Journal of Functional Foods 48 (2018) 643–653

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Analysis of health-related biomarkers between vegetarians and non-vegetarians: A multi-biomarker approach

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ARTICLE INFO

Keywords:
Vegetarians
Non-vegetarians
Dietary habits
Oxidative stress
Genomic instability
Health-related biomarkers

ABSTRACT

This study was performed in a group of adult vegetarians (N = 40) and matched non-vegetarian subjects (N = 40) in order to analyse differences in health-related biomarkers. Obtained results revealed differences in various biomarkers between subjects on a traditional mixed and vegetarian diet, indicating that vegetarians have a lower nutritional status of some nutrients (Ca, Cu and Zn, and vitamins B_{12} and D) accompanied with a lower antioxidant defence system (glutathione) and higher homocysteine and genome damage (micronuclei and DNA strand breaks), along with shorter telomeres. This suggests that the supplementation of animal derived nutrients to this particular dietary group would be beneficial for the improvement of some measured health-related biomarkers. However, the level of certain toxic metals (As and Hg) was higher in non-vegetarians. The presented multi-biomarker approach implies the necessity of evaluating a large number of different health-related biomarkers in order to obtain clear insight into dietary preferences and health outcomes.

1. Introduction

Diet is one of the key environmental factors affecting the incidence of various chronic health disorders and there is growing evidence that a vegetarian diet as well as specific components of a vegetarian diet promote health and longevity and lower the incidence of many chronic diseases including cancer. The different vegetarian diets show the variability in the extent to which animal products are avoided which can then influence micronutrient intake (Kazimírová et al., 2006; Krajcovicová-Kudlácková, Valachovicová, Pauková, & Dusinská, 2008; Majchrzak et al., 2006). Vegetarian diets are classified according to whether they contain no animal products (vegan) or if they include dairy products (lacto (L)-vegetarian), eggs (ovo (O)-vegetarian) or both (lacto-ovo (LO)-vegetarian) (Gaby, 2013). A vegetarian diet may result in a higher intake of some vitamins and micronutrients, which provide antioxidant defence, but it may also lead to a deficiency in others involved in DNA metabolism and stability, such as B group vitamins (Kazimírová et al., 2006).

Antioxidants from food have an important role in cellular antioxidant defences. Antioxidant substances in a diet enhance the DNA, protein and lipid protection by modulating several signalling pathways and gene expression, protecting and repairing DNA damage and increasing the free radical scavenging ability that occurs during metabolic reactions (Khuda-Bukhsh, Das, & Saha, 2014; Nosrati, Bakovic, & Paliyath, 2017; Varoni, Lo Faro, Sharifi-Rad, & Iriti, 2016). It has been shown that a human diet with a high intake of fruits or vegetables rich in antioxidants decreases the level of oxidative DNA damage (Duthie, Ma, Ross, & Collins, 1996; Key, Appleby, & Rosell, 2006; Pool-Zobel,

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https://doi.org/10.1016/j.jff.2018.07.054
Received 23 May 2018; Received in revised form 26 July 2018; Accepted 26 July 2018

Bub, Müller, Wollowski, & Rechkemmer, 1997). Since DNA damage is the initiating event in carcinogenesis, this supports the idea that fruits and vegetables might be beneficial in cancer protection by preventing free radical attacks on macromolecules (Kazimírová et al., 2006; Wasson, McKelvey-Martin, & Downes, 2008). Antioxidants of plant origin such as vitamins C and E, carotenoids and flavonoids are involved in protection from diseases by decreasing the level of oxidative damage (Smolková et al., 2004). Folate is an essential B vitamin that occurs naturally in a wide variety of foods, such as broccoli, cabbage, cauliflower, fruit and nuts. Its synthetic oxidized form, folic acid, is used in fortified foods and vitamin supplements because it is more stable than the natural reduced glutamated form. Since mammals are unable to synthesize folate *de novo*, they are bound to obtain it from dietary sources. Inadequate folate intake is associated with increased risk of neural tube defects, Down's syndrome, cardiovascular disease, Alzheimer's disease and various cancers (Beetstra, Thomas, Salisbury, Turner, & Fenech, 2005). These findings may indicate that vegetarianism increases antioxidant protection, implying a possible reduction in the risk of cardiovascular diseases and cancer (Kazimírová et al., 2004).

On the other hand, meats and dairy products are sources of vitamin B_{12}. Vitamin B_{12} is necessary for cell division and erythropoiesis and its deficiency can cause anaemia, lesions of the nervous system and an increased level of homocysteine related to increased risks for cardiovascular disease, Alzheimer's disease and atherosclerosis (Herrmann, Schorr, Purschwitz, Rassoul, & Richter, 2001; Obersby, Chappell, Dunnett, & Tsiami, 2013). Vitamin D, important in calcium metabolism, is present mostly in food of animal origin. However, humans get vitamin D from exposure to sunlight, from their diet, and from dietary supplements. Dietary sources of vitamin D include fatty fish, cod liver oil, egg yolks, portabella mushrooms, beef liver, and fortified foods such as breakfast cereal, milk (dairy and non-dairy), infant formula, cheese, and orange juice (Holick, 2007; Pfotenhauer & Shubrook, 2017). In most vegetarians, iron, calcium and total protein concentrations are lower, which may lead to DNA damage and oxidative stress (Ames, 1999, 2001). Therefore, minimising or eliminating animal products from the diet decreases the intake of some essential nutrients. Hence, the lack of balance between the amount of "unhealthy" and "healthy" food also leads to the accumulation of unrepaired damage, initiating DNA instability and the possibility of inducing cancer development (Kapiszewska, 2006).

Exposure to different chemicals is also an issue that has to be considered when assessing differences in the dietary intake of a certain population. The assessment of the diet should also include various pesticides, herbicides, and other potentially toxic agricultural chemicals, mycotoxins, food additives, heavy metals, environmental contaminants and compounds formed during food processing. Exposure to pesticides and other toxic compounds may be lower in vegetarian diets due to their accumulation in animal tissues (Gaby, 2013). On the other hand, higher intake of fruit, vegetable and cereals may result in higher exposure to pesticide residues and mycotoxins (Leblanc, Tard, Volatier, & Verger, 2005; Van Audenhaege et al., 2009).

Therefore, the aim of our study was to compare the effects of vegetarian *vs.* non-vegetarian diet using a large number of health-related biomarkers including haematological, biochemical and oxidative stress parameters, and to see whether differences in one's diet influence genomic instability and impact telomere length. Essential and toxic elements, pesticides and mycotoxins were also measured in biological samples of both groups as well as bone mineral density (BMD). The results of the present study offer complex insight into the differences of selected biomarkers related to specific dietary preferences, which could directly benefit clinicians and nutritionists in patient counselling regarding nutrition and dietetics.

