# HRANA U ZDRAVLJU I BOLESTI
# FOOD IN HEALTH AND DISEASE

ZNANSTVENO-STRUČNI ČASOPIS ZA NUTRICIONIZAM I DIJETETIKU
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# PRACTICES OF DIETARY SUPPLEMENTATION AMONG FOOTBALL PLAYERS

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*Original scientific paper*

**Summary**
Dietary supplements are foodstuffs, medicinal herbs, plant or animal extracts and concentrates containing active compounds the purpose of which is to supplement the normal diet with the aim of gaining power and endurance as well as muscular weight. The active compounds in dietary supplements are nutrients (vitamins and minerals, fatty acids, proteins) or other substances with a physiological effect (enzymes, microorganisms, hormones). The most often used dietary supplements among athletes are fatty acids, whey proteins, fat burners, creatine and isotonic drinks. Football players use dietary supplements as ergogenic aids to meet elevated dietary needs and hasten recovery. The aim of this study was to assess dietary supplementation practices among football players. A cross-sectional study encompassed 20 active football players from Sarajevo. Data were collected using a short questionnaire which included the general characteristics of the study participant (age, height, weight, residence), information on the frequency of dietary supplement use, type of supplement and dosage, as well as the reasons for supplementation and source of recommendation to use it. Study participants most often reported taking omega-3-fatty acids, magnesium, whey proteins and branched amino acids. Performance was the most frequently stated reason for supplementation. Supplementation was supervised and recommended by a nutritionist or self-initiated and practiced in dosages as recommended. In conclusion, dietary supplements can be ergogenics and enhance performance on one the hand and help in recovery on the other. Still, their usage should always be recommended and supervised by a specialist.

*Keywords*: dietary supplements, football players, recommendations

## Introduction

Supplementation for athletes has become commonplace. Demanding training processes and the desire for high scores are the most common reasons for taking nutritional supplements for athletes (Burke, 2007). Food supplements are concentrated active ingredients that are taken to enrich the diet, improve strength and increase muscle mass. The active ingredients may be vitamins, minerals, fatty acids, protein concentrates, enzymes, plant extracts, cultures of microorganisms, etc. The sport supplements commonly used are dietary supplements based on proteins (branched chain amino acids, whey proteins), fat-burning products, and various vitamin and mineral complexes. By taking dietary supplements, athletes strive to achieve better endurance and faster muscle recovery after training (Jager et al., 2017).
Athletes in Bosnia and Herzegovina mostly rely on dietary advice that they get from their coaches. Not many clubs employ nutritionists. This research collected data on the frequency of consuming dietary supplements among football players in the Sarajevo area. The aim of the research was to collect data on the types of dietary supplement the subjects consume and on whose recommendation they decided to use them.

## Subjects

A cross-sectional study, using a specially designed questionnaire, collected data about the consumption of dietary supplements by 20 active athletes training football (Table 1).

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**Table 1.** Subjects

| Average age | Average height | Average weight | Average BMI |
|---|---|---|---|
| 26.4 | 181.7 | 77.55 | 23.49 |

## Methods

The survey questionnaire is divided into two parts. The first part contains general information about respondents (age, height, weight, place of residence). In the second part of the questionnaire, data were collected on the frequency and types of dietary supplements taken, and the reasons and recommendations based on which the preparations were consumed.

## Results and discussion

Of the 20 respondents/football players surveyed, the majority takes supplements regularly and occasionally (80%), while only 20% of respondents do not take dietary supplements (Table 2).

**Table 2.** Frequency of taking dietary supplements

| | **Frequency of taking dietary supplements** | | | **At whose recommendation:** | |
|---|---|---|---|---|---|
| | Periodically | Regularly | I do not take anything | Self-initiated | Nutritionist's |
| N | 8 | 8 | 4 | 4 | 12 |
| % | 40 | 40 | 20 | 25 | 75 |

When it comes to the type of dietary supplements consumed, protein and amino acid preparations are taken by the largest number of subjects (Table 3).

**Table 3.** Type of dietary supplements that the respondents consume

| | **Protein or amino acids** | **Minerals (Ca, Mg and complexes)** | **Omega- 3** | **L-carnation** | **vitamin D** |
|---|---|---|---|---|---|
| N | 19 | 12 | 5 | 1 | 2 |
| % | 95 | 60 | 25 | 5 | 10 |

The above fact probably stems from the belief that taking pre-training protein can help improve performance and ease recovery of muscles after exercise. However, the scientific community has different opinions when it comes to this. According to the International Society for Sport Nutrition, on the basis of the above evidence, protein consumption before and after exercise can stimulate muscle synthesis (Jager et al., 2017). Taking protein from artificial sources, however, can produce consequences such as iron, zinc, niacin and thiamine deficiency. Very often, isolated amino acids such as arginine and lysine may result in less absorption of other amino acids (Mahan and Raymond, 2017).

That is why it is important to pay attention to the composition, origin and dose of dietary supplements taken. The general recommendation is 1.4 to 2.0 g protein / kg body weight per day to obtain muscle mass. To reduce body weight and lose fat then the intake should be more than 3 g protein / kg body weight per day (Jager et al., 2017). Caution should be exercised with such an intake, however, as it could put a strain on the kidneys (Mahan and Raymond, 2017). That is why intake recommendations must take into account all factors, in particular the extent of the efforts which the athlete is exposed to, body weight and the target to be achieved.

The highest number of respondents (68.75%) adhered to the doses indicated on the product declaration (Table 4).

**Table 4.** The dose of dietary supplements

| | **Which daily dose are you taking:** | | |
|---|---|---|---|
| | According to the declaration | According to the doctor's recommendation | Higher doses than recommended |
| N | 11 | 4 | 1 |
| % | 68.75 | 25 | 6.25 |

Instructions on the use of supplements list general information and are not a good source of information for a person who is professionally engaged in sports (Table 5). To ensure proper implementation and maximum effects, an individual approach is required. A disturbing factor is the deliberate intake of larger doses in order to achieve better results. Generally, doses above 40 g per day can be taken by older athletes, while doses above 70 g per day are associated with weakening of the muscles (Jager et al., 2017; Kim et al., 2016). It is also important how protein supplements are consumed. Studies dealing with the number and distribution of the meals showed that the best effect was achieved by eating 4-5 meals a day with 20-40 g protein, with or without training (Kinsey et al, 2016).

**Table 5.** Nutritional supplements based on proteins and amino acids that are used by respondents

| | Name | Composition | Instructions on consuming |
|---|---|---|---|
| 1 | ISO GO | Glucose, maltodextrin, potassium chloride, sodium chloride, magnesium salts, citric acid, L-glutamine, BCAA, calcium, vitamin premixes: L-ascorbic acid, nicotinic acid, D-alpha tocopherol, d-calcium pantothenate, riboflavin, hydrochloride, thiamine mononitrate, folic acid, D-biotin, cyanocobalamin, aroma, acetyl L-carnitine, sucralose sweetener. | Do not exceed 3 servings a day. |
| 2 | Muscle On | Five fractions of high quality proteins: whey protein, mycelia casein, milk protein isolate. | For maximum performance, it is recommended to take a 30 g dose 30-60 minutes after training or an additional meal. When increasing muscle mass, to maintain a positive nitric balance, it is recommended to take it as a dietary supplement during the evening and during the day. |
| 3 | PreRace powder | It contains L-taurine, citrulline malignant 2: 1, neuro stimulative mixture: dimethyl amino ethanol, caffeine anhidrus, theobromine (cocoa (Theobroma cacao)), catechin extract from green tea (Camellia sinensis), quercetin and malic acid (citrulline malate). | Mix 1 vial (4.9 g) in EFS or a favourite electrolyte drink. Take 30-45 minutes before exercising. Start by using half a dose of the preparation and then evaluate your endurance. |
| 4 | Ultragen | A unique formula that holds whey protein isolates, whey protein hydrolysates, glutamine, branched chain amino acids, vitamins (A, B and D) and minerals (calcium, magnesium, zinc, sodium and potassium). | Mix 1 container in 1.8 dl of water and drink after completion of physical activity, or as recommended by a professional. |
| 5 | Whey protein | Hydrolyzed Whey Protein Isolate (from Milk, Emulsifier: Soy lecithin), Hydrolyzed Whey Protein Concentrate (from Milk, Emulsifier: Soy lecithin), Taste, Cocoa Powder (20-22%), Sweetener (Sukralose, Acesulfame K), Sodium Chloride, Xanthan Gum), Microgranulized β-galactosidase (Tolerase ™ L Lactase Enzyme), 28 g protein per portion. | Mix 1 serving (1 measuring = 35 g) in 350 ml of water. If possible, use a shaker with a mesh and then shake well. The best time to use is after training. |
| 6 | BCAA | 5000 mg of branched-chain amino acid (BCAA) in dose | Mix one dose with 300 - 500 ml of water and drink it during training. You can also use 1-3 doses daily, before meals or after training or before bedtime. The product is best dissolved if the mixture is left to stand for a while. |

The quality of the protein supplements taken is also very important. Protein quality is defined as protein efficacy to stimulate muscle protein synthesis and muscular hypertrophy (Lemon, 2000). Animal protein has a higher percentage of essential amino acids that helps improve muscle hypertrophy and protein synthesis in relation to plant protein sources (Campbell et al., 1999; Tang et al., 2009,). For essential amino acids to induce muscle protein synthesis, doses of 6 to 15 g are required. Up to 3 g per serving should be leucine, which is essential for protein synthesis in the body (Jager et al., 2017).

Leucine can also facilitate recovery after exercise. For this reason, athletes are advised for each meal to eat high-protein foods that are rich in leucine (Wilson et al., 2011). Essential branched-chain amino acids (BCAA), isoleucine, leucine and valine, play an important role in protein metabolism, neural function, and regulation of glucose and insulin levels (Norton and Layman, 2006, Blomstrand, 2006). That is why they are emphasized as vital ingredients in dietary supplements for athletes. Indeed, a dietary supplement is very often purchased because it contains BCAAs, as indicated by the types of supplements used by the study subjects (Table 3). BCAAs are recommended in the recovery phase of the organism after the training process in the amount of 3-5 g (Mahan and Raymond, 2017). Interestingly, even though a product declaration recommends taking one 5 g dose of BCAA, the respondents consume up to three doses per day. In this way it is suggested to consume more BCAAs than recommended in the literature.

The origin of protein and amino acids in dietary supplements differs. They are most often derived from milk and whey. Each protein has its advantages and disadvantages. The protein value is estimated based on the content of individual amino acids, fat and micronutrients. An important criterion is the leucine content as well as the rate of digestion (Jager et al., 2017). Whey proteins are digested faster than other proteins and have a higher amount of leucine. However, some research suggests that a protein blend could have more beneficial effects on the training process. The key ingredients in such mixtures are the leucine content, the content of essential amino acids, and the level of bioactive peptides and antioxidants (Jager et al., 2017).

In addition to protein-based dietary supplements, the subjects used L-carnitine. L-carnitine is an amino acid derivative synthesized from lysine and methionine in the human body. The food is contained in foodstuffs of animal origin. Metabolism plays a key role in energy production, helping to transfer fatty acids into mitochondria. It is very common in dietary supplements whose purpose is to better "burn" fat (Rebouche, 1992). Although there are numerous studies to confirm the positive effect of carnitine in dietary supplements for athletes, more comprehensive research is still required to demonstrate efficacy and the ergogenic role in supplementation (Luckose et al., 2015).

Omega-3 fatty acids, which are active against inflammation, help muscle recovery after intense exercise. It is therefore recommended that omega-3 fatty acids be supplemented, especially after injury (Mahan and Raymond, 2017). The 2014 study reveals a higher risk of injury for athletes who had deficiencies of iron, vitamin D and calcium (McClung et al., 2014). Vitamin D is associated with better muscle strength, endurance, stronger bones, smaller inflammations, increased testosterone secretion and faster recovery after training (Dahlquist et al., 2015; Farrokhyar et al., 2015; Maroon et al., 2015). Therefore, the proper supplementation of vitamins and minerals is essential.

In the study, 70% of respondents (Table 3) use vitamin and mineral nutrition supplements. This is a commendable fact, especially considering that the nutritional supplements are taken in recommended doses and under professional supervision. It is also positive that the majority of respondents (12 or 75%) take nutritional supplements at a nutritionist's recommendation, while only 4 respondents (25%) decided to self-initiate supplementation (Table 2).

## Conclusion

Nutritional supplements can contribute to greater strength and endurance of athletes, and help faster recovery after demanding sports preparation and competition. The choice of dietary supplements should be carried out under the supervision of an expert and should not be taken according to an athlete's own discretion. When consuming nutritional supplements, the recommended doses should not be exceeded.

