holes, and the color is pale yellowish. Its aroma is clean, milk-specific, and it is moderately saline and easily soluble.
According to Gerber, the fat content is about 32%, the water content is about 41% while the dry matter is 59% (Table 2).
Some authors mention that the Trappist cheese is produced in the form of a block (Kirin, 2002). All types of cheeses which are made by Trappist monks are done in the form of a wheel. The authors note that this is cheese with bark which is usually protected by a coating, or as cheese without bark, if it ripens and ships as a cheese packaged in foil or vacuum packed plastic bag, thereby reducing the manufacturing abatement (Dorušić et al., 1976; Kirin, 2002). This was typical for forgeries, or for semi-hard cheeses that were made in dairies of former system, or for those that today illegally use this name. The Trappist cheese has a natural rind, and a special coating is used, which is acceptable in terms of hygiene and health and which gives a yellowish color of the rind.

Table 2. Chemical composition of Trappist cheese

Water (%)	Dry matter (%)	Milk fat in dry matter (%)	Milk fat according to Gerber	NaCl (%)
41.26	58.74	53.56	33.00	2.00

In earlier papers, the authors state that the cheese has small holes once it is cut. The scent and taste of the cheese are described almost as in the original Saint-Paulina, as well as in the illustrated versions (Miletić, 1969; Sabadoš and Rajšić, 1980; Sabadoš, 1981). The original Trappist cheese, once it is cut, does not have holes because of the production technology and the quality of milk which is used for its production.

The technological process of trappist cheese

For obvious reasons, it is difficult to describe the technological process of Trappist cheese production. The quality of the raw materials out of which the cheese is produced and their hygienic and microbiological safety is of utmost importance. Furthermore, the production conditions must be of a high standard.After the milk is delivered, a low pasteurization is done after which the milk is cooled and cultures and rennet have been added. Unfortunately for all, and fortunately for the cheese, the secret of the quantity and order of culture is known only to monks but not to all of them. Lay people do not know the quantities and types of cultures so they cannot describe this process. The written recipe is only in the Port-du-Salut monastery in France and is available only to the chosen monks.
After adding cultures, the cheese is left resting to create a cheese curd after which the cutting starts. The cheese is moved to the cheese making table and it undergoes pre-pressing to separate the whey. Often, semi-hard cheese types undergo the rinse of the

curd with water which is not the case with the Trappist cheese. After pre-pressing, the cheese is cut into an appropriate form, it is placed into a cheese mould and goes under pressing again. The cheese is pressed under certain pressure and after some time it is rotated and goes back under the pressure.
After completion of this process, the cheese is left to rest for some time and afterwards goes into brine which consists of water and salt concentration.
Once this phase is finished, the cheese is left on a shelf to drip and afterwards it is put into a pre-chamber. The first phase of cheese ripening has been done in this pre-chamber under adequate moisture and temperature conditions. After 40 to 50 days, the cheese is moved into another chamber with altered ripening conditions: lower temperature and slightly higher humidity. The ripening process ends with the optimal 75 to 90 days.
During the ripening process, the cheese is covered by the coating and it is rotated and cleaned in the chambers daily. The cheese is cleaned and coated regularly, as well as the wooden holders and shelves on which the cheese ripens. Hygiene has a great influence on the ripening and the quality of Trappist cheese.

INSTEAD OF A CONCLUSION

Considering everything mentioned above, it remains to hope that the community of the monks of Marija Zvijezda monastery will continue to produce their cheese and that finally in Bosnia and Herzegovina the conditions will be set to protect the originality of the product according to the European standards.
This is also very important for other indigenous types of cheese that are made in BiH because they represent a significant potential for the development of tourist and gastronomic offer. The protection of cheese will enable the milk production to increase and a greater value will be achieved. In addition to this, it will lead to hiringmore people, either through direct or indirect arrangement in agricultural production, tourism and other related industries.

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