2. Subjects and methods

2.1. Participants

The study was performed in a group of 40 healthy adult vegetarians (average age 31.93 ± 7.23, range 19–55 years), 24 women and 16 men, and a group of matched (by age, gender and smoking habit) healthy adult non-vegetarians (average age 31.58 ± 7.67, range 22–59 years). The percentage of active smokers was 17.5% in both groups. There were more women than men in this study, which is expected as there are generally more women who practice a vegetarian diet than men (Phillips, 2005). In the vegetarian group, there were 30 LO-vegetarians and 10 vegans. The average period of vegetarianism was 8.85 ± 4.69 years (range 3–20 years). Semi-vegetarians who consumed small quantities of chicken and fish were excluded from the study. Both vegetarians and non-vegetarians were recruited from the general Croatian population. All of the subjects lived in the same region (Zagreb and surroundings), had similar patterns of physical activity and similar levels of education (high school and university). None of the assessed subjects had been exposed to ionizing radiation or steroid therapy for at least 6 months, or antibiotics for at least 3 months before the study. The weight and height of the volunteers varied between 47 and 116 kg and 155 and 200 cm, respectively with a mean body mass index (BMI) of 22.85 ± 3.04 kg/m^2. Height and weight were measured using a portable stadiometer and electronic scale. BMI was calculated as weight (kg) divided by the square of height (m^2).

A detailed questionnaire with general (age, gender) and anthropometric data (weight, height) as well as life style (smoking, alcohol), health status and dietary habits was filled in for each subject. The section regarding dietary habits was based on the non-quantitative food frequency questionnaire (FFQ). Population characteristics are given in Table 1 and dietary factors for both groups gathered by the questionnaire in Table 2. The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The subjects were informed about the aim and experimental details of the study and all gave their written informed consent. No private details on the subjects involved in the study have been or will be disclosed in public.

2.2. Sampling

Blood was collected by venipuncture from fasting subjects under aseptic conditions between 8 and 9 am. Details regarding the blood sampling collection tubes are given in Supplementary material (Table S1). Blood samples were immediately used for common biochemistry and haematology parameters, as well as cytogenetic assays and oxidative status measurements, while blood, plasma and serum for other tests were stored at −80 °C until analysis. After collection, all of the blood samples were handled in the same manner. They were blindly coded,

Table 1
Population characteristics in vegetarians compared to non-vegetarians.

	Non-vegetarians		Vegetarians	
	mean ± SD	range	mean ± SD	range
Gender (M:F)	16:24	–	16:24	–
Age (years)	31.58 ± 7.67	22–59	31.93 ± 7.23	19–55
Height (cm)	173 ± 10	155–198	174 ± 10	158–200
Weight (kg)	70.48 ± 15.82	47–116	68.65 ± 13.06	50–99
BMI (kg/m^2)	23.29 ± 3.38	18.11–29.60	22.42 ± 2.63	18.31–28.04
Smokers (%)	17.50	–	17.50	–
Vegetarianism (years)	–	–	8.85 ± 4.69	3–20

M – male; F – female; BMI – body mass index.
Results are presented as means ± SD (standard deviation of the mean) including their ranges.

Table 2
Dietary factors in vegetarians compared to non-vegetarians covered by the questionnaire.

Question	Answer											
	Non-vegetarians						Vegetarians					
	Daily	Few times a week	Weekly	Few times per month	Rarely	Never	Daily	Few times a week	Weekly	Few times per month	Rarely	Never
Meat consumption												
Red meat	7	22	5	1	5	0	0	0	0	0	0	40
Poultry	0	27	4	5	4	0	0	0	0	0	0	40
Fish consumption												
Fish	0	8	11	14	7	0	0	0	0	0	0	40
Sea food	0	1	4	19	15	1	0	0	0	0	0	40
Milk and dairy products consumption												
Milk	20	11	2	0	3	4	8	9	1	2	7	13
Dairy products	31	9	0	0	0	0	17	8	4	1	0	10
Eggs consumption	0	6	9	17	8	0	0	8	4	7	7	14
Vegetables consumption	33	7	0	0	0	0	39	1	0	0	0	0
Fruits consumption	23	11	6	0	0	0	22	17	1	0	0	0
Honey consumption	4	7	9	8	11	1	3	14	3	5	5	10
Olive oil consumption	13	11	4	6	3	3	15	19	3	2	1	0
Coffee consumption	25	3	1	3	0	8	15	9	2	2	4	8
Tea consumption												
Green tea	3	8	0	5	17	7	2	11	3	10	10	4
Other teas	6	11	8	9	6	0	12	12	5	7	4	0
Alcohol consumption												
Beer	0	6	9	4	12	9	0	6	3	4	13	14
Vine	0	5	7	19	7	2	0	3	3	10	10	14
Spirits	0	3	6	9	17	5	0	1	1	4	22	12
Dietary supplements	Yes	No					Yes	No				
Vitamins	15	25					21	19				
Minerals	9	31					17	23				
Probiotics	34	6					25	15				
ω-3 fatty acid	4	36					4	36				

stored in ice, protected from light and processed as quickly as possible, usually within 2 h from sampling.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.07.054.

2.3. Haematological and biochemical analysis

The routine haematology profile – leukocytes with 5-parameter differential, erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), red blood cell distribution width (RDW), thrombocytes and mean platelet volume (MPV) was assessed with an automated haematology analyser (Advia120, Siemens Diagnostic Solutions, USA).

The routine serum biochemistry profile – sodium, potassium, total and ionized calcium, inorganic phosphate, ferritin, urates, creatinine, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), albumin, glucose, total cholesterol, high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-cholesterol, triglycerides, as well as ferritin and high-sensitivity C-reactive protein (hs-CRP) was performed on an automated biochemistry analyser, using dedicated reagents (AU680, Beckman Coulter, USA). Validated chemiluminescent immunoassays (Advia Centaur XP, Siemens Diagnostic Solutions) were used for the measurement of plasma homocysteine (La'ulu, Rawlins, Pfeiffer, Zhang, & Roberts, 2008), vitamin B_{12} (Vogeser & Lorenzl, 2007) and total vitamin D levels (Hermida et al., 2012), respectively.