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# KORIŠTENJE DODATAKA PREHRANI MEĐU NOGOMETAŠIMA

*Izvorni znanstveni rad*


## Sažetak

Dodaci prehrani su hrana, biljke ili ekstrakti podrijetlom iz biljaka ili životinja koji sadrže aktivne sastojke za nadopunu prehrane a sa ciljem povećavanja snage, izdržljivosti ili mišićne mase. Aktivne komponente u njima su hranjive tvari (vitamini, minerali, masne kiseline, bjelančevine) ili ostale komponente koje mogu imati fiziološko djelovanje (enzimi, mikroorganizmi, hormoni). Najčešće korišteni dodatci među sportašima su masne kiseline, proteini sirutke, sredstva za izgaranje masti, kreatin i izotonični napitci. Nogometaši koriste dodatke prehrani sa ciljem zadovoljenje povećanih energetskih potreba, kao ergogena sredstva ili za ubrzavanje oporavka. Cilj ovog rada je procijeniti naviku korištenja dodataka prehrani među nogometašima. Presječna studija je obuhvatila 20 aktivnih nogometaša iz Sarajeva. Podaci su prikupljeni korištenjem kratkog upitnika koji je uključivao opće značajke ispitanika (dob, visina, težina, mjesto stanovanja), podatak o učestalosti korištenja dodataka, vrstu dodataka i korištenu količinu, razloge za korištenje kao i podatak o tome tko im je preporučio korištenje dodataka prehrani. Rezultati su pokazali da ispitanici najčešće koriste omega-3 masne kiseline, magnezij, proteine sirutke i razgranate aminokiseline. Najčešći razlog uzimanja je bila želja za boljom sportskom izvedbom. Preporuka i nadzor nad korištenjem provodili su nutricionisti ali i sportaši su se samostalno odlučivali za korištenje preporučenih količina. Može se zaključiti da dodaci prehrani mogu biti ergogena sredstva i poboljšati sportsku izvedbu s jedne strane dok s druge strane pomažu oporavku, ali korištenje mora biti pod nadzorom stručnjaka.


*Ključne riječi*: dodaci prehrani, nogometaši, preporuke

# INFLUENCE OF DIETARY PATTERN AND METHYLENTETRAHYDROFOLATE REDUCTASE C677T POLYMORPHISM ON THE PLASMA HOMOCYSTEINE LEVEL AMONG HEALTHY VEGETARIANS AND OMNIVORES

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*Original scientific paper*

## Summary

Elevated total plasma homocysteine level (Hcy) is associated with physiological and dietary factors as well as the genetic defect of enzymes involved in Hcy metabolism. The objectives of the study were to examine (1) differences between healthy vegetarian and omnivorous subjects in relation to biochemical parameters, prevalence of the MTHFR (methylentetrahydrofolate reductase) T/T genotype, and the plasma Hcy level, and (2) the effects of the MTHFR C677T polymorphism and dietary pattern on the plasma Hcy level. In 47 vegetarian and 53 omnivorous subjects the plasma level of Hcy, folate, vitamin $B_{12}$, glucose, total cholesterol, triglycerides, HDL and LDL-cholesterol and creatinine were measured. MTHFR C677T polymorphisms were analyzed using the PCR-RFLP method. Obtained results have shown that vegetarians had lower vitamin $B_{12,}$ total cholesterol, LDL-cholesterol and creatinine status. The plasma Hcy level was higher among vegetarians compared with omnivore subjects (14.10±6.69 *vs.* 10.49±2.41 µmol/L) and negatively correlated with vitamin $B_{12}$ status and folate. The plasma Hcy level did not differ between the given MTHFR C677T genotypes among either vegetarians or omnivores. Unlike the MTHFR C677T polymorphism, the effect of dietary pattern on plasma Hcy level was confirmed. It could be concluded that vegetarians tend to have lower vitamin $B_{12}$ status and a higher plasma Hcy level. The MTHFR 677C/T polymorphism has no effect on plasma Hcy level, in contrast to dietary pattern which indicates the importance of adequate vitamin $B_{12}$ and folate status in bypassing the mutation.

*Key words:* folate, homocysteine, MTHFR C677T polymorphism, vegetarian diet, vitamin $B_{12}$

*Abbreviations:* HDL - High Density Lipoprotein-cholesterol, LDL—Low Density Lipoprotein-cholesterol, Hcy – homocysteine, MTHFR- methylentetrahydrofolate reductase

## Introduction

A review of epidemiological reports has established a high plasma homocysteine (Hcy) level (hyperhomocysteinemia, hHcy) as a risk factor for cardiovascular diseases, cerebrovascular diseases, dementia-type disorders, and osteoporosis -associated fractures (Maron and Loscalzo, 2009). Also, important meta-analyses on the connection of hHcy with type 2 diabetes and mental disorders such as schizophrenia and depression have recently been published (Huang et al., 2013; Nishi et al., 2014).
It has been found that inadequate nutrition is associated with hHcy. Insufficient vitamin $B_{12}$, $B_6$ and folate intake, as well as low ω-3 polyunsaturated fatty acids (ω-3 PUFA) status, are associated with elevation of the plasma Hcy level (Huang et al.,2012; Li, 2013; Stabler and Allen, 2004). Lower levels of those nutrients are com-

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monly found among vegetarians. An excessively energy restrictive and monotonous vegetarian diet may result in nutrient deficiency, mainly in fat and saturated fatty acids (SFA), heme iron ($Fe^{(2+)}$), zinc, vitamin A, $B_{12}$, D and especially in ω-3 PUFA. Even though morbidity and mortality are generally lower in vegetarians than omnivores (Appleby et al., 2001), vitamin $B_{12}$, folate and Hcy status could be better thus lowering the risk of hHcy associated diseases.

Besides by nutritional deficiencies, hHcy can also be genetically induced. One of the significant genetic factors contributing to hHcy is MTHFR (methylentetrahydrofolate reductase) C677T polymorphism. When the MTHFR C677T polymorphism is accompanied by nutritional deficiencies the effect on the plasma Hcy level is even more accentuated.

The aim of this study was to examine the differences between healthy vegetarian and omnivorous subjects in relation to biochemical parameters, the prevalence of the MTHFR T/T genotype, and the plasma Hcy level, and its association with the B12 and folate status. In the second step, the effects of the methylentetrahydrofolate reductase (MTHFR) C677T polymorphism and the dietary pattern on the plasma Hcy level between healthy vegetarian and omnivorous subjects were examined.

## Subjects and methods

*Subjects*

The examined group consisted of 100 healthy subjects between 18 and 69 years of age. Among them, 47 were vegetarians and 53 omnivores. Regarding vegetarian subgroups, 46 vegetarian subjects were lacto-vegetarians and one was vegan. Subjects with history of hormonal intake or other medical treatments as well as present chronic diseases were excluded to eliminate the possible effect on blood nutrient levels.

*Methods*

All the molecular analyses were done at the Laboratory for Molecular Diagnostics at the Department of Pathology, University of Rijeka. The procedures were approved by the medical ethics committee of the University Hospital Center Rijeka and Faculty of Medicine at the University of Rijeka and in accordance to the World Medical Association Declaration of Helsinki and the Nürnberg codex regarding ethical principles for medical research. All subjects gave consent to participate in the study, being motivated by general health, Hcy, vitamin $B_{12}$ and folate status feedback. Fundamental bioethical principles (autonomy, beneficence, nonmaleficence and justice) as well as privacy and protection of personal data were assured throughout the whole study.

*Dietary pattern data*

Self-reported meat-eating habits were the basis for categorizing the subjects into the vegetarian or omnivorous group, regardless of the length, types or levels of the vegetarian diet. The vegetarian dietary patterns were determined according to reported eating of no meat for at least two years. Furthermore, the type and level of vegetarianism was not specified.

*Biochemical analyses*

Several hematological and biochemical parameters were observed. Hematological parameters were obtained from whole blood samples while glucose, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, creatinine, total Hcy, folate and vitamin $B_{12}$ were obtained from plasma or serum. Routine hematological parameters were detected on a hematological analyzer Cell Dyn 3700 (Abbott, Illinois, USA), biochemical parameters on an analyzer COBAS 6000 (Roche, Manheim, Germany) while Hcy (Architect Homocysteine Reagent, Abbott, Illinois, USA), folate (Architect Folate Reagent, Abbott, Illinois, USA) and vitamin $B_{12}$ levels (Architect $B_{12}$ Reagent, Abbott, Illinois, USA) were measured using the chemiluminescence method (CMIA) on an automated immunoassay analyzer Architect (Abbott Laboratories, Illinois, USA). For C677T MTHFR polymorphism analysis, DNA from peripheral blood leukocytes was isolated with a NucleoSpin Blood kit (Machery-Nagel, Duren, Germany), according to the manufacturer's recommendations. Target fragment of MTHFR gene (198 base-pairs) was amplified by polymerase chain reaction (PCR). MTHFR C677T genotype was determined by digestion of a PCR product with the restriction enzyme Hinf I, as recommended by the manufacturer (Takara, Illinois, Japan) and the obtained DNA fragments were separated on a 3% agarose gel and visualized by ethidium bromide dye.

*Statistical analysis*

Data were presented as mean (and standard deviation, SD) and percentages. The one-way analysis of variance and the Chi-square ($\chi 2$) test were used for comparisons between the groups. To determine the frequency of MTHFR C677T polymorphism, the Hardy-Weinberg equilibrium was used. A $\chi 2$ analysis was used to test the significant differences in the prevalence of MTHFR C677T genotypes among vegetarian and omnivores. Pearson correlation coefficients were used to assess the correlations between the vitamin B12, folate and the plasma Hcy level. To test differences in the plasma Hcy levels between the vegetarian and omnivorous subjects regarding all three MTHFR C677T genotypes, the one-way analysis of variance and nonparametric Kruskal-Wallis test were performed. The factorial ANOVA was performed to compare means across two independent variables (MTHFR C677T genotypes and dietary pattern) in the Hcy level. The differences were considered significant at the 5% level in this study and data were interpreted with a 1% and 5% confidence level. The SPSS software 19.0 was used in the statistical analyses.

## Results and discussion

The demographic characteristics, together with hematological and biochemical parameters of both of the dietary pattern groups and total population, are listed in Table 1.

**Table 1**. Characteristics of the subjects in total and two subgroups

| Variable | Total population[1] (*n* = 100) | Vegetarians[1] (n=47) | Omnivores[1] (n=53) | P[2] |
|---|---|---|---|---|
| Age (y) | 42.8±11.9 | 42.3±11.7 | 43.3±12.2 | |
| Male (n) | 27 | 16 | 11 | |
| Female (n) | 73 | 31 | 42 | |
| $B_{12}$ (pmol/L) | 281.50±117.00 | 237.21± 117.81 | 320.77± 102.16 | 0.001 |
| Folate (g/mL) | 8.70±4.70 | 8.17± 4.04 | 9.16± 5.21 | 0.295 |
| Hcy (µmol/L) | 12.19±5.21 | 14.10± 6.69 | 10.49± 2.41 | 0.001 |
| Glucose (mmol/L) | 4.88±0.48 | 4.88±0.53 | 4.89±0.44 | 0.922 |
| Cholesterol (mmol/L) | 5.08±1.30 | 4.58±1.46 | 5.53±0.94 | 0.001 |
| Triglycerides (mmol//L) | 0.94±0.37 | 0.90±0.38 | 0.98±0.35 | 0.277 |
| HDL (mmol/L) | 1.58±0.41 | 1.52±0.35 | 1.63±0.46 | 0.164 |
| LDL (mmol/L) | 3.11±0.96 | 2.76±0.99 | 3.43±0.81 | 0.001 |
| Creatinine (µmol/L) | 73.16±13.42 | 69.83±12.23 | 76.11±13.84 | 0.019 |
| Iron (µmol/L) | 15.72±6.75 | 15.76±7.20 | 15.76±6.40 | 0.950 |
| UIBC (µmol/L) | 46.20±11.61 | 47.35±12.89 | 45.18±10.36 | 0.353 |
| TIBC (µmol/L) | 61.37±8.65 | 63.00±8.41 | 59.92±8.67 | 0.074 |
| Hcy>15 µmol/L (%) | 19 | 15 | 4 | 0.002 |
| MTHFR T/Tgenotype (%) | 13 | 4 | 9 | 0.209 |

[1]Values are means±SD, or percentages. UIBC - Unsaturated iron binding capacity; TIBC - Total iron binding capacity

[2]ANOVA or chi-square test between vegetarians and omnivores

In this predominantly female population, 47% consumed a vegetarian diet. Vegetarians statistically had a lower vitamin $B_{12}$ level and a higher Hcy level than omnivores. A statistically significantly higher prevalence of hHcy among vegetarians was shown, while, in total, 19% of subjects had hHcy (Hcy level >15 µmol/L). The folate level was lower among vegetarians but did not differ significantly between the two groups, as was expected. A statistically significant difference was found among vegetarians having lower total cholesterol, LDL-cholesterol and creatinine levels, compared with omnivores.

The MTHFR C677T genotype distribution was compatible with Hardy-Weinberg equilibrium (Table 2). The prevalence of the MTHFR C677T T allele frequency is 13%, which corresponds to an average of 12.5%, as well as C/C and C/T genotype.