2.4. Mineral and trace element analysis

The concentration of all elements in blood and serum samples was determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500cx (Agilent Technologies, Germany) with the collision/reaction cell in normal/no gas (B, Al, Hg), helium (Na, Mg, Ca, Mn, Co, Cu, Zn, As, Sr, Mo, Cd, Pb) or hydrogen mode (Fe, Se). Details on instrumental operating conditions and analysis parameters are given in Supplementary material (Table S2). Samples for analysis were prepared by 20-fold (serum) or 80-fold (blood) dilution of samples with a solution containing 0.7 mM ammonia, 0.01 mM EDTA, 0.07% (v/v) Triton X-100 and internal standards (2 µg/L of Ge, Rh, Lu and Ir) in ultrapure water. Standard addition method (i.e. matrix-matched calibration using pool sample of blood and serum) was used for the quantification of concentration of metals in blood and serum. To confirm the accuracy of the measurements, the following reference materials for trace elements in blood and serum/plasma were used: ClinChek® Serum Controls (Level I and II), ClinChek® Plasma Controls (Level I and II), ClinChek® Whole Blood Controls (Level I, and II) (Recipe, Germany), SeronormTM Trace Elements Serum (L-1-2) and SeronormTM Trace Elements Whole Blood (L-1) (Sero AS, Norway). Results are presented in Supplementary material (Table S3).

2.5. Pesticide analysis

For pesticide analysis, the serum sample was placed in a centrifuge tube. After addition of 2 × 5 mL of *n*-hexane solvent the sample was shaken for 1 min and centrifuged for 5 min at 3000 *g*. The upper layer of *n*-hexane was decanted in a test tube, iso-octane was added and concentrated with a stream of nitrogen. The extract was purified at the prepared Florisil SPE column (Phenomenex, CA, USA). Sodium sulphate (1 cm) was added to the Florisil cartridge and the column was washed with 10 mL n-hexane. The extract was transferred to the prepared and washed columns. The eluates were collected in test tubes with added iso-octane and concentrated (Matos Lino, Azzolini, Nunes, Silva, & da Silveira, 1998). Identification and quantification was performed on a

GC–MS (Shimadzu Corporation, Japan) using the selective ion monitoring (SIM) mode assay based on a main ion and two confirmatory ions and the retention time. The concentration was calculated automatically using a GC–MS-software solution based on the ratio of the peak area of the analyte divided by the peak area of standards. A total of 77 active pesticide substances were analysed. A detailed list of tested pesticides is given in Supplementary material.

2.6. Mycotoxin analysis

For the mycotoxin analysis, ochratoxin A (OTA) was chosen because it often contaminates various foods, particularly grains in a temperate climate, and has long persistence in the organism which makes it suitable for the evaluation of mycotoxin exposure. This mycotoxin was measured in the Croatian population previously (Peraica et al., 2001). Plasma samples were extracted and purified according to Beker and Radić (1991). A total of 500 μL of plasma samples were added to 5 mL of mixture of $MgCl_2$ and HCl (1:1) and 2.5 mL chloroform. After 20 min of rotation, tubes were centrifuged 10 min at 3000 rpm. Chloroform phase was taken, washed twice with distilled water (2 mL) and evaporated under a stream of nitrogen. Before injecting to the HPLC, the sample was dissolved in a 500 μL mobile phase. The HPLC consisted of a degasser, isocratic pump, column oven and fluorescence detector (Shimadzu Corporation). The guard column and analytical column were C-18 reverse-phase (LiChrospher, Merck, Germany) with 5 μm particles (4.0 × 4.0 and 4.0 × 125.0 mm, respectively). The mobile phase consisted of acetonitrile:water:acetic acid (500:500:5) and the flow rate was 0.5 mL/min. The fluorescence detector was set at 336 nm λ_{ex} and 464 nm λ_{em}. OTA was quantified according to the calibration curve prepared from OTA standards.

2.7. Malondialdehyde (MDA) analysis

The measurements of malondialdehyde (MDA) in plasma were based on the method by Drury, Nycyk, and Cooke (1997). To a 50 μL sample or standard (2.5 μM 1,1,3,3-tetraethoxy propane), 5 μL of butilatedhydroxyltoluene (BHT) (0.2%, w/v), 750 μL of phosphoric acid (1%, v/v), 250 μL thiobarbituric acid (TBA) (0.6%, w/v) and 445 μL of water were added. Samples were mixed and incubated in a boiling water bath for 30 min. The MDA analysis was performed on HPLC consisting of a degasser, isocratic pump, column oven and UV detector (Shimadzu Corporation). The guard column and analytical column were C-18 reverse-phase (LiChrospher, Merck) with 5 μm particles (4.0 × 4.0 and 4.0 × 125.0 mm, respectively). The mobile phase consisted of 50 mM KH_2PO_4 and methanol (60:40, v/v, pH 6.8) and the flow-rate was 1 mL/min. The UV detector was set at 532 nm. The injection volume was 20 μL and the temperature in the column oven was set to 32 °C. Identification and quantification of MDA was based on MDA standards.

2.8. Glutathione (GSH) analysis

Glutathione (GSH) was analysed by the method from Ellman (1958). To a 100 μL H_2O (blank solution), standards or samples (plasma), 850 μL of phosphate buffer and 50 μL of 5,5′-ditiobis-2-nitrobenzoat (DTNB) were added. Absorbance of blank solution, standards and samples were measured against blank spectrophotometrically (Cecil 9000, UK) at 412 nm. GSH was quantified by GSH standards.

2.9. Cell viability (cytotoxicity) assay

Cytotoxicity was determined by differential staining with acridine orange/ethidium bromide (AO/EtBr) using fluorescence microscopy (Duke & Cohen, 1992). Lymphocytes were isolated by Histopaque density gradient centrifugation method and stained. A total of 100 cells per repetition were examined using an epifluorescence microscope (Olympus BX51, Japan). Quantitative assessments were made by a determination of the percentage of viable and dead cells.

2.10. Alkaline comet assay

The alkaline comet assay was done on white blood cells according to Singh, McCoy, Tice, and Schneider (1988) with minor modifications. Briefly, whole blood was embedded in agarose matrix, and afterwards the cells were lysed (2.5 M NaCl, 100 mM $EDTANa_2$, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH10) at 4 °C. After the lysis, the slides were placed into alkaline solution (300 mM NaOH, 1 mM $EDTANa_2$, pH13) for 20 min at 4 °C and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5), stained with EtBr (10 μg/mL) and analysed at 250× magnification using an epifluorescence microscope (Zeiss, Germany) with an image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). One hundred randomly captured comets from each of the duplicate slides were examined. To quantify DNA damage, the following comet parameters were evaluated: tail length, tail intensity (% of tail DNA), and tail moment.

2.11. Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was done according to Fenech and Morley (1985) with minor modifications. Briefly, whole blood (500 μL) was incubated in an RPMI medium at 37 °C in an atmosphere of 5% CO_2. Cytochalasin-B was added at a final concentration of 6 μg/mL 44 h after the culture was started. The cultures were harvested at 72 h. The lymphocytes were fixed in the methanol–acetic acid solution (3:1), air-dried and stained with 5% Giemsa solution. One thousand binuclear lymphocytes were analysed at 400× magnification using a light microscope (Olympus CX41). Micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were counted in 1000 binucleated cells according to criteria published by Fenech (2006). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei (Kirsch-Volders et al., 2003).

2.12. Telomere length analysis

For telomere length analysis, a subpopulation of individuals from both groups, non-vegetarians (N = 18; 12 women and 6 men; average age 29.20 ± 4.10, range 22–38 years) and vegetarians (N = 21; 11 women and 10 men; average age 29.90 ± 5.20, range 19–42 years) was chosen. Genomic DNA was isolated from peripheral blood leukocytes with the QIAamp Blood Maxi Kit (Qiagen, USA). Mean telomere length was quantified by Q-PCR method using a 7900HT Fast Real-Time PCR System (Applied Biosystems) as previously described (Laganović et al., 2014). TaqMan Copy Number Reference Assay RNase P (Applied Biosystems) was used to measure the single copy gene copy number (S). To measure telomere repeat copies (T) primers telg – 5′-ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T-3′ and telc – 5′-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA-3′ were added (0.5 μmoL/L) to SsofastEvaGreenSupermix with low ROX (BioRad, Denmark). Cycling conditions were: 2 min at 50 °C, 2 min at 95 °C, followed by two cycles of 95 °C for 15 s, 52 °C for 15 s and 36 cycles of 95 °C for 15 s, 62 °C for 15 s and 71 °C for 15 s. Following amplification, T/S ratios were derived from a standard curve using the 7900HT Sequence Detection System version SDS2.3 (Applied Biosystems). The results were presented as T/S ratios. Samples were run twice in triplicate and the mean of these runs were calculated. T/S ratios were calibrated to control samples on each plate to minimize batch effect. Samples that did not yield coefficient of variability (CV) below 15% in at least two runs were excluded. Finally, samples with DNA concentrations outside the optimal range were excluded.

2.13. Bone density measurement

Quantitative ultrasound (QUS) measurements of the heel (non-dominant side) were performed using a "Sahara" sonometer ("Hologic", USA). The primary parameters measured with ultrasound are broad-band ultrasound attenuation (BUA) and speed of sound (SOS). BUA (dB/MHz) is the attenuation of sound waves as they pass from the transmitting transducer to the receiving transducer. SOS (m/s) is the speed at which the ultrasound signal travels from one transducer to the other. These two parameters are combined to form the quantitative ultrasound index (QUI) referred as ultrasound stiffness, used to evaluate bone mineral density (BMD). T scores for QUI were calculated with Croatian QUS normative data (Kraljevic et al., 2007) according to the formula: (P-YA)/SDya (P = patient result, YA = young adult mean value, SDya = standard deviation of young adult population). The instrumental quality control was performed daily by scanning the manufacturer-provided, temperature-sensitive phantom.

2.14. Statistical analysis

The data were analysed using descriptive statistics and the results are presented as means ± SD (standard deviation of the mean) or medians (5th/95th percentiles). Statistical analysis was performed using the data analysis software system Statistica, version 13.2 (Dell Inc., USA). The normality of variables was tested by the χ^2 test and Kolmogorov-Smirnov test and skewed variables were normalized by logarithmic transformation before statistical analysis. The mean and SD of telomere lengths (obtained as T/S ratios) were used to describe the distribution of continuous variables. Differences between groups were tested with either independent sample *t*-test or non-parametric Mann-Whitney *U* test. All of the results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Haematology and biochemistry parameters

Vegetarians displayed significantly ($P < 0.05$) lower values of thrombocytes and vitamins B_{12} and D (244.70 ± 140.50 × 10^9/L, 273.54 ± 140.50 pmol/L and 38.66 ± 12.86 μg/L, respectively) compared to non-vegetarians (267.50 ± 51.90 × 10^9/L, 314.11 ± 83.60 pmol/L and 43.99 ± 15.05 μg/L, respectively). On the contrary, the homocysteine level was significantly ($P < 0.05$) higher in vegetarians than non-vegetarians (12.30 ± 3.80 μmol/L *vs.* 10.70 ± 2.60 μmol/L) (Table 3).

3.2. Essential and toxic elements

Vegetarians had significantly ($P < 0.05$) higher concentrations of boron, cobalt and molybdenum (32.84 (20.09/65.18) μg/L, 0.29 (0.17/0.82) μg/L and 1.01 (0.64/2.00) μg/L, respectively) compared to non-vegetarians (23.74 (12.28/91.65) μg/L, 0.20 (0.14/0.51) μg/L and 0.74 (0.45/1.38) μg/L). At the same time, vegetarians had lower concentrations of calcium, copper and zinc (94.34 ± 3.03 mg/L, 969.45 ± 265.59 μg/L and 820.85 ± 119.64 μg/L) compared to non-vegetarians (96.68 ± 3.11 mg/L, 1145.95 ± 393.53 μg/L and 885.00 ± 112.66 μg/L, respectively). Furthermore, non-vegetarians exhibited significantly ($P < 0.05$) higher concentrations of arsenic and mercury (1.40 (0.62/9.55) μg/L and 1.65 (0.26/8.79) μg/L, respectively) compared to vegetarians (0.68 (054/6.58) μg/L and 0.10 (0.02/4.56) μg/L, respectively) (Table 4).

3.3. Pesticide residues

From all of the pesticide residues analysed in both groups, only azinphos ethyl (0.08 ± 0.005 mg/60 kg) and dieldrin (0.04 ± 0.001 mg/60 kg) were identified in vegetarians and were significantly ($P < 0.05$) elevated compared to non-vegetarians (Table 5). All of the other pesticide residues were not detected in either of the

Table 3
Common haematology and biochemistry parameters including hs-CRP, homocysteine and vitamins (B_{12} and D) in vegetarians compared to non-vegetarians.