**Table 2**. MTHFR C677T genotype distribution according to dietary pattern

| MTHFR C677T | $f_{ob}$ | $f_{ex}$ | $(f_{ob}-f_{ex})^2/f_{ex}$ | Vegetarian n (%) | Omnivores n (%) | P[1] |
|---|---|---|---|---|---|---|
| T/T | 13 | 11.90 | 0.101 | 4 (8.3) | 9 (17.3) | 0.209 |
| C/T | 43 | 45.20 | 0.107 | 22 (47.9) | 21 (38.5) | 0.897 |
| C/C | 44 | 42.90 | 0.028 | 21 (43.8) | 23 (44.2) | 0.469 |
| Total | 100 | 100.00 | 0.236 | 47 (100) | 53(100) | 0.431 |

$f_{ob}$ - observed frequency; $f_{ex}$ - expected frequency by Hardy-Weinberg equilibrium; [1]Chi-square test

As shown in Table 2, there was a higher frequency of homozygous MTHFR 677T/T mutation carriers in the omnivore group (17.3% *vs* 8.3%) and a higher frequency of heterozygous MTHFR 677C/T mutation carriers in the vegetarian group (47.9% *vs* 38.5%), but no significant difference in prevalence of the MTHFR genotypes regarding dietary pattern has been found. The plasma Hcy level was not significantly different between the vegetarian and omnivorous subjects regarding all three MTHFR genotypes (Table 3.).

**Table 3**. Plasma Hcy level according to the MTHFR polymorphism genotype in vegetarians and omnivores

| MTHFR genotype | Homocysteine level (µmol/L) | | | |
|---|---|---|---|---|
| | Vegetarian | Omnivores | Total | P[2] |
| T/T type | 17.08±9.71[1] | 10.07±2.16 | 12.23±6.17 | 0.209 |
| C/T type | 13.95±6.34 | 10.35±2.25 | 12.19±5.08 | 0.469 |
| C/C type | 13.69±6.69 | 10.79±2.69 | 12.17±5.16 | 0.897 |

[1]Data are expressed as mean±S.D.;[2]Statistical significance was analyzed by one-way analysis of variance and checked with the nonparametric Kruskal-Wallis test.

As shown in Table 4, the vegetarian diet has significant effect on the plasma Hcy level ($F(1.94) = 13.58$, $p<0.001$), while the effects of MTHFR genotype ($F(2.94)=0.383$, $p>0.05$) and the interaction of vegetarian diet and MTHFR genotype ($p=0.471$) were not significant.

**Table 4.** Factorial ANOVA for the effect of vegetarian diet and MTHFR genotype on the plasma Hcy level

| | SS | df | MS | F | p | Eta |
|---|---|---|---|---|---|---|
| MTHFR | 18.86 | 2 | 9.44 | 0.383 | 0.683 | 0.008 |
| Vegetarian | 334.92 | 1 | 334.92 | 13.58 | 0.000 | 0.126 |
| MTHFR/ Vegetarian | 37.49 | 2 | 18.74 | 0.76 | 0.471 | 0.016 |
| Error | 2318.60 | 94 | 24.67 | | | |
| Total | 17542.92 | 100 | | | | |

MTHFR genotype did not correlate with the plasma Hcy level (Table 5.). Significant inverse correlations were found between plasma Hcy and vitamin $B_{12}$ status (r =-0.559 among vegetarian, r =-0.411 among omnivores), and between plasma Hcy and folate (r =-0.420 among vegetarian. r =-0.502 among omnivores).

**Table 5.** Pearson correlation coefficients for MTHFR genotype, $B_{12}$ and folate in the subgroups and total population

| Parameters | Homocysteine level (µmol/L) | | |
|---|---|---|---|
| | Vegetarian | Omnivores | Total |
| MTHFR C677T | -0.105 | 0.114 | -0.004 |
| $B_{12}$ (pmol/L) | -0.559** | -0.411** | -0.548** |
| Folate (g/mL) | -0.420** | -0.502** | -0.387** |

Statistical significance level *P<0.05, **P<0.01, ***P<0.001

This is a study investigating the influence of both dietary pattern and MTHFR C677T polymorphism on the plasma Hcy level among healthy vegetarians and omnivores. Results have confirmed the association of the vegetarian diet with an elevated plasma Hcy level, but not with MTHFR C677T polymorphism.

The importance of these findings lies in the fact that an elevated plasma Hcy level is an independent risk factor for cardiovascular diseases. Unfortunately, a detailed list of diseases associated with a Hcy level higher than 15 µmol/L is much longer: coronary artery disease, stroke, fracture, venous thrombosis, retinal artery and vein occlusion, nonarteritic anterior ischemic optic neuropathy, abdominal aortic aneurysm, diabetes and diabetes induced peripheral neuropathy and nephropathy, cancer, depression, schizophrenia, Parkinson's and Alzheimer's diseases, as well as inflammatory bowel diseases. pregnancy complications and poor pregnancy outcomes (Nishi et al., 2014; Wu and Wu, 2002).

Our results have shown that vegetarians had a higher plasma Hcy level than omnivores (14.10±6.69 *vs* 10.49±2.41 µmol/L). As mentioned above, the cut-off value for defining hHcy is 15 µmol/L and 20 µmol/L for the elderly (Refsum et al., 2004). Based on that criterion, 31.91% of our vegetarian and 7.55% of our omnivorous subjects had hHcy (in total 19%). We also had 3 vegetarian and 7 omnivorous subjects older than 60 years but none of them had Hcy levels higher than 20 µmol/L. If taken into account that some European societies define the 12 µmol/L as a cut-off value, our vegetarian subjects indeed are at risk of developing hHcy associated diseases (Stanger et al., 2003). According to data from Slovakia and Austria, 29% and 53% of vegetarians, respectively, had Hcy levels higher than 15 µmol/L (Krajcovicova-Kudlackova et al., 2000; Majchrzak et al., 2006). Chinese scientists came to a similar conclusion examining 103 vegetarian men whose average Hcy level was 13.99±4.63 µmol/L. According to them, the prevalence of hHcy (plasma Hcy ≥14 µmol/L) in vegetarians is 26.47%, in contrast to 13.28% seen in an omnivore group (Huang et al., 2013). Furthermore, a study of vegetarian families conducted in India on 300 subjects from 119 families showed that hHcy (>15 µmol/L) was present in 75% of male and 35% of female family members (Deshmukh et al., 2010).

There are several environmental and disease-related factors considered to raise the plasma Hcy level, such as lack of B vitamins, ω-3 PUFA, alcoholism, smoking, kidney disease, hypothyroidism, estrogen deficiency and certain medications (Huang et al., 2012; Li, 2013; Maron and Loscalzo, 2009). However, elevation in plasma Hcy levels among healthy individuals is generally caused by two factors: nutritional deficiencies of vitamin cofactors, and genetic defects in key enzymes involved in Hcy metabolism (Lovričević et al., 2004).

*A vegetarian diet and the Hcy level*

The World Health Organization (2012) states that vitamin $B_{12}$ and folate deficiency, together with iron, iodine and vitamin A deficiency, is recognized as a global public healthcare problem. Vitamin $B_{12}$ and folate deficiency are primarily the result of inadequate dietary intake. Individuals who regularly do not consume fruits rich in folate, green leafy vegetables, legumes, enriched cereals and breads are at risk of developing folate deficiency, while those excluding or reducing the intake of animal source foods are at risk from developing vitamin $B_{12}$ deficiency (de Benoist, 2008).

In the Eastern part of the world, vegetarianism is a long-known traditional dietary pattern, while in Western countries many people turn to vegetarianism to achieve better health. A diverse and planned vegetarian diet is considered to be appropriate and can provide all the nutrients needed. Depending on the type of vegetarian diet and the ability to plan and design their diet properly, people can either enjoy health benefits or be at risk of the previously mentioned diseases. For instance, hematological and biochemical parameters from our study showed that vegetarians (mainly lacto-vegetarians) had significantly lower total cholesterol ($p<0.001$), LDL-cholesterol ($p<0.001$) and creatinine ($p<0.019$). On the other hand, a poorly designed vegetarian diet usually resulted in the lack of vitamin $B_{12}$, folate and ω-3 PUFA, all being nutrients important for Hcy level regulation (Li, 2013).

Results from our study confirmed that vegetarians have a lower vitamin $B_{12}$ and folate level. Regarding folate status, there was no statistically significant difference between the two groups, even though vegetarians in average did have a lower folate level than omnivores (8.17±4.04 g/mL *vs* 9.16±5.21 g/mL). A particularly low folate level is a risk factor for many diseases associated with Hcy status. Folate (tetrahydrofolate) is a methyl donor needed for the conversion of sufficient amounts of plasma Hcy back to methionine. A decrease of folate level below 4 ng/mL causes Hcy to accumulate in the blood, leading to hHcy (WHO, 2012). Almost three decades ago, Kang et al. (1987) found that a folate level below 4 ng/mL, found in 65% of the subjects, was related to Hcy accumulation (Kang et al., 1987).

In addition to folate being an essential substrate in the reaction of Hcy transmethylation, vitamin $B_{12}$ is a cofactor of transmethylation enzyme i.e. methionine sinthase. Vitamin $B_{12}$ deficiency impairs Hcy metabolism and leads to its accumulation in the plasma. An elevated Hcy level was found in more than 95% of individuals with clinically and laboratory confirmed vitamin $B_{12}$ deficiency (Selhub et al., 2007).

Vitamin $B_{12}$ deficiency is considered a global public health problem (Allen, 2009). Borderline values for defining deficiency differ from one study to another, ranging between 150 – 350 pmol/L. Together with different sensitivity and specificity of serum vitamin $B_{12}$ tests (13% - 75% and 45% - 100%), this is the reason why epidemiological data on vitamin $B_{12}$ deficiency are inconsistent (Willis et al., 2011). Last year's review revealed that 5% - 7% of young people, 10% - 30% of people older than 65, 63% lacto and lacto-ovo vegetarians and over 86% vegans were vitamin $B_{12}$ deficient. The review also presumes that vitamin $B_{12}$ deficiency data were understated (Groeber et al., 2013). The average vitamin $B_{12}$ level in the group of our subjects was 237.21±117.81 pmol/L among vegetarians and 320.77±102.16 pmol/L among omnivores. Another Croatian study on vitamin $B_{12}$ status among vegetarians found a slightly higher vitamin $B_{12}$ level (271.7 pg/mL in average) (Šatalić, 2009). In addition, a study from four Indian states demonstrated that 49% of the subjects had less than 220 pg/L vitamin $B_{12}$, and 30% of those subjects had hHcy (>15 μmol/L). Based on their results, they concluded that individuals with an optimum vitamin $B_{12}$ and folate status generally have a low Hcy level, even in an hHcy related risk genotype (Sukla et al., 2012). Our results showed strong inverse correlation of both vitamin $B_{12}$ and folate status, but not the MTHFR C677T polymorphism, with plasma Hcy level.

*MTHFR C677T polymorphism and the Hcy level*

MTHFR is an enzyme that facilitates the conversion of Hcy to methionine. When the most common genetically inherited mutation at nucleotide 677 (C677T) occurs, the MTHFR enzyme activity becomes impaired and can result in increasing the Hcy level by 25%. Homozygous T/T genotype is an independent risk factor for hHcy (Maron and Loscalzo, 2009). The prevalence of MTHFR C677T gene mutation varies between population groups. A Croatian study from 2004, performed on a sample of 228 subjects, found 9.21% homozygous (T/T), 44.74% heterozygous (C/T) and 46.05% wild type mutation (C/C) carriers (Lovričević et al., 2004). Our study showed a similar distribution, finding 13% total homozygous (T/T), 43% heterozygous (C/T) and 44% wild type (C/C) carriers. A South-East Bosnian study on 207 healthy subjects found 11.11% homozygous (T/T) and 44.44% heterozygous (C/T) carriers. All of these results are in correlation with mutation frequency for the European population.

Fodinger et al. (2000) found the association of MTHFR C677T polymorphism with elevated plasma Hcy. In contrast to these findings, our study did not confirm that association; there was no statistically significant difference in the Hcy level between different genotypes either among vegetarians or omnivores. Vegetarian homozygous (T/T) carriers indeed did have higher Hcy levels but without the expected statistical significance. Lea et al. (2009) from Australia had similar findings, showing no statistically significant effect of the T/T genotype on plasma Hcy level (Lea et al., 2009). A possible explanation could be related to the small sample size (group of 50). The effect of MTHFR C677T genotype on the plasma Hcy level is dependent on the folate status and is smaller in areas like Croatia and Australia with folate fortification, compared with low folate regions like Asia (Holmes et al., 2011).

Overall, it was shown that MTHFR C677T polymorphism alone, as well as the interaction of MTHFR C677T polymorphism with vegetarian diet, had no effect on plasma Hcy level. On the other hand, a vegetarian diet had a strong and statistically significant influence and effect on plasma Hcy level. Therefore, a lower level of vitamin B12 and the folate status in vegetarians are indicators that supplementation is needed in order to bypass methylation and influence on genetic variations of the MTHFR enzyme essential in Hcy metabolism.

## Conclusion

Vegetarians had lower vitamin $B_{12}$ and higher Hcy levels than omnivores. The lower vitamin $B_{12}$ and folate status was correlated with higher plasma Hcy levels. The MTHFR C677T genotype was not associated with plasma Hcy level either among vegetarians or omnivores. Nevertheless, the MTHFR C677T polymorphism had no effect on plasma Hcy level. To conclude, the effects of a vegetarian diet on plasma Hcy level are evident and indicate the significance of adequate vitamin $B_{12}$ and folate status in bypassing the MTHFR C677T polymorphism. The limitations of this study are the sample size and the absence of data on dietary intake of vitamin $B_{12}$ and folate. Further clinical trials on a larger number of participants are recommended.

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# UTJECAJ PREHRAMBENOG MODELA I POLIMORFIZMA C677T METILENTETRAHIDROFOLAT REDUKTAZE NA RAZINU HOMOCISTEINA U PLAZMI MEĐU ZDRAVIM VEGETARIJANCIMA I OMNIVORAMA

**Sažetak**
Povišena razina homocisteina u plazmi (Hcy) povezana je s fiziološkim i prehrambenim čimbenicima, kao i genetskim defektom enzima koji su uključeni u metabolizam Hcy. Ciljevi ove studije bili su (1) utvrditi razlike između zdravih vegetarijanca i omnivora u odnosu na biokemijske parametare, prevalenciju MTHFR genotipa i razinu Hcy, i (2) utvrditi učinke polimorfizma metilentetrahidrofolat reduktaze (MTHFR) C677T i načina prehrane na razinu Hcy u plazmi. U 47 vegetarijanaca i 53 omnivora izmjerena je razina Hcy, folata, vitamina B12, glukoze, ukupnog kolesterola, triglicerida, HDL i LDL kolesterola i kreatinina u plazmi. Polimorfizam MTHFR C677T analiziran je pomoću PCR-RFLP metode. Dobiveni rezultati su pokazali da su vegetarijanci imali niži vitamin B12, ukupni kolesterol, LDL-kolesterol i status kreatinina. Razina Hcy u plazmi bila je veća kod vegetarijanaca u usporedbi s omnivorima (14,10 ± 6,69 *vs* 10,49 ± 2,41 µmol/L) i negativno je korelirala sa statusom vitamina B12 i folatom. Razina plazme Hcy nije bila različita u odnosu na genotipove MTHFR C677T, ni među vegetarijancima ni omnivorima. Za razliku od MTHFR C677T polimorfizma, potvrđen je utjecaj načina prehrane na razinu Hcy u plazmi. Može se zaključiti da vegetarijanci obično imaju niži status vitamina B12 i višu razinu Hcy u plazmi. Polimorfizam MTHFR C677T nema utjecaja na razinu Hcy plazme, za razliku od prehrambenog uzorka koji ukazuje na važnost adekvatnog vitamina B12 i statusa folata u zaobilaženju mutacije.

*Ključne riječi*: homocistein, MTHFR C677T polimorfizam, vegetarijanska prehrana, vitamin B12,

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# NITRATES AND NITRITES, METABOLISM AND TOXICITY

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*Review*

## Summary

Nitrates naturally occur in the environment and are involved in the nitrogen cycle. Nitrates and nitrites are chemical compounds used in fertilizers, rodenticides and as food preservatives. They can be found in the air, soil, water and food (especially in vegetables) and can be synthesized in the human body. Nitrates play a significant role in the diet and metabolism of plants. Nitrates are formed by oxidation of organic waste by the action of nitrogen-binding bacteria. Humans are exposed to nitrates and nitrites via consumption of vegetables and processed meat products, and to a lesser extent via water and other food. Consumption of drinking water with an increased concentration of nitrate may affect the human body in two ways: (i) acutely, most often manifested as methemoglobinemia (where nitrates in the digestive system are reduced to nitrites, which then oxidize iron in the hemoglobin of the red blood cells forming methemoglobin unable to transmit oxygen in the body), resulting in blue skin, and (ii) chronic, manifested by the occurrence of cancer as a result of organism exposure to nitrosamines (formed during the reaction of nitrates with amines in the body).

## Introduction

Nitrogen is essential for all living beings as it is a component of proteins. Nitrogen exists in the environment in many forms and changes forms as it moves through the nitrogen cycle (Oram, 2015). There is a widespread concern over exposure to nitrate from dietary and environmental origins and its increased risk to negative impact on human health (Wagner et al., 1983; Rivett et al., 2008). Nitrates and nitrites are ubiquitous within environmental, food, industrial and physiological systems (Moorcroft et al., 2001; Santamaria, 2006). Nitrates and nitrites are present in different forms, but usually are white or crystalline powder (US EPA, 2007). These chemicals play a significant role in the nutrition and function of plants. Nitrates are formed by oxidation of organic waste by the action of nitrogen-binding bacteria (HAH, 2014). Nitrate is an important component of vegetables due to its potential for accumulation that can affect many biotic and abiotic factors (about 80% of nitrate in human nutrition comes from vegetables). Elevated levels of nitrate are usually found in leaves, while in small amounts they are found in seeds or bulbs. Humans are exposed to nitrates via consumption of vegetables, and to a lesser extent by water or other food, and in addition, nitrates are formed endogenously (Lundberg et al., 2008; EFSA, 2008; Hord et al., 2009).

Inorganic anions are the most common pollutants found in high concentrations in drinking water. In addition, there is always a need to find new and eco-friendly technologies to remove such pollutants. Elevated nitrate concentrations in drinking water can threaten human health because nitrates are reduced to nitrites in gastrointestinal tract. Furthermore, nitrates can cause methemoglobinemia, while nitrites and nitrates have the potential to form carcinogenic *N*-nitroso compounds (Katan, 2009; Rangabhashiyam et al., 2014). However, some studies have demonstrated that inert anions ($NO_3^-$ and $NO_2^-$) can be recycled *in vivo* to form nitrogen oxide (NO), representing an important alternative source of NO to the conventional synthesis in L-arginine-NO-synthase pathway, especially in hypoxic states (Lundberg et al., 2008).

Nitrates and nitrites are naturally occurring substances in fruits and vegetables, which humans are encouraged to consume because of their beneficial health effects. On the other side, nitrates and nitrites are used as food additives such as sausages, ham and other processed meat. Such processed food is linked with gastric cancer risk in humans consuming it in high amounts (Song et al., 2015). Therefore, the objective of this

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work is to review the most common sources of nitrates and nitrites, their occurrence in food and water for human consumption, as well as explain their metabolism, and finally, to show the effect of ingested nitrate and nitrite on human health.

**Sources of nitrates and nitrites**

Nitrates and nitrites are the most common pollutants in the environment. The highest concentrations of these compounds have been found in areas of intensive agricultural production where they are accumulated in the soil and groundwater. By using nitrogen fertilizers, nitrates are irrigated from the groundwater because the nitrate ions do not bind to the soil adsorption complex and are prone to rinsing from the soil to groundwater (Sofilić, 2014). However, the total contamination of water with nitrates, contributes the fecal water from septic tanks and inadequately built sewage systems, the application of organic fertilizers and atmospheric deposition (Filipović et al., 2013).

*Nitrates and nitrites in food*

Plant species that can accumulate nitrates include Brassica plants, are green cereals grains (oats, wheat, rye and maize), sorghum and Sudan grass, corn, beets, sweet clover and raspberry (Wrathall, 2002).
Vegetables and cured meat products are the largest sources of nitrates and nitrites in food, but small amounts may be present in fish and dairy products. Meat products may contain <2.7-945 mg nitrate/kg and <0.2-6.4 mg nitrite/kg; dairy products usually contain <3-27 mg nitrate/kg and <0.2-1.7 mg nitrite/kg. Food processing, use of fertilizers and growth conditions (especially soil temperature and daytime light intensity), influence on the quantity of nitrate in vegetables. Crops such as beetroot, green salad, radish and spinach often contain nitrates at concentrations above 2500 mg/kg, especially when grown in greenhouses. The amount of nitrite in food is very low (generally below 10 mg/kg) and rarely above 100 mg/kg. An exception is noticed in vegetables that are damaged, inadequately stored or stored for a long time, as well as grilled or fermented vegetables. Under such conditions, up to 400 mg/kg nitrite was determined (WHO, 2011). The main intake estimates for nitrate and nitrite in the United States (US) and Europe differ by researcher, but are comparable. The international estimates of nitrate intakes from food is 31-185 mg/day in Europe and ≈40-100 mg/day in the US. Nitrate intake from foods other than vegetables, drinking water and cured meat products, has been estimated to be about 35-44 mg/person/ day for a human with 60 kg (Hord et al., 2009).
Nitrites are often added to meat products as an antimicrobial factor against *Clostridium botulinum* bacteria, and to obtain a characteristic red-pink color of meat (Abid et al., 2014). In the European Union, only certain processed foods (meat products) may contain added nitrites (European Council, 2011) in amounts up to 150 mg/kg (maximum amount that can be added during production). The maximum permissible concentration (MPC) for nitrites in "traditional" meat products is 50-175 mg/kg, and for nitrates 10-300 mg/kg (without added nitrite). Nitrates are also allowed in hard, semi-hard and semi-sweet cheeses, and MPC for cheeses is up to 150 mg/kg (HAH, 2014). Nitrates and nitrites from processed meat and smoked cheeses can lead to the formation of *N*-nitroso compounds that occur when the nitrogen reacts with secondary amines and *N*-alkylamides, a process that is primarily carried out endogenously. The International Agency for Research on Cancer concluded that nitrates and nitrates ingested under conditions that cause endogenous nitrosation are "probable human carcinogens (2A)". However, the formation of *N*-nitroso compounds can be changed by other nutritional factors. Heme iron, of which red meats are a rich source, can act as a catalyst in the formation of *N*-nitroso compounds in the gut (Abid et al., 2014).
In the NIH-AARP study (nutrition and health study developed at the National Cancer Institute, USA), the intake of nitrates and nitrites from processed meat products was associated with stomach cancer, esophagus, bladder, pancreas, thyroid (only in men), prostate and ovarian cancer, as well as with the death caused by chronic liver disease (Abid et al., 2014). The International Agency for Research on Cancer (IARC), clas-

sified processed meat as carcinogenic hazard to humans (Group 1), with the formation of carcinogenic nitrosamines as contributing factor (EFSA, 2017a).

In order to improve the health status by vegetable consumption, certain measures should be taken to reduce exposure to nitrates and nitrites, but to maintain the recommended amount of vegetables. Also, the excessive use of nitrogen fertilizers should be avoided as nitrates are incorporated into soil and then into vegetables. Vegetables should be stored and processed to prevent contamination caused by bacteria, and thus reduction of nitrates to nitrites. Removal of stems result in a reduction of nitrate content by 30-40% in lettuce and spinach. Peeling of potatoes and beetroot decreases the nitrate content by 20-62%. By cooking peas, cabbage, beans, carrots, potatoes, spinach and endives in water, nitrate levels decrease by 16-79% (Chan, 2011). Hord et al. (2009) conducted nitrate and nitrite analysis on a convenience sample of vegetables, fruit, vegetables and fresh and processed meats shown in Table 1.

**Table 1.** Nitrate and nitrite contents of a convenience sample of some fruits, vegetables and eat products* (Hord et al., 2009)

| | Nitrates | Nitrites |
|---|---|---|
| | (mg/100 g) | |
| **Fruits** | | |
| Apple sauce | 0.3 | 0.008 |
| Banana | 4.5 | 0.009 |
| Fruit mix | 0.9 | 0.08 |
| Orange | 0.8 | 0.02 |
| **Vegetables** | | |
| Broccoli | 39.5 | 0.07 |
| Carrots | 0.1 | 0.006 |
| Cole slaw | 55.9 | 0.07 |
| French fries | 2.0 | 0.17 |
| Ketchup | 0.1 | 0.13 |
| Mustard greens | 116.0 | 0.003 |
| Salad mix | 82.1 | 0.13 |
| Spinach | 741 | 0.02 |
| Tomato | 39.2 | 0.03 |
| Vegetable soup | 20.9 | 0.001 |
| Desiccated vegetable dietary supplement** | 27,890 | 10.5 |
| **Meats/processed meats** | | |
| Bacon | 5.5 | 0.38 |
| Bacon, nitrite-free | 3.0 | 0.68 |
| Ham | 0.9 | 0.89 |
| Hot dog | 9.0 | 0.05 |
| Pork tenderloin | 3.3 | 0 |

*Nitrate and nitrite concentrations were quantified by ion exchange chromatography (ENO 20 Analyzer; Eicom, Kyoto, Japan). Analysis of food reflects the mean value from triplicate or quadruplicate analysis.

**Nature's Way Garden Veggies (1 capsule; 900 mg desiccated vegetables; Nature's Way Products Inc, Springville, UT)

L'hirondel et al. (2006) stated that although the MPC in the United States for nitrate in drinking water is 45 mg/L, nitrate concentration in vegetables can be 50 times higher (vegetables often contain between 2000-3000 mg of nitrate per kilogram). However, vegetables rich in nitrates are good for health. The authors pointed out that the intake of dietary nitrates have a lower tendency to increase nitrosation due to the presence of nitrosation inhibitors in vegetables.

*Nitrates and nitrites in drinking water*

Determination of nitrogen in the form of ammonia, nitrate or nitrites is very important because these compounds are generated by the decomposition of organic substances (proteins, urea, etc.) by spontaneous chemical reactions or by bacterial action.