	Non-vegetarians	Vegetarians
Leukocytes (×10^9/L)	6.25 ± 1.44	5.84 ± 1.38
Neutrophils (×10^9/L)	3.46 ± 1.10	3.35 ± 0.99
Lymphocytes (×10^9/L)	2.06 ± 0.72	1.80 ± 0.50
Monocytes (×10^9/L)	0.37 ± 0.10	0.35 ± 0.10
Erythrocytes (×10^9/L)	4.69 ± 0.43	4.55 ± 0.50
Haemoglobin (g/L)	139 ± 13	136 ± 17
Haematocrit (L/L)	0.40 ± 0.04	0.39 ± 0.04
MCV (fL)	85.50 ± 4.12	86.10 ± 7.37
Thrombocytes (×10^9/L)	267.50 ± 51.90	244.70 ± 140.50*
MPV (fL)	8.10 ± 0.76	8.42 ± 0.90
Na (mmol/L)	140.40 ± 1.50	140.30 ± 1.70
K (mmol/L)	4.50 ± 0.40	4.50 ± 0.40
Ca-ionized (mmol/L)	1.22 ± 0.03	1.23 ± 0.03
Phosphate (mmol/L)	1.15 ± 0.14	1.14 ± 0.16
Glucose (mmol/L)	5.00 ± 0.40	4.90 ± 0.50
Albumin (g/L)	45.50 ± 2.20	45.00 ± 1.90
ALT (IU/L)	25.10 ± 12.63	17.20 ± 7.26
AST (IU/L)	25.80 ± 12.00	22.90 ± 5.40
GGT (IU/L)	22.30 ± 15.70	17.50 ± 8.40
Total cholesterol (mmol/L)	4.77 ± 1.05	4.57 ± 0.86
HDL-cholesterol (mmol/L)	1.55 ± 0.36	1.57 ± 0.36
LDL-cholesterol (mmol/L)	2.74 ± 0.84	2.59 ± 0.71
Triglycerides (mmol/L)	1.06 ± 0.55	0.90 ± 0.44
Creatinine (μmol/L)	85.50 ± 13.50	79.90 ± 9.60
hs-CRP (mg/L)	0.55 (0.10/7.63)	0.40 (0.10/1.89)
Ferritin (μg/L)	39.50 (8.00/177.60)	26.50 (9.00/86.80)
Urate (μmol/L)	249.10 ± 80.58	225.98 ± 62.51
Bilirubin (μmol/L)	15.10 ± 11.59	14.73 ± 11.20
Homocysteine (μmol/L)	10.70 ± 2.60	12.30 ± 3.80*
Vitamin B_{12} (pmol/L)	314.11 ± 83.60	273.54 ± 140.50*
Vitamin D (μg/L)	43.99 ± 15.05	38.66 ± 12.86*

MCV – mean corpuscular volume; MPV – mean platelet volume; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT – gamma-glutamyltransferase; HDL – high-density lipoprotein; LDL – low-density lipoprotein; hs-CRP – high-sensitivity C-reactive protein.
Results are presented as means ± SD (standard deviation of the mean) or medians (5th/95th percentiles) according to data distribution.
* Statistically different ($P < 0.05$; *t*-test or Mann-Whitney *U* test).

Table 4
Element concentration in plasma or whole blood in vegetarians compared to non-vegetarians.

Medium-element	Non-vegetarians	Vegetarians
P-Na (mg/L)	3158.65 ± 99.38	3111.23 ± 68.00
P-Ca (mg/L)	96.68 ± 3.11	94.34 ± 3.03*
P-Mg (mg/L)	20.95 ± 1.62	20.72 ± 1.55
P-Fe (mg/L)	1.50 ± 0.66	1.43 ± 0.52
P-Cu (μg/L)	1145.95 ± 393.53	969.45 ± 265.59*
P-Zn (μg/L)	885.00 ± 112.66	820.85 ± 119.64*
P-B (μg/L)	23.74 (12.28/91.65)	32.84 (20.09/65.18)*
P-Se (μg/L)	95.68 ± 13.54	90.67 ± 20.00
P-Co (μg/L)	0.20 (0.14/0.51)	0.29 (0.17/0.82)*
P-Mo (μg/L)	0.74 (0.45/1.38)	1.01 (0.64/2.00)*
P-Sr (μg/L)	20.89 ± 8.00	23.33 ± 7.46
P-Al (μg/L)	7.13 ± 1.02	7.13 ± 1.19
B-Mn (μg/L)	9.73 ± 3.35	10.01 ± 3.25
B-As (μg/L)	1.40 (0.62/9.55)	0.68 (0.54/6.58)*
B-Cd (μg/L)	0.25 (0.11/4.25)	0.42 (0.13/2.57)
B-Pb (μg/L)	13.20 (6.46/31.5)	11.4 (5.59/38.73)
B-Hg (μg/L)	1.65 (0.26/8.79)	0.10 (0.02–4.56)*

P – plasma; B – blood.
Results are presented as means ± SD (standard deviation of the mean) or medians (5th/95th percentiles) according to data distribution.
* Statistically different ($P < 0.05$; *t*-test or Mann-Whitney *U* test).

Table 5
Pesticide and mycotoxin concentrations in vegetarians compared to non-vegetarians.

	Non-vegetarians	Vegetarians
Azinphos ethyl (mg/60 kg)	0.00 ± 0.00	0.08 ± 0.005*
Dieldrin (mg/60 kg)	0.00 ± 0.00	0.04 ± 0.001*
Ochratoxin A (ng/mL of plasma)	0.56 ± 1.31	0.20 ± 0.44

Results are presented as means ± SD (standard deviation of the mean).
* Statistically different ($P < 0.05$; Mann-Whitney *U* test).

groups.

3.4. Ochratoxin A

There was no significant difference in OTA concentrations between the groups (Table 5). The mean concentration of OTA in both groups was 0.38 ± 0.98 ng/mL of plasma.

3.5. Oxidative stress parameters

There were no significant differences in MDA level between the groups while vegetarians exhibited significantly ($P < 0.05$) lower GSH levels compared to non-vegetarians (0.77 ± 0.54 μg/mg proteins and 1.15 ± 0.79 μg/mg protein, respectively) (Table 6).

3.6. Cell and genome damage

Mean cell viability was 96.81 ± 2.50% in both of the assessed groups. Vegetarians had significantly ($P < 0.05$) higher comet assay parameters (tail length, tail intensity and tail moment; 14.75 ± 1.48 μm, 2.22 ± 0.92% and 0.29 ± 0.13, respectively) compared to non-vegetarians (13.75 ± 1.10 μm, 1.51 ± 0.65% and 0.19 ± 0.09, respectively). Genomic instability was further evaluated by the CBMN assay. According to the results, the mean MNi frequency observed in the binucleated lymphocytes was significantly ($P < 0.05$) higher in vegetarians (7.35 ± 3.61) compared to non-vegetarians (4.33 ± 2.39) (Table 6).

3.7. Telomere length

Mean telomere length was measured by Q-PCR and was calculated as the amount of telomeric DNA (T) divided by the amount of a single-copy control DNA (S) (T/S ratio). When compared against each other within the subpopulation, telomere length in non-vegetarians showed a higher but statistically insignificant T/S value (1.50; N = 18) compared to vegetarians (1.34; N = 21) (Fig. 1).

Table 6
Oxidative and cytogenetic status of vegetarians compared to non-vegetarians.