In the last few years nitrate contamination of groundwater is an increasing problem all over the world. The highest nitrate concentrations in groundwater were recorded in areas of intensive agricultural production (Filipović et al., 2013). Natural levels of nitrate in groundwater depend on the type of soil and geology. Bacteria from the soil translate various forms of nitrogen into nitrate, which is a desirable process, because most of the nitrogen used by plants is absorbed in the form of nitrate. However, nitrate are highly leachable and easily moves with water through the soil profile. If the precipitation is strong, or if irrigation is frequent and abundant, the nitrate will leach below the plant's root zone and eventually reach groundwater (Oram, 2015). In Croatia, the limit value of nitrate in groundwater, prescribed by the Ordinance on compliance parameters and methods of analysis of water for human consumption (Ministry of Health of the Republic of Croatia, 2013), is 50 mg/L expressed as $NO_3^-$, which is also the limit value prescribed by the Nitrates Directive in the EU (European Commission, 1991). In the United States, nitrate concentrations are in range from 4 to 9 mg/L, while agricultural activities can result in increased levels (up to 100 mg/L). Drinking water rich in nitrates is associated with private wells, especially shallow wells (<15 m deep) in areas with permeable soils (Fewtrell, 2014).

Under aerobic conditions, nitrate can leach in relatively large amounts in the aquifer when there is no plant material absorbing the nitrate. Degradation or denitrification occurs only in a small extent in the soil and rocks forming the aquifer. Under anaerobic conditions, nitrate can be completely degraded to nitrogen. The presence of high or low groundwater level, the quantity of rainwater, the presence of other organic material with different physico-chemical characteristics are important in determining the fate of nitrate in the soil. In surface waters, nitrification and denitrification may also occur, depending on the temperature and pH. However, nitrate absorption by plants is the most common reason of nitrate reduction in surface waters (WHO, 2011).

*Nitrates and nitrites in the body*

Although nitrate and nitrite are present in food and therefore their intake into the body is unavoidable, their excessive amount in the body can cause harmful effects on health.

However, the metabolism of nitrates in the human body should be emphasized. Namely, nitrate in saliva (not in food), are converted to nitrite by oral microflora. In fact, plasma nitrate are extracted by the salivary glands and excreted in high concentrations in saliva. Healthy adults have a salivary conversion of nitrate to nitrite of 5 to 7% of the total nitrate intake, while infants and patients with gastroenteritis, who have a higher pH, can have a greater conversion rate (Alexander at al., 2008).

The primary harmful effect to human health from drinking water with nitrate nitrogen occurs when nitrate is converted to nitrite in the digestive system (Oram, 2015).

Nitrate and nitrite in humans are present due to diet or are produced by the action of endogenous L-arginine-NO synthase. Nitric oxide, generated by NOS (NO synthase) enzymes, is oxidized in the blood and tissue to form nitrate and nitrite. The reaction of NO with oxyhemoglobin produces nitrate and methemoglobin, while the oxidation of NO forms nitrite, a process catalyzed in plasma by the multi-copper oxidase and NO oxidase ceruloplasmin. The level of nitrate is considerably lower in populations lacking ceruloplasmin. The same was confirmed in experi-

mental mice. Regular exercise increases the expression and activity of endothelial NOS (eNOS) which results in higher circulating level of nitrate (Lundberg et al., 2008).
Lundberg et al. (2006) gave an overview of studies based on the correlation between fruit and vegetable intake and cardiovascular disease. It is supposed that a large intake of fruits and vegetables reduce the risk of coronary heart disease and stroke. Namely, this group of authors propose that the high content of inorganic nitrate is a major factor related to the positive health effects of certain vegetables via bioconversion to nitrite, nitric oxide, and other secondary reaction products (nitroso/nitrosyl compounds), all of which may have positive effect on the cardiovascular system. Although toxicological studies in rats have not confirmed that nitrate or nitrite are carcinogenic, these considerations have not diminished the public's concerns about current amounts in drinking water and food. However, numerous papers indicate that nitrite is the key physiological regulator of hypoxic vasodilation and mitochondrial respiration (Lunberg et al., 2006).

**Metabolism of nitrate and nitrite in humans**

There are two major sources of nitrate in human body. One is endogenous NO formation, which is spontaneously oxidized in blood and tissues forming nitrite, and the other is the diet. In the diet, nitrate exists mostly in the form of precursor nitrate. Only a small portion is taken up directly as nitrite via ingestion, for example, cured meat products (bacon and sausages), where it is used as a food preservative in combination with vitamin C (Lundberg et al., 2006).
Understanding the risk assessment of dietary nitrate and nitrite does not only imply description of the intake and removal of these ions, but also knowledge of their distribution in the body. The action of oxyhemoglobin in oxidizing nitrite to nitrate, maintains blood nitrite concentrations at very low levels. Hence, endogenous nitrosation is most likely to occur in the gastrointestinal lumen (Schultz et al., 1985).
The metabolism of inhaled, injected and endogenously formed NO, $NO_2$, $N_2O_3$, $NO_2^-$ and $NO_3^-$ in blood depends on diffusion, convection, distribution coefficient and chemical reactions between different compartments of human blood (Kelm, 1999).
Nitrates are rapidly absorbed in the stomach and small intestine. Orally administered nitrate salt reaches a peak plasma concentration within 1 hour. Approximately 25% of peroral nitrate is concentrated and excreted via the salivary glands, so that the concentration of salivary nitrate is about ten times that of plasma. Part of this salivary nitrate is reduced by facultative anaerobic bacteria found on the surface of the tongue. These bacteria use nitrate in the absence of oxygen and act as terminal electron acceptor and produce nitrite as a byproduct. It is important to note that the concentration of nitrite in the saliva is 1000 times higher than in plasma in fasting state. Furthermore, a small amount of the nitrite is reduced to NO by bacteria or peridontal acidity, but most will be swallowed and will react with stomach acid to form a complex mix of nitrogen oxides, including nitrous acid, nitrogen dioxide, dinitrogen trioxide and nitrogen oxide (EFSA, 2008; Gilchrist et al., 2010).
Ingested nitrate is absorbed from the small intestine into the circulatory system. Blood nitrate is either concentrated in the saliva glands where it is reduced to nitrite or removed by the kidney and excreted in the urine. Sodium nitrite is absorbed unchanged from the stomach of rats and mice. In blood, nitrite is rapidly and irreversibly oxidized to nitrate, which is then eliminated by excretion. Nitrite is unstable in acidic media and is spontaneously decomposes to nitrate and nitrogen dioxide. In acidic conditions and in the presence of food, nitrite disappeared with a half-life of 2.2 h and pH 4.5; 0.93 h and pH 3.5 and 0.42 h at pH 2.5. There is no data on the absorption, distribution, metabolism or excretion of sodium nitrite in humans (US Department of Health and Human Services, 2001).
Wagner et al. (1983) investigated the fate of nitrate (3.5 mmol $^{15}NO_3^-$) in 12 healthy young adults. Samples of urine, saliva, plasma and feces were collected over a period of 48 hours. The subjects received either 60 mg of ascorbic acid, 2 g of ascorbic acid, or 2 g of sodium ascorbate per day. About 60% of $^{15}NO_3^-$ appeared in urine as nitrate within 48 hours. Less than 0.1% appeared in the feces. The $^{15}N$ label

of nitrate was found in urine (3%) and feces (0.2%) in the form of ammonia or urea. The fate of the remaining 35% $^{15}NO_3^-$ is unknown. Also, the effect of ascorbic acid or sodium ascorbate on the level of nitrate and nitrite in plasma, saliva, urine or feces was not observed. Wagner et al. (1983) stated that the diet is not the only source of nitrate, but that nitrate is endogenously synthesized. Therefore, not the total amount of nitrate excreted in the urine is from food, although during the period of high nitrogen intake, the contribution of endogenous nitrate biosynthesis to urinary nitrate is small.

*Toxicity of nitrate and nitrite*

Concerns about nitrate emerged in 1940s when first report on infant methemoglobinemia ('blue child syndrome') was associated with high concentrations of nitrate in well water. Case studies of methemoglobinemia showed that such cases were rare when the concentration of nitrates in well water was below 44 mg/L. Nitrate alone is not the primary cause, and without bacterial contamination it is unlikely that nitrates will cause methemoglobinemia, therefore, a limit of 45 to 50 mg/L in drinking water is not necessary (Lundberg et al., 2008; Gilchrist et al., 2010).

In 2017, EFSA updated and improved the exposure assessment to dietary nitrate and nitrite used as food additives. Experts evaluated that the exposure to nitrates from food additives was less than 5% and did not exceed the safe levels. Unfortunately, the safe levels could be exceeded (for individuals if all sources of nitrates are considered, such as food additives, nitrates naturally occurring in foods, etc.), especially for individuals of all age groups. The exposure to nitrates used as food additives is higher for children, who might also exceed the ADI (EFSA, 2017a).

*Acute toxicity*

The primary health hazard of drinking water with an elevated nitrate concentration occurs when nitrate is transformed into nitrite in the digestive system. The nitrate ion oxidizes iron in hemoglobin of red blood cells forming methemoglobin that has no ability to transmit oxygen to cells in the body, resulting in blue color of the skin. The potential risk of cancer due to nitrates exists when nitrates react with amines in the body and thus produce nitrosamines that are known to be carcinogens. In this case, the nitrates must be converted into nitrites before nitrosamine is formed (Oram, 2015). Some studies confirmed the concern about cancer-causing potential of nitrates and nitrites used as preservatives and color-enhancing agents in meats. Elevated risk of non-Hodgkin's lymphoma and cancers of the esophagus, bladder, colon and thyroid have been reported. Additionally, an increase of stomach cancer was observed in workers manipulating with nitrate fertilizers. However, epidemiological and human toxicological studies have not shown and unambiguous relation between nitrate intake and the risk of cancer (ATSDR, 2013).

Plants absorb nitrates converted into nitrite by nitrate reductase and are incorporated into amino acids and proteins. Genetic or environmental factors that interfere with or inhibit the function of nitrate reductase, allow for nitrate to accumulate in the plant. In response to stress conditions, certain types of sorghum species reduced the activity of reductase. Drought and conditions with reduced sunlight will also reduce the activity of the nitrate reductase system. Despite the decrease in activity, the plants continuously absorb nitrate and accumulate them in abnormally high amounts. Ruminants, by consuming such plants, convert nitrate to nitrite and finally to ammonia. The nitrate toxicity is a function of the amount of consumed nitrate. When an animal consumes conventional feed with a large amount of nitrate, the conversion of nitrite to ammonia becomes a limiting factor that allows the accumulation of nitrite in toxic amounts. Toxicity occurs by absorption of nitrite into the blood that oxidizes iron to hemoglobin from ferrous (+2) to ferric (+3) state. The resulting methemoglobin has a low affinity for oxygen transfer capacity of red blood cells. Death due to anoxia may occur if 70-80% of hemoglobin is converted to methemoglobin (Wrathall, 2002). In Table 2 some methemoglobin-inducing substances are listed.

**Table 2.** Substances that promote the formation of methemoglobin (Knobeloch et al., 2000)

| **Substance** | **Utilization** |
|---|---|
| Aniline dyes | Laundry inks, markers |
| Benzocaine, lidocaine | Local anesthetics |
| Chlorates | Matches |
| Isobutyl nitrite | Roomdeodorizers |
| Naphtalene | Moth balls |
| Nitrate/nitrite | Drinking water, fruits, vegetables, cured meats |
| Nitric oxide | Inhalant used to treat pulmonary hypertension in newborns |
| Nitrobenzene | Metal cleaners |
| Nitroethane | Nail care products |
| Nitrogen oxides | Auto emissions, wood smoke, gas-burning appliances |
| Nitroglycerine | Angina drug, explosives |
| Resorcinol | Antipruritic, over-the-counter medications |
| Sodium nitrite | Pickling salts, boiler conditioners, cleaning solutions |
| Sulfonamides | Antibiotics |

Intoxication of nitrate is caused by the consumption of groundwater with high concentration of nitrate. Recorded lethal doses of nitrate ions for humans range from 67 to 833 mg of nitrate ions per kg of body weight. Toxic amounts - with the formation of methemoglobin as a criterion for toxicity - ranged from 33 to 350 mg of nitrate ions/kg of body weight. The oral lethal dose for humans is estimated to vary from 33 to 250 mg of nitrate ions per kg of body weight (lower doses are applicable for children and elderly). Doses of 1 to 8.3 mg of nitrate ions per kg of body weight lead to the induction of methemoglobinemia. Comparison of these results for humans with the results for experimental animals, shows that nitrite toxicity is similar for humans and experimental animals (Table 3). Compared to humans, rats are 10 to 100 times less sensitive to nitrate because they do not have mechanism to convert nitrate to nitrite (Boink and Speijers, 2001).

**Table 3.** Toxicity of nitrate/nitrite in rats and humans (Boink and Speijers, 2001)
*Chronic toxicity*

| | Lowest observed effect level: nitrate |
|---|---|
| Rat | 360-2000 mg/kg/day |
| Human* | 30-150 mg/kg |
| | Lowest observed effect level: nitrites |
| Rat | 10-200 mg/kg/day |
| Human* | 1-250 mg/kg |

As it is already mentioned, after nitrate is converted to nitrite in the body, it can react with amines from food thus forming nirosamines. Nitrosamine formation, however, can be inhibited by antioxidants from food (Vitamin C and E). Several long-term toxicity/carcinogenicity studies have been conducted on nitrate. The rats were given 0, 0.1, 1, 5 and 10% sodium nitrate through diet for two years, which is 0, 50, 500, 2500 and 5000 mg/ kg/body weight/day. A No observed effect level (NOEL) of 500 mg/kg/ body weight/day was determined for sodium nitrate based on a slight decrease in growth rate. No histological changes or increase in tumor frequency were observed. Furthermore, rats were given water with 0 or 0.5% sodium nitrate for 84 weeks, which is 0 and 500 mg/kg/ body weight/day, and no histopathological effects were observed at that time. Another two-year study was conducted where 0, 2.5 and 5% sodium nitrate in drinking water (0, 2500 and 5000 mg/kg body weight/day) were given to rats. This study found a no adverse effect level (NOAEL) of 2500 mg/ kg body weight/day. All studies have found low chronic toxicity of nitrate (EFSA, 2008).
L'hirondel et al. (2006) consider that the cancer risks of nitrate is unjustified because, if drinking water with 10-20 ppm of nitrate-nitrogen ($NO_3$-N) is toxic, then vegetables (with extremely high levels of nitrate) would also be toxic, in spite the presence of a known nitrosation inhibitor.

The effect of nitrite on carcinogenicity in rats was tested with nitrite in drinking water (doses were 0, 10, 100, 200 and 300 mg/kg body weight/day), where no significant differences were observed between control and treated groups for growth, mortality and total hemoglobin level. At the highest three doses, methemoglobin increased to 5, 12 and 22% and lung toxicity was observed. Focal degeneration, fibrosis of the heart and dilatation of coronary arteries were observed at the highest dose (EFSA, 2008).

*Research on humans*

An ecological study of nitrate in drinking water and non-Hodgkin lymphoma (NHL) and digestive and urinary tract cancer was conducted in the Slovak Republic. Data on nitrate in villages using public water supplies were correlated with the occurrence of cancer for the period 1986-1995. There was an elevated rate of colorectal cancer in women, as well as non-Hodgkin lymphoma in women and men. There were no indications of kidney and bladder cancer. In addition, exposure to nitrate from drinking water is associated with increased risk of colorectal cancer (EFSA, 2008).

*Acceptable daily intake*

The concept of acceptable daily intake (ADI) was defined by the Joint Expert Committee of the Food and Agriculture Organization of the United Nations/World Health Organization – JECFA for substances deliberately added to food or to contaminants (additives, residues of pesticides, etc.). Due to the lack of data on the possible effects of vegetable matrices on the bioavailability of nitrate, JECFA considered it inappropriate to compare the exposure to nitrate from vegetables with ADI. JECFA and the European Commission's Scientific Committee on Food (SCF) have set an ADI for $NO_3$ of 0 to 3.7 mg/kg body weight, which is actually 277 mg of nitrate per person (75 kg). The US Environmental Protection Agency (US EPA) has set a reference dose (RfD) for nitrate nitrogen ($N\text{-}NO_3$) of 1.6 mg/kg body weight/day, equivalent to 7 mg $NO_3$/kg body weight/day (Santamaria, 2006). In 2017, EFSA re-evaluated sodium and potassium nitrate as food additives. It was stated that even using the highest nitrate-to-nitrite conversion factor of 9% a dose corresponding to ADI of 3.7 mg/kg body weight/day will be converted to 0.25 mg nitrite ion/kg body weight/day. They recommended additional studies on humans measuring the excretion of nitrate into the saliva and its conversion to nitrites, as well as further studies on the levels of nitroso compounds formed in different meat products with known amounts o nitrates/nitrites added (EFSA, 2017b).

The European Union sets limits of 2500-3000 mg/kg of nitrates in spinach (fresh product), and 2000 mg/kg of processed product (frozen spinach). Taking into account the consumption of 2 liters of water per day and 100 g of vegetables per day, the total nitrate intake can vary between 200 and 400 mg. The statistical model of exposure has shown that ADI is usually exceeded by 15% and in children even by 45% (Boink and Speijers, 2001).

**Conclusions**

High levels of nitrate in drinking water and fruits and vegetables are frequently reported. The potential risk of nitrate poisoning is from its conversion to nitrite before and after ingestion. For prevention of unnecessary exposure to nitrate, regulatory standards and advisory levels have been established. Some efforts have been made in order to protect consumers from poisoning, for example, monitoring the water quality helps to ensure that nitrate levels are known, taking measures to reduce nitrate and nitrite from vegetables in order to maintain the recommended intake of fruits and vegetables (reduction of nitrogen fertilizers, appropriate storage and preparation of fruits and vegetables, etc.), and education and outreach to the public. The ADI concept gives a very high degree of protection to the consumers against exposition to nitrate and nitrite, except if high nitrate amount in vegetables or drinking water is exceeded over a longer period of time. Regarding this, it is highly desirable to produce and consume foods with reduced amounts of nitrate.

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## NITRATI I NITRITI, METABOLIZAM I TOKSIČNOST

*Pregledni rad*

**Sažetak**

Nitrati i nitriti su kemijski spojevi koji se koriste kao gnojivo, rodenticidi ili konzervansi. Mogu se naći u zraku, tlu, vodi ili hrani (posebice povrću) a mogu se i stvarati u ljudskom tijelu. Nitrati imaju važnu ulogu u hranjenju i metabolizmu biljaka. Nastaju oksidacijom organskog otpada djelovanjem dušičnih bakterija. Ljudi mogu biti izloženi nitratima i nitritima preko konzumacije povrća i mesnih prerađevina, a manjim dijelom preko vode ili ostale hrane. Pijenje vode sa većom koncentracijom nitrata može djelovati na ljudsko tijelo na dva načina: (i) akutno, što se najčešće manifestira methemoglobinemijom (nitrati u probavnom sustavu se reduciraju u nitrite koji potom oksidiraju željezo u hemoglobinu crvenih krvnih stanica stvarajući methemoglobin koji nije sposoban prenositi kisik po tijelu), a koja se manifestira pojavom plave boje kožw, i (ii) kronično, što se manifestira pojavom karcinoma kao rezultata izloženosti nitrozaminima (koji nastaju tijekom reakcije nitrata sa aminima u tijelu).

*Ključne riječi: nitrati, nitriti, toksikolokogija*

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# NUTRITIONAL PROPERTIES OF VIRGIN OLIVE OIL WITH EMPHASIS ON PHENOLIC COMPOUNDS

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*Review*

**Summary**
Foods have been used historically, across cultures and for centuries, in treating disease and curing illness, owing to the role of antioxidants found naturally in foods. Also, it is known that in the process of historical development, virgin olive oil with a high content of antioxidants was consumed not only as food but also was used as a therapeutic agent in the treatment of various diseases, applied by rubbing onto the skin. Olives and the oil extracted from them by physical means are key nutrients in the Mediterranean diet pattern. They contain high amounts of bioactive compounds (polyphenols, tocopherols, phospholipids, carotenoids, chlorophylls, sterols and squalene) and possess high antioxidant potential. The supplementation of diets with virgin olive oil positively affects human health. The main objective of this review is to describe the physicochemical and nutritional characteristics of virgin olive oil and the influence of its physiologically active compounds, with special emphasis on the effect of phenolic compounds on human health.

*Keywords*: bioactive compounds, public health, promoting the consumption of virgin olive oil

## Introduction

Across centuries and civilisations, many fruits and vegetables and other plant foods have been used, recommended and/or avoided for their supposed medicinal properties. These types of foods, which have their own antioxidant properties, are essential for health and wellbeing and play an important role in treating/preventing disease. Olives and virgin olive oil (VOO) are chief components of the Mediterranean diet and they contain many compounds useful for human health. Olive fruit, which is particularly interesting, contains over 230 antioxidant compounds. As olive oil is made from olive fruit, it also contains a lot of these compounds. The best olive oil is extra virgin olive oil (EVOO), which is produced by the first pressing or by other physical processes under conditions that do not lead to changes in the oil, which has not undergone any process other than washing, decantation, centrifugation or filtration. It is considered to be high-quality oil for health and nutrition. Depending on its chemical and organoleptic properties, olive oil is classified into different grades that also serve as guidelines for the consumer in the choice of the preferred kind of oil. Based on Trade Standard applying to Olive Oils and Olive -Pomace Oils (International Olive Council, 2016), virgin olive oils are the oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decanting, centrifuging and filtration. The aim of this review is to reveal the multiple nutritional properties of VOO, and the correlation between polyphenols and other bioactive compounds, and their antioxidant activities.

*Nutritional properties of virgin olive oil*

The olive flesh components are passed on to the oil, which mainly consists of two components, namely saponifiables and unsaponifiables. The former, comprising triacylglycerols, partial glycerides, esters of fatty acids or free fatty acids and phosphatides, represent nearly 98% of the oil's chemical composition, while the latter, consisting of mainly minor components, account

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for 1%-2% of the oil composition (Viola and Viola, 2009). In particular, oleic acid (18:1 n-9) ranges from 56% to 84% of total fatty acids, while linoleic acid (18:2 n-6), the major essential fatty acid and the most abundant polyunsaturate in our diet, is present in concentrations between 3% and 21% (Tiscornia et al., 1982). The nutritional value and health functions of VOO are ascribed to the presence of large amounts of monounsaturated fatty acids (MUFAs) such as oleic acid and valuable minor components, including aliphatic and triterpenic alcohols, sterols (mainly β-sitosterol), hydrocarbons (squalene), volatile compounds, tocopherols (chiefly α-tocopherol), pigments such as chlorophylls, carotenoids (β-carotene and lutein) and antioxidants (Ghanbari et al., 2012).

In addition to triglycerols and free fatty acids, olive oil contains a variety of nonsaponifiable nonglyceridic compounds that add up to 0.5%-2% of the oil and are important for its oxidative stability and unique flavour and taste (Lee et al., 2006; Pellegrini et al., 2001). Also, the composition of the bioactive compounds is affected by many agronomical and technological factors. Factors affecting the bioactive compounds content of olive oil are cultivar, climate, ripeness of the olives at harvesting and the processing system for the type of olive oil (Cimato et al., 1992).

There is an increasing interest in the phenolic compounds in olive oil due to their biological properties (Ross and Kasum, 2012). Polyphenols are plant secondary metabolites present in all plant tissues and their primary role is to protect plants from insects, ultraviolet radiation and microbial infections, and to attract pollinators (Del Rio et al, 2013). At least 30 phenolic compounds belonging to the hydrophilic group have been detected in olive oil (Tuck and Hayyball, 2002).

Low phenolic content of common olive oil ranges from 10 to 70 mg/kg, while high phenolic content of VOO ranges from 150 to 400 mg/kg. Phenolic compounds are at the top of antioxidant substances naturally present in VOO (Boskou, 2009a). Phenolic compounds of VOO belong to different classes: phenolic acids, phenyl ethyl alcohols, hydroxy-isochromans, flavonoids, lignans and secoiridoids, which are the main compounds of the phenolic fraction of *Oleaceae* plants (Boskou, 2009b).

Beside polyphenols, other bioactive compounds in olive oil are oleuropein, squalene, tyrosol, lignanes, hydroxytyrosol (3,4-dihydroxyphenylethanol), caffeic acid, phytosterols, vanillic acid, flavonoids, syringic acid, rutin, protocatechuic acid, leuteolin and p-hydroxyphenylacetic acid (Ghanabari et al., 2012). Such components also contribute to the unique flavour and taste of olive oil.

*Health benefits of virgin olive oil consumption*

The benefits of consuming olive oil have been known since antiquity. Olive oil was used not only for cooking/lighting but also as medicine, mainly as the basic ingredient for ointments (Efe et al., 2011). It has been the subject of several studies around the world, especially with regard to reporting its role in human nutrition, given its strong physiological effects on human health. However, it is now well established that health benefits are not attributed just to oleic acid but also to the phenolic fraction of olive oil with its anti-oxidant, anti-inflammatory and anti-microbial activities (Martín-Peláez et al., 2013).

The role of antioxidants, naturally occurring in foods and taken into the human body through diet, in the treatment of diseases and in disease prevention is one of the subjects of research (Howard and Kritchevsky, 1999; Visioli et al., 2000). Bioactive compounds which have antioxidant characteristics are put forward as the main reasons for a lower incidence rate of cholesterol and cardiovascular disease, atherosclerosis, neurodegenerative diseases, certain types of cancer, blood pressure in hypertensive patients, rheumatoid arthritis and improved cerebral and visual function among populations which have the habit of regular virgin olive oil consumption (Kiortsis and Simos, 2014; Khalatbary, 2013; Martín-Peláez et al., 2013; Bach-Faig et al., 2011; Cicerale et al., 2009).

In their studies, Owen and co-workers (2000a, 2000b, 2000c) stated that at high rates squalene and oleic acid as well as other phenolic compounds in olive oil have protective effects against bowel, breast and skin cancers, coronary heart disease, oxidative stress and aging and they highly recommended the consumption of VOO.