	Non-vegetarians	Vegetarians
MDA (nmoL/mg proteins)	0.13 ± 0.06	0.12 ± 0.08
GSH (μg/mg proteins)	1.15 ± 0.79	0.77 ± 0.54*
TL (μm)	13.75 ± 1.10	14.75 ± 1.48*
TI (%)	1.51 ± 0.65	2.22 ± 0.92*
TM	0.19 ± 0.09	0.29 ± 0.13*
MNi	4.33 ± 2.39	7.35 ± 3.61*
NPBs	1.59 ± 1.79	1.75 ± 1.78
NBUDs	3.81 ± 1.49	3.00 ± 2.07
NDI	2.02 ± 0.14	1.96 ± 0.14

MDA – malondialdehyde; GSH – glutathione; TL – tail length; TI – tail intensity; TM – tail moment; MNi – micronuclei; NPBs – nucleoplasmic bridges; NBUDs – nuclear buds; NDI – nuclear division index.
Results are presented as means ± SD (standard deviation of the mean).
* Statistically different ($P < 0.05$; Mann-Whitney *U* test).

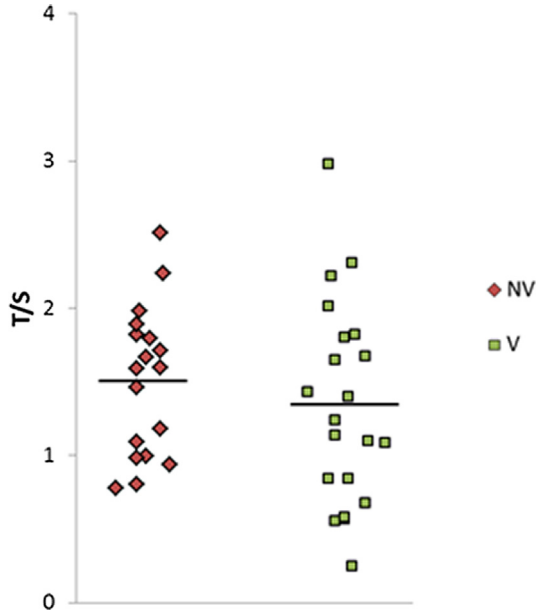

Fig. 1. Mean telomere length expressed as T/S ratio in vegetarians (V) compared to non-vegetarians (NV).

Table 7
Bone mineral density parameters in vegetarians compared to non-vegetarians.

	Non-vegetarians	Vegetarians
QUI	106.13 ± 18.97	102.20 ± 16.57
QUI T-score	0.24 ± 1.00	0.13 ± 1.00
BUA (dB/MHz)	89.37 ± 15.78	87.90 ± 13.57
SOS (m/s)	1560.78 ± 32.64	1554.80 ± 28.60

QUI – quantitative ultrasound index; BUA – broadband ultrasound attenuation; SOS – speed of sound.
Results are presented as means ± SD (standard deviation of the mean).
note: no statistical difference observed ($P < 0.05$; *t*-test).

3.8. Bone mineral density parameters

There were no significant differences in any of the bone density parameters between the groups, including the QUI T-score (Table 7). An equal number of vegetarians and non-vegetarians (four) had a QUI T-score lower than −1.0.

4. Discussion

It is still a matter of debate whether the vegetarian diet is healthier than a traditional one and if that is so, a difference in a variety of health-related biomarkers is to be expected. This study aimed to investigate differences in a large number of various health-related biomarkers associated to the dietary habits of vegetarians and non-vegetarians. The presented multi-biomarker approach was meant to give insight into the differences in selected health-related biomarkers based on the specific dietary intake and could consequently distinguish between those that are relevant and/or are not in assessing the dietary differences between various dietary groups.

According to population characteristics, both groups had a similar level of education and pattern of physical activity. They also exhibited similar values for height, weight and BMI indicating that the traditional diet does not necessarily induce excessive weight nor does vegetarianism induce lower weight presuming that both groups have healthy and balanced dietary habits. The range of BMI was similar in vegetarians (18.31–28.04 kg/m^2) and non-vegetarians (18.11–29.60 kg/m^2). However, higher number of overweight subjects (cut-off point

≥ 25.00 kg/m^2) belonged to non-vegetarians (35%) compared to vegetarians (20%) and more underweight subjects (cut-off point < 18.50 kg/m^2) belonged to the group of vegetarians (7.5%) compared to non-vegetarians (2.5%). As expected, BMI was higher in males of both groups (24.63 ± 2.77 kg/m^2 in males compared to 21.66 ± 2.66 kg/m^2 in females) (Table 1). It has to be pointed out that none of the subjects were obese according to BMI values (WHO Expert Consultation, 2004). Although both groups reported alcohol consumption, this habit was less frequent in vegetarians than in non-vegetarians. Lower BMI and less frequent alcohol consumption among vegetarians have already been noticed (Burkert, Muckenhuber, Großschädl, Rásky, & Freidl, 2014).

In our study, vegetarians used dietary supplements more often, particularly those with vitamins B and D, iodine and selenium together with ω-3 fatty acids, calcium, iron, and zinc which could be deficient in their diet (Craig, 2010). Although the vegetarian diet may lead to a deficiency of micronutrients involved in DNA metabolism and stability, such as vitamins B or D, a higher intake of other vitamins and micronutrients may provide a better antioxidant defence (Kazimírová et al., 2006, 2004). Despite the supplementation, vegetarians in the present study displayed significantly lower values of both vitamins, B_{12} and D compared to non-vegetarians that could be expected because the dietary sources of these vitamins are primarily meat and dairy products (Kazimírová et al., 2004). On the contrary, the homocysteine level was significantly higher in vegetarians compared to non-vegetarians, which was also the case in other studies evaluating homocysteine level based on specific dietary habits (Fenech, Dreosti, & Rinaldi, 1997; Kazimírová et al., 2006). Cobalamin deficiency is a well-known inflicting factor of pernicious anaemia and neuropathy, while hyperhomocysteinaemia has been recognized as a risk-factor of atherosclerosis and cardiovascular morbidity (Elmadfa & Singer, 2009). Also, both elevated homocysteine and low levels of vitamins B_{12} and D are considered as important risk factors for increased chromosome damage and maintenance of DNA integrity (Fenech et al., 1997; Nair-Shalliker, Armstrong, & Fenech, 2012). Thus, vegetarian dietary habits may inflict health risks, unless careful monitoring and appropriate supplementation is provided on a regular basis.