*Health-related properties of phenolic compounds in virgin olive oil*

It has been found that a linear relationship exists between the phenolic content and oxidative stability of olive oil. Polar phenolic compounds, found in small amounts in olive oil, are important ingredients because they affect both the stability and the biological properties of the oil (Visioli et al., 2004).

The major phenolic compounds in olive oil are: (1) simple phenols (e.g., hydroxytyrosol, tyrosol, vanillic acid); (2) secoiridoids: oleuropein glucoside, and SIDs which are the dialdehydic form of oleuropein (SID-1) and ligstroside (SID-2) lacking a carboxymethyl group, and the aglycone form of oleuropein glucoside (SID-3) and ligstroside (SID-4); and (3) polyphenols: lignans (e.g., (+)-pinoresinol and (+)-1-acetoxypinoresinol) and flavonols (Owen, 2000a; Covas et al., 2006). Tyrosol and hydroxytyrosol are the most representative phenolics of olives and olive oil where they occur as such or in the form of esters of the secoiridoid elenolic acid. Tyrosol, hydroxytyrosol, and their secoiridoid derivatives make up around 90% of the total phenolic content of a VOO.

The phenolic compounds have been widely studied for their effects on coronary heart disease, specifically for their ability to reduce blood pressure and LDL cholesterol. Also, the Mediterranean Diet with extra-VOO significantly reduced the risk of atrial fibrillation, and related morbidity and mortality (Lou-Bonifante et al.;2012, Martínez-González et al., 2014)

Polyphenols isolated from olive oil include oleuropein and hydroxytyrosol which have displayed antioxidant activity in *in vitro* models of LDL oxidation. In addition, α-tocopherol has antioxidant activity that protects against LDL oxidation. Consumption of phenols-rich olive oil as a source of fat provides additional benefits not just against the cardiovascular risk profile but also against oxidative DNA damage (Martín-Peláez et al., 2013).

New evidence suggests that phenolic compounds found in extra-VOO have a role in cancer prevention. So, olive oil consumption has benefits for colon and breast cancer prevention. Antioxidant phenolic compounds present in olive oil are potent inhibitors that act directly in the colon to reduce oxidative damage (Martín-Peláez et al., 2013; Waterman and Lockwood, 2007).

Phenolic compounds have been also demonstrated to inhibit or delay the rate of growth bacteria because oleuropein, hydroxytyrosol and tyrosol have antimicrobial effects against several bacterial strains which are causal agents of intestinal or respiratory tract infections in humans (such as *Salmonella typhi, Vibrio parahaemolyticus, Vibrio cholerae, Staphylococcus aureus and Moraxella catarrhalis*) (Waterman and Lockwood, 2007).

Studies (human, animal, *in vivo* and *in vitro*) have demonstrated that olive oil phenolic compounds have positive effects on certain physiological parameters (Cicerale et al., 2010).

**Conclusions**

After summarizing the current knowledge on the phenolic compounds of virgin olive oils, this paper gives an overview of scientific literature available on multiple nutritional and pharmacological properties of virgin olive oil. The consumption of virgin olive oil may deliver greater health benefits through the supply of natural antioxidants for its high antioxidant potential, so the supplementation of diets with virgin olive oil is important.

In sum, biologically active metabolites, in particular those derived from virgin olive oil, possess high antioxidant potential. Therefore, continuous efforts should be made to promote the consumption of virgin olive oil and raise awareness of its public health benefits. This was the purpose of this review. Knowing that olive oil contains high amounts of bioactive components will also be useful to consumers in planning rich antioxidant diets, and to nutritionists in estimating the daily intakes of phenolic antioxidants and their impact on health.

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# BREAD AS INDICATOR OF AGE-CHANGING DIETARY HABITS AMONG YOUNG PEOPLE

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*Professional paper*

**Summary**
Recognized as a staple food consumed in large quantities in the Balkans region, bread is suitable for the investigation of dietary habits. This survey was conducted to gain insight into the dietary habits, related to intake and knowledge about various bread types, of the young population (14 to 26 years of age). The total number of surveyed respondents was 173 (37% males and 63% females). They were randomly selected among students from an elementary school (30), high school (45) and university (98) in the Novi Sad Municipality. A correlation between the respondents' age and the practice of "healthy food" intake was observed, as whole wheat bread consumption increased from 15% in elementary school to 26% among high school and university students. Considering gender, whole wheat bread intake among female respondents was 9% higher in comparison with male respondents. Still, white bread was most commonly consumed by all respondents (56%-69%), as a result of a habit adopted in the family. However, the obtained results indicate a strong correlation between age-changing dietary habits ("healthy" food awareness) and the impact of the environment rather than the impact of the family. Namely, the greater impact of family and tradition is noticed among elementary school pupils (54%) while among high school and university students the corresponding impact was significantly less pronounced (34%).

*Keywords*: bread intake, dietary habits, healthy diet, young population, whole wheat bread

## Introduction

Throughout the school-age years, an adequate food and nutrient intake to support health as well as brain development and function is critical. Healthful dietary habits formed during childhood are associated with the prevention of diseases of modern-day society (obesity, type 2 diabetes, cardiovascular diseases, obstipation and cancer) and with positive health outcomes in the later life stages (Hilger et al., 2017; Meşe et al., 2017). Changes in dietary habits, coupled with insufficient physical activity, have a great influence on the increased prevalence of disease development (Lupi et al., 2015). Profound changes in dietary habits can occur in different life stages but they usually appear along with changes of social environment, like the start of elementary, high-school or university education (Bagordo et al., 2013). The food choices of individuals in these life stages are affected by many factors such as newly formed habits, study pressure, social activity as well as sufficient information and knowledge about healthy food (Meşe et al., 2017). Other factors that have a major influence on populations of university students are separation from families, costs and financial resources, as well as market diversity and the availability of stores (Bagordo et al., 2013). As a consequence of adaptation to the above factors, young people often tend to negatively modify their diet in terms of fruit and vegetable consumption, diet diversity, and time and frequency of meal intake (Lupi et al., 2015). Healthy diet recommendations presented by the WHO (2016) include high intake of fruits, vegetables and whole grains combined with low intake of refined carbohydrates, saturated fats and salt. As known from the food pyramid, bread, grains or pasta are a part of every meal and one of the

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main energy sources. Bread has always been widely consumed and considered as one of the major constituents of human diet due to the high content of carbohydrates (mainly starch) as well as the presence of proteins, lipids, dietary fibres and micronutrients. Furthermore, on the growing market, breads made from different grain flours (wheat, rye, barley, oat and rice), with or without the addition of other functional ingredients, are being introduced. A partial substitution of wheat flour with whole grain flour in bread formulations is of nutritional interest considering its lower glycaemic index (GI) and content of health-related compounds such as dietary fibre, minerals, vitamins and antioxidants (Conte et al., 2016). However, in order to achieve positive health outcomes, these kinds of breads have to be available, sensory acceptable and eaten by consumers.

Since bread has been recognized as a staple food consumed daily in large quantities in Serbia, it provides a good basis for monitoring the age-changing dietary habits among the young population. The respondents' subjective assessments of their intake and knowledge regarding various bread types were captured through a survey and the responses obtained were analysed and interpreted.

## Subjects and methods

*Study population*

The total number of surveyed respondents was 173 (37% males and 63% females), ranging in age from 14 to 26. The respondents were selected by random sampling method among students from elementary schools (30), high schools (45) and the university (98) in the Novi Sad Municipality. Survey participation was voluntary and anonymous, and prior to filling in the survey, respondents were informed of the study's aims as well as that responses would be used only for research purposes and the given information would be treated as confidential. Once their willingness to participate was verified, instructions for completing the survey were given to the respondents. The basic characteristics of the studied population are presented in Table 1.

*Survey characteristics*

Bread-related dietary habits were surveyed using a questionnaire, designed by the authors, which included two major sections: demographic, social and cultural characteristics and information on bread-eating habits. The demographic data included gender and age, while social and cultural data focused on education level (elementary school, high school or university) and degree course, place of residence (town or village) and permanency of the residence (while attending school/faculty or permanent place of residence). The second section considered the existing knowledge of the respondents and the environmental aspects in order to understand their impact on bread-related dietary habits. In this section, respondents completed the questionnaire by marking or ranking the offered responses.

Ethical approval for conducting the survey was granted by the Ethical Committee of the Faculty of Technology.

**Table 1.** Basic characteristics of the studied population

| | Male | | Female | |
|---|---|---|---|---|
| | n | % | n | % |
| **Education level** | | | | |
| Elementary school | 19 | 30 | 11 | 11 |
| High school | 12 | 19 | 33 | 30 |
| University | 33 | 51 | 65 | 59 |
| **Place of residence** | | | | |
| Urban | 50 | 78 | 104 | 95.5 |
| Rural | 14 | 22 | 5 | 4.5 |
| **Total** | 64 | 37 | 109 | 63 |

*Data analysis*

The answers provided by the survey were collected in a database using Microsoft Excel 2010. Survey results were expressed in percentages (%) of the total number of respondents based on education level and gender and presented using the Statistica 13.2 software (Dell Inc.)

## Results and discussion

University students were among the majority of the surveyed population (56%), followed by high school (26%) and elementary school (18%)

students. With respect to gender, most of the respondents were female (63%). Survey results of the studied population's dietary habits related to intake and knowledge about various bread types and with regard to education level and gender are presented using figures.

Regardless of their education level, a preference for white bread was observed among all respondents since more than 55% of them reported white bread as the most commonly eaten bread type (Fig. 1a). Nevertheless, a difference of 10% in the corresponding answer was observed between respondents with a low (elementary and high school) and a high (university students) education level. The assumption is that university students are more aware of the existence of healthier bread types since 27% of them selected whole wheat bread. Furthermore, 26% of high school students reported whole wheat bread as their choice, indicating an age-related influence on food preference (Fig. 1a).

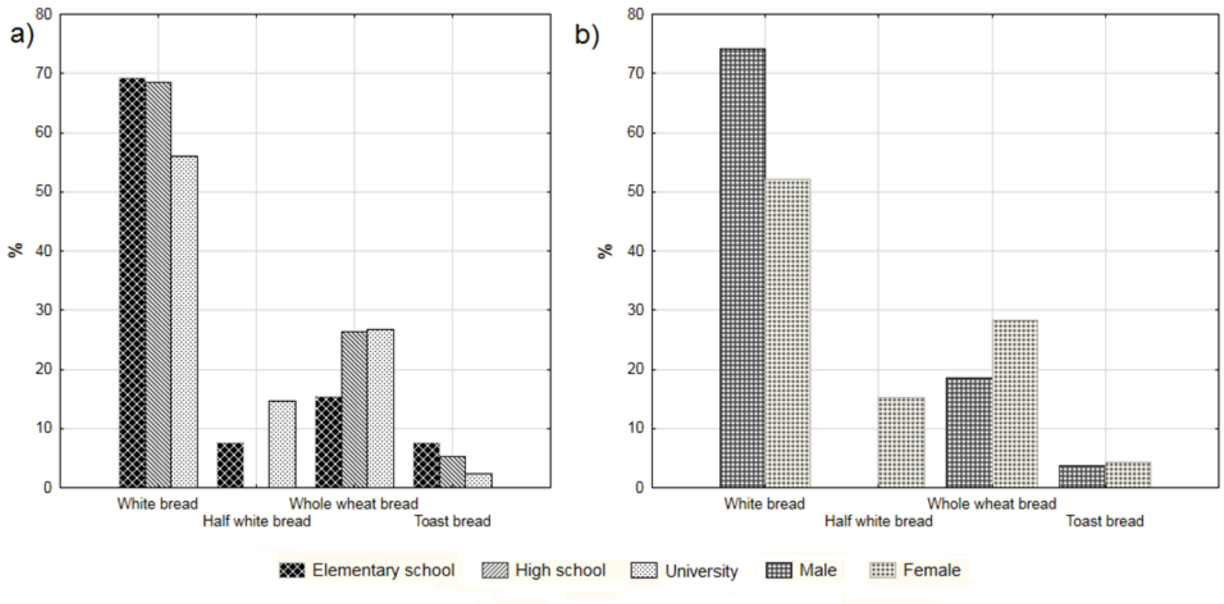


**Fig. 1.** Respondents' preference for bread type with regard to education level (a) and gender (b)

The connection between age, and thus education level, and whole grain products consumption was previously confirmed since lower whole grain intake was associated with younger age groups whereas the opposite was seen for white bread (Lang and Jebb, 2003; Sandvik et al., 2014). In the study of Simovska-Jarevska et al. (2012) conducted in Macedonia, only 20% of the surveyed population confirmed the consumption of whole grain bread, especially participants older than 50, while 76% consumed white and/or half-white bread. Looking at the consumption of bread in Turkey, the study of Meşe et al. (2017) showed that 47.8% of respondents ate white bread daily, while 47% of the respondents responded in the negative regarding whole wheat bread intake. Results reported in these studies addressing whole grain bread intake lead to the conclusion that the young population in Serbia is more aware of "healthy food" practice. Gender appeared to influence the preference of bread type (Fig. 1b). Females demonstrated to consume more whole wheat bread (28%) compared with males (19%), probably because they are better informed about the nutritional value of the corresponding bread type or simply because they are more attentive to weight control.