When measuring different elements in the blood of both groups we noticed significant differences according to dietary habits. Vegetarians had lower values of iron, cooper, zinc, calcium and selenium compared to non-vegetarians although a significantly lower level compared to non-vegetarians was found only for calcium, copper and zinc, which may be the result of either their lower bioavailability from this type of diet or the fact that some of the accessed micronutrients are present in lower levels in the vegetarian diet (Craig, 2010; Foster, Chu, Petocz, & Samman, 2013; Gibson, 1994). Essential trace elements such as zinc, copper and selenium are considered antioxidant micronutrients since they play a key role in free radical defences and their lower status caused by their inadequate intake may lower antioxidant defence which in turn may contribute to a broad spectrum of so-called free radical diseases (Kadrabová, Madaric, Kováciková, & Ginter, 1995). On the other hand, lower iron stores have even been hypothesized to reduce the risk of chronic diseases (Hunt, 2003). Furthermore, significantly higher concentrations of boron, cobalt and molybdenum were found in vegetarians compared to non-vegetarians. On the contrary, higher levels of toxic metals such as arsenic, mercury, cadmium and lead were found in non-vegetarians with arsenic and mercury being significantly elevated compared to vegetarians. This higher level of mercury and arsenic could be attributed to fish and sea food consumption because fish are at the top of the food chain and contain heavy metals as well as other toxins (Gaby, 2013; Oehlenschläger, 2012). However, a large number of evidence suggests that the benefits of moderate fish consumption outweigh the risks (Gaby, 2013), especially if we have in mind that arsenic in food mostly occurs as organic arsenic species and as such does not represent a health risk (Molin, Ulven, Meltzer, & Alexander, 2015).

A healthy diet should include a wide variety of whole, unprocessed foods free of additives and, if possible, without pesticides and other potentially toxic chemicals including mycotoxins (Gaby, 2013). Unfortunately, this is hard to achieve and can sometimes even be impossible. Therefore, we wanted to assess differences in vegetarians and non-vegetarians based on the pesticide and mycotoxin exposure that can act as potential pro-oxidants and eventually as DNA damaging agents (Mostafalou & Abdollahi, 2013; Sorrenti et al., 2013). Of more than 70 pesticide residues analysed in both groups, only azinphos ethyl and dieldrin were detected to be significantly elevated compared to non-vegetarians. This could be explained by the fact that vegetarians may be more exposed to pesticides than the general population due to specific dietary habits that include a higher fruit, vegetable and cereal intake (Van Audenhaege et al., 2009). On the contrary, the concentration of OTA in plasma of non-vegetarians was higher, but not significantly different from vegetarians. Mycotoxin OTA was chosen because of its long half-life in blood of humans as well as because of previous studies in the Croatian population. The mean concentration of OTA in both groups was 0.38 ± 0.98 ng/ml of plasma, which is similar to our previous findings for the Croatian population (0.30 ng/ml) (Peraica et al., 2001).

Oxidative stress was assayed by its well-established biomarkers GSH and MDA (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013). A significantly lower GSH concentration was measured in vegetarians indicating lower antioxidant defence in this group. This was probably due to lower protein quality and quantity that the vegetarian diet may provide, which leads to an insufficient intake of sulphur-containing amino acids that may subsequently affect GSH values (Krajcovicová-Kudlácková et al., 1999). The same was noticed by Nagyova, Ginter, and Kovacikova (1995) and Krajcovicová-Kudlácková et al. (1999) where vegetarians had lower GSH levels. Furthermore, our results showed that the MDA concentration, as a marker of lipid peroxidation, is similar in both groups. Although we did not notice differences in lipid peroxidation as measured by MDA, vegetarians could have lower antioxidant defence due to a lower level of antioxidant micronutrients (Zn, Cu, Se, Fe), GSH, GGT and uric acid.

Based on the literature, the vegetarian type of diet helps to improve antioxidant status, lowers oxidative stress, and reduces blood lipid levels (Kim, Cho, & Park, 2012; Rauma & Mykkänen, 2000). However, based on the answers collected through our questionnaire it is clear that non-vegetarians also consume fruits and vegetables on a daily basis. Our questionnaire showed that a large number of non-vegetarians preferentially ate white meat, fish and sea food avoiding red meat which makes their diet a “carnivorous diet rich in fruits and vegetables” (Burkert et al., 2014). Moreover, a recent PURE (Prospective Urban Rural Epidemiology) study (www.phri.ca/pure/) showed that fat intake from animal products as well as intake of fruits and vegetables was inversely associated with mortality, while processed carbohydrates were suspected as potential drivers that affect human longevity (Dehghan et al., 2017; Miller et al., 2017; Ramsden & Domenichiello, 2017).

Our results showed that the vegetarian diet does not provide a better antioxidant defence nor that this type of diet lowers oxidative stress, which confirms results from Kazimírová et al. (2006, 2004). In the mentioned study, there was no significant difference in total antioxidant capacity between vegetarians and non-vegetarians although some authors have indicated that the vegetarian type of diet can provide protection against oxidative damage especially in aging (Krajcovicová-Kudlácková et al., 2008), where a reduction in blood lipid levels was shown with non-vegetarians having higher total cholesterol, LDL cholesterol and triglycerides compared to vegetarians, while their HDL cholesterol was slightly lower compared to vegetarians. These results were explained by consumption of red meat. Similar evidence was provided by several studies where non-vegetarians had higher levels of total cholesterol and LDL cholesterol, but a difference in levels of HDL cholesterol was not observed (Chen et al., 2008; Sambol,

Stimac, Orlić, & Guina, 2009; Zhang et al., 2014). Our study revealed no diet-associated difference in the lipid profile, indicating that the diet may have limited value as a lipid-lowering tool, while consuming reasonable amounts of red meat as a part of a well-balanced mixed diet, as was the case in our subjects, is not-inferior to the vegetarian diet in maintaining a normal lipid balance in young and healthy adults.

Nutrition antioxidants have an important role in cellular antioxidant defences. Antioxidant substances such as vitamin C, β-carotene, and vitamin A increase DNA, protein and lipid protection by increasing the free radical scavenging ability that occurs during metabolic reactions. Consequently, a human diet with an increased intake of fruits and/or vegetables rich in antioxidants favours a decrease in chromosome damage and oxidative DNA damage (Duthie et al., 1996; Kazimírová et al., 2004; Key et al., 2006; Pool-Zobel et al., 1997). Vitamin D is absent in vegetables and there is a higher occurrence of deficiencies in iron, calcium, total proteins and vitamin B_{12} in vegetarians. Deficiencies of vitamins B_{12}, B_6, C, E, folate, niacin, or iron mimic radiation in causing single- and double-strand breaks and/or oxidative lesions in DNA, whereas adequate levels of vitamin D may also be beneficial in maintaining DNA integrity. Low levels of folic acid and vitamin B_{12} are related with elevated chromosome damage and high concentrations of homocysteine in blood. Additionally, they are significantly correlated with increased MNi formation in lymphocytes (Ames, 1999; Fenech et al., 1997; Fenech, 2001; Kazimírová et al., 2004; Nair-Shalliker et al., 2012). Based on our results, vegetarians displayed significantly lower values of both vitamin B_{12} and D, and significantly elevated homocysteine levels.