The results obtained concerning the impact of different factors on the respondents' choice of bread are graphically illustrated in Fig. 2. The participants were asked to rank the impact of the following factors, from the most important ($1^{st}$) to the least important ($4^{th}$): habit adopted in family, taste of bread, health benefits provided by bread consumption, and store proximity. The results indicate that "habit adopted in family" is ranked first, regardless of education level, although some differences are noted (Fig. 2a). Namely, more than 52% of elementary school and university students reported "habit adopted in family" as having the strongest influence on their bread choice, compared with only 32% of high school students. "Taste of bread", "health benefits" and "store proximity" were ranked second, third and fourth, respectively, by elementary and high school students, indicating that they are still under the influence of family and tradition.

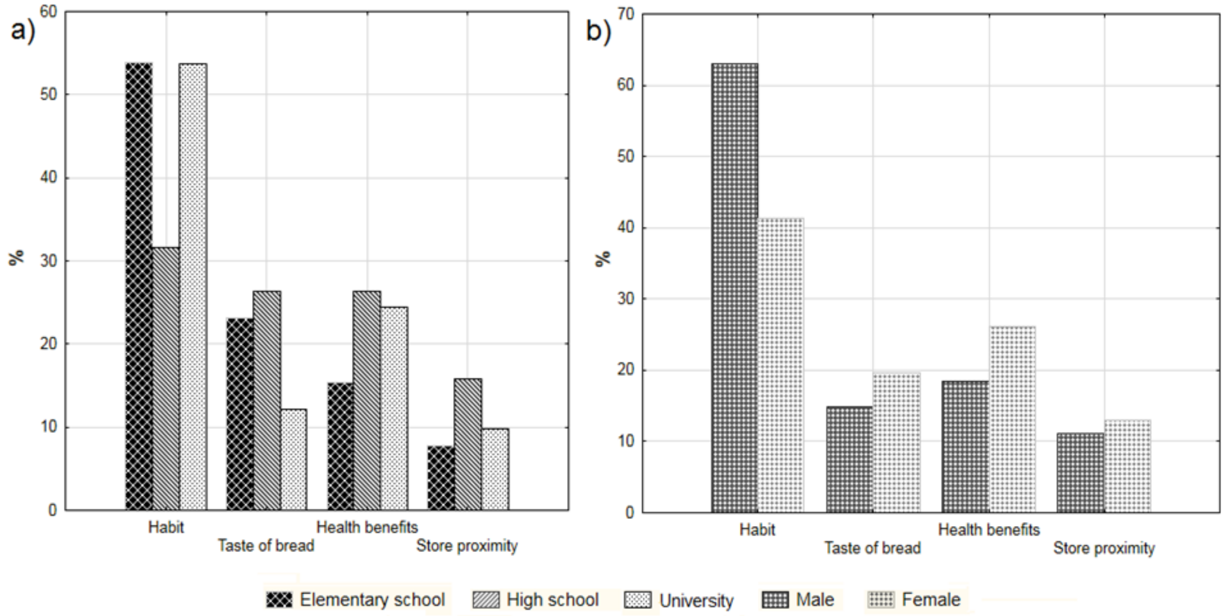


**Fig. 2.** Factors influencing respondents' bread choice with regard to education level (a) and gender (b)

"Health benefits provided by bread consumption" was ranked second by university students, and followed by "taste of bread" and "store proximity", revealing their greater familiarity with the positive outcomes provided by the consumption of different bread types. Food preferences, unconsciously and unintentionally learned and established in childhood, have been shown to be important in predicting preferences in the later life stages (Köster, 2009). Moreover, the cultural and socioeconomic environments are playing one of the primary roles in creating preferences for particular sensory experiences (Mela, 2001). Regardless of gender, the primary factor influencing the respondents' bread choice was "habit adopted in the family", followed by "health benefits", "taste of bread" and "store proximity" (Fig. 2b). However, female respondents seem to pay more attention to health benefits and the taste of the consumed bread, in comparison with their male counterparts, indicating their greater awareness.

When the question addressed the level of bread intake, 66% of university students and 53% of high school students stated that they consume 100 g of bread daily (Fig. 3a).

An equivalent percentage of elementary school students reported that they consume 100 g and 200 g of bread daily. Considering that bread is a staple food traditionally present as a part of each meal in Serbia, an unexpected result was that only 8%-16% of respondents, across all education levels (the majority being from high school), consume 300 g of bread daily (Fig. 3a). Furthermore, none of the surveyed respondents replied affirmatively about bread consumption higher than 300 g per day. These findings go against the trend regarding frequency of bread consumption reported in a recent study by Lošić and Čačić Kenjerić (2015) conducted among a young population in Bosnia and Herzegovina. A decrease in bread consumption among young people in Serbia is noticed, probably due to the greater exposure to and influence of advertisements and market trends. The observed trend is not desirable in view of the importance of bread in diet as one of the major sources of carbohydrates. Approximately two-thirds (≥55%) of the respondents, regardless of gender, consumed 100 g of bread daily (Fig. 3b). However, a slightly higher percentage of male respondents, in comparison with female respondents, consumed 200 g and 300 g of bread daily.

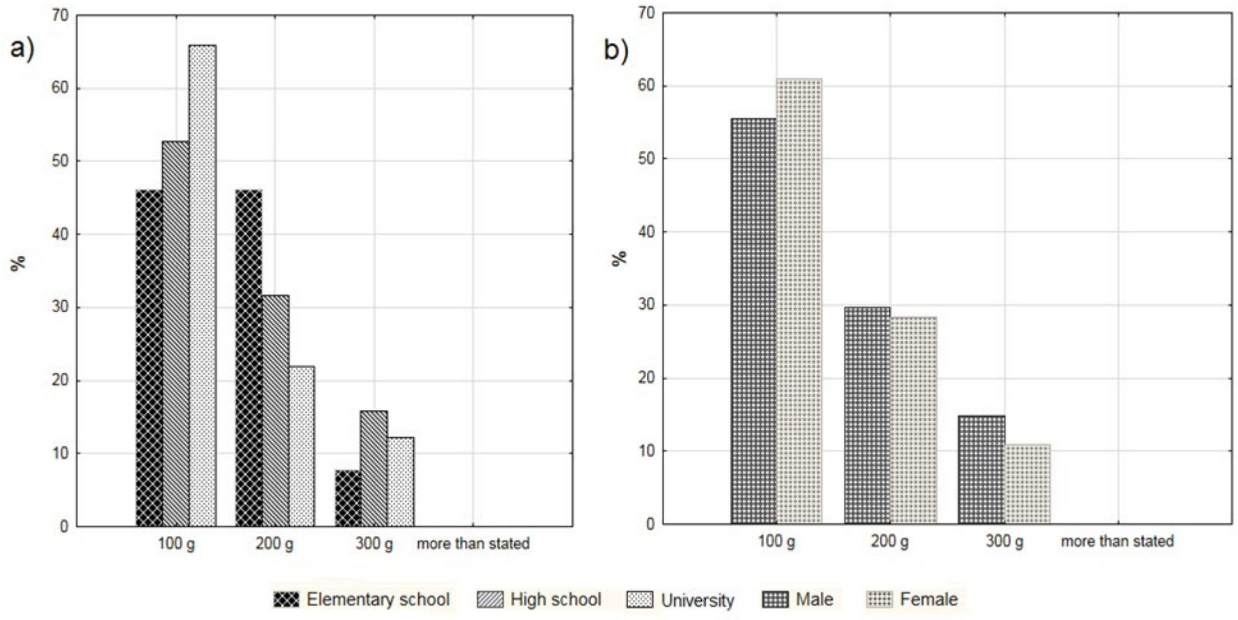


**Fig. 3.** Respondents' bread intake with regard to education level (a) and gender (b)

Figure 4 presents responses given to the question regarding preference of fresh bread or toast bread. The majority of high school and university students (53%-68%) selected fresh bread rather than toast, while 54% of elementary school students demonstrated no preference in their choice (Fig. 4a). In comparison with elementary school students, a higher percentage of high school and university students (>20%) selected integral toast. These choices may be attributed to the lack of knowledge among the elementary school students as well as to family dependence.

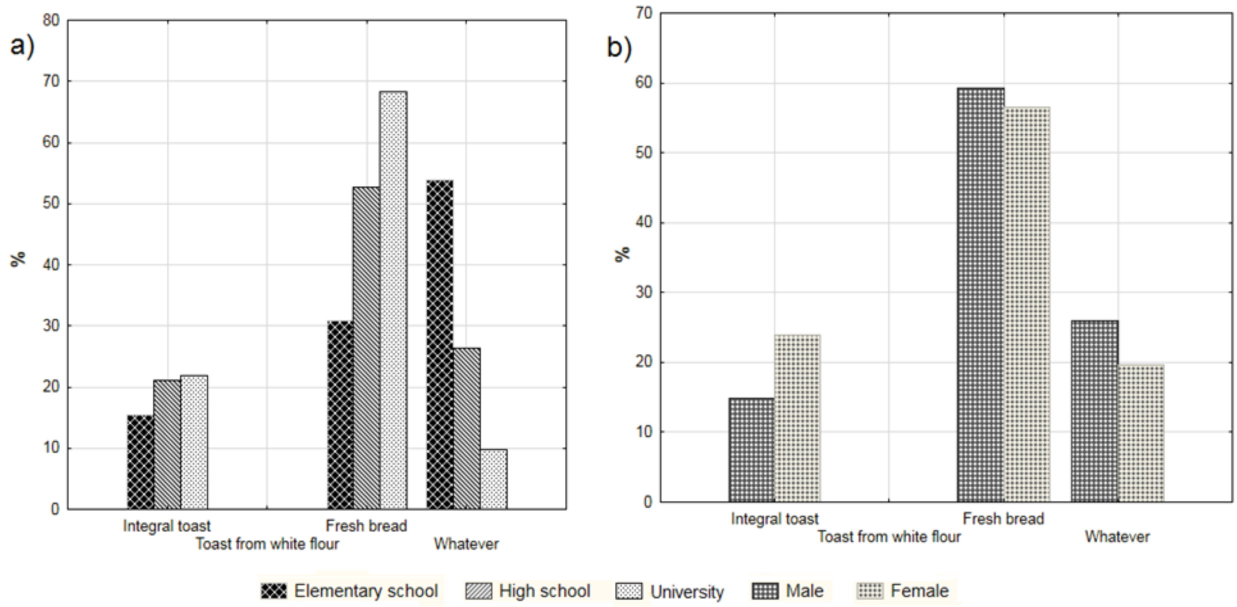


**Fig. 4.** Respondents' preferences for fresh bread or toast bread with regard to education level (a) and gender (b)

Consumption of fresh bread was confirmed by more than 50% of respondents regardless of gender. Males reported slightly higher consumption of fresh bread (59%), compared with females. However, 27% of them showed no preference in their choice (Fig. 4b). Furthermore, a high percentage of females (24%) confirmed consumption of integral toast, implying greater awareness of improvement in health status associated with components in whole grains.

## Conclusions

White bread was most commonly consumed by all respondents, elementary school students in particular, as a result of family impact and tradition. As their education level rises, the young population becomes more aware of health-related benefits provided by whole grain products consumption. Female respondents appeared to be more interested in healthy diet practices, in comparison with males. A discouraging observation is the decrease in bread consumption among the young population since bread is one of the main sources of energy which is crucial for their further development. Further education about healthy food choices should be provided to the young population because only a well-informed population can take advantage of the health benefits provided by the intake of such food.

**Acknowledgements**
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# KONZUMACIJA KRUHA MEĐU MLADIMA KAO INDIKATOR PROMJENA PREHRAMBENIH NAVIKA S ODRASTANJEM

**Sažetak**

Kruh je često konzumirana hrana na Balkanu te je samim time pogodan za istraživanje prehrambenih navika. Cilj ovog rada je istražiti prehrambene navike populacije mladih (14-26 godina) obzirom na konzumaciju različitih vrsta kruha. Ukupno je u istraživanje bilo uključeno 173 ispitanika (37% mladića i 63% djevojaka), nasumično odabranih među učenicima osnovne škole (30), srednje škole (45) i fakulteta (98) sa područja Novog Sada. Uočen je porast konzumacije kruha od cjelovitih žitarica od 15% među učenicima osnovne škole do 26% među učenicima srednje škole i studentima. Obzirom na spol, konzumacija kruha od cjelovitih žitarica je bila 9% veća među djevojkama u usporedbi sa mladićima. Svi ispitanici su pod utjecajem obiteljskih prehrambenih navika najviše konzumirali bijeli kruh (56-69%). Dobiveni rezultati pokazuju povezanost promjena prehrambenih navika sa odrastanjem i jači utjecaj okoline nego obitelji. Najveći utjecaj obitelji i običaja je uočen među osnovnoškolcima (54%) dok je među srednjoškolcima i studentima bio slabije izražen (34%).

*Ključne riječi*: kruh od cjelovitih žitarica, mladi, pravilna prehrana, prehrambene navike, unos kruha

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