Nutritional genomics studies the interactions between nutrition and individuals genomes at molecular, cellular and systemic level (Davis & Milner, 2004) including also dietary effects on genome stability (Fenech, El-Sohemy et al., 2011). Thus, the present study revealed an increased DNA damage as measured by the comet assay in vegetarians compared to non-vegetarians and this increase was significant for all the parameters tested. CBMN assay parameters also showed increased values of MNi and NPBs in vegetarians compared to non-vegetarians, the MNi frequency being significantly elevated. MNi can originate during anaphase from lagging acentric chromosome or chromatid fragments caused by misrepair of DNA breaks or unrepaired DNA breaks. NPBs originate from dicentric chromosomes, which may occur due to the misrepair of DNA breaks, telomere end fusions, and might also be observed when defective separation of sister chromatids at anaphase happens due to failure of decatenation (Fenech, Kirsch-Volders et al., 2011; Kopjar et al., 2010). On the contrary, non-vegetarians showed a higher frequency of NBUDs but this increase was insignificant. NBUDs represent the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells (Fenech, Kirsch-Volders et al., 2011).

MacGregor (1990) suggested that some dietary and nutritional factors might exert a quantitatively significant influence on spontaneous chromosomal damage frequencies in human populations. Although there is strong evidence that the antioxidants present in fruits and vegetables can increase the resistance of cellular DNA to an oxidative attack (Dusinska & Collins, 2008), our results showed that vegetarians had a higher frequency of DNA strand breaks and genomic instability. This could be explained with lower urate levels, vitamin B_{12} and D, GSH level, calcium and several antioxidant micronutrients (Zn, Cu, Se, Fe), and/or higher homocysteine and even pesticide levels in addition to the fact that the non-vegetarians in our group practiced a carnivorous diet that is also rich in fruits and vegetables. It has been hypothesised that DNA damage from micronutrient deficiencies is likely to be a major cause of cancer and that improving micronutrient deficiencies should lead to a major improvement in health and an increase in longevity (Ames, 2001). Moreover, moderate alcohol consumption, especially wine drinking that was more prominent in the non-vegetarians from our study, could also have beneficial effects on oxidative and DNA damaging status. It has also been hypothesized that regular consumption of moderate doses of wine could provide health benefits. Wine's beneficial effect has been attributed principally to its non-alcoholic portion, which has antioxidant properties (Biasi et al., 2014).

These results are in accordance with the results of Fenech and Rinaldi (1995) who found that vegetarians did not have a lower genetic damage rate compared to non-vegetarians. On the other hand, Gaziev et al. (1996) showed that the consumption of a mixture of antioxidant vitamins favours a decrease in the chromosome damage produced by endogenous and exogenous factors in human lymphocytes. Controversial results were also found with regard to genome instability between these two dietary groups. Using the comet assay, Dhawan, Mathur, and Seth (2001) found significantly elevated DNA strand breaks in non-vegetarians. Similarly, in a study by Kotova et al., (2015), a higher frequency of MNi in human transferrin-positive reticulocytes was associated with a traditional diet. Kazimírová et al. (2006, 2004) found no differences in the percentage of cells with chromosome aberrations or in the frequency of MNi between vegetarians and non-vegetarians while non-vegetarians had significantly higher levels of oxidative DNA damage compared to vegetarians. Krajcovicová-Kudlácková et al. (2008) found no difference in the oxidative DNA damage of young individuals between vegetarians and non-vegetarians. On the contrary, significantly reduced values of oxidative DNA damage were found in an older vegetarian group compared to non-vegetarians, suggesting that the increase of oxidative damage in aging may be prevented by vegetarian nutrition. These differences could be explained by geographical distribution, different dietary patterns of the selected studies, sensitivities of methods and the sample/cell types used.

Telomere length reflects biological aging and may be influenced by a large number of environmental factors including lifestyle changes dependent on dietary interventions (Ornish et al., 2013, Ornish et al., 2008). We can define the vegetarian diet to be a major intervention, widespread in developed countries, into one's lifestyle designed to improve general health and health span. Surprisingly, there are very few reports describing telomere dynamics in vegetarians *vs.* non-vegetarians and this study is among the first to compare telomere length between these two dietary groups. The presented results revealed that the mean telomere length of both subpopulations was about the same. This points to the conclusion that while vegetarians avoid certain negative factors influencing meat-eaters, they may be increasingly exposed to herbicides and pesticides. They may also lack some vitamins, like vitamin D, which is an important factor in telomere maintenance and therefore affects general health and health span (Vidaček Škrobot et al., 2018). Greater longevity has so far been established only for the Mediterranean diet (Sofi, Cesari, Abbate, Gensini, & Casini, 2008; Trichopoulou, 2004). The possible reasons for this may be low meat consumption and moderate fish consumption, preferential use of low-fat milk products, seasonal vegetables, fruits, nuts and seeds, the use of olive oil, and a moderate intake of alcohol. Moreover, the Mediterranean diet lowers the level of oxidative stress markers and inflammation due to a high abundance of antioxidant compounds such as ω-3 fatty acids and resveratrol and has a direct positive effect on telomere length. In northerly countries, a change in dietary patterns has also resulted in a significant increase in life span. Although longevity is determined by a healthy diet, it is also influenced by a whole range of other factors such as genetics, absence of severe diseases, etc. (Rabast, 2008; Vidaček Škrobot et al., 2018). Another observed beneficial dietary effect is the so-called "French paradox" with its observation of low coronary heart disease death rates despite a higher intake of dietary cholesterol, saturated fat and red wine. The French paradox suggests that the promotion of primary prevention, based on an optimal diet rich in fruit and vegetables, regular physical exercise, and life without smoking is beneficial (Ferrières, 2004). In terms of health, epigenetics should also be considered since nutrients can modify physiologic and pathologic processes through epigenetic mechanisms critical for gene expression. The modulation of such processes through diet may prevent diseases and maintain health although it remains a complex issue to deal with and

requires further research (Park, Friso, & Choi, 2012).

In regard to bone mineral density, there was no statistically significant difference between the two groups although vegetarians exhibited slightly lower values of all of the tested parameters (QUI, BUA, SOS) compared to non-vegetarians, which was expected based on their dietary habits. Several studies have evaluated differences in bone density between vegetarians and non-vegetarians with inconclusive results. Siani et al. (2003) and Wang, Chiu, Chuang, Chiu, and Lin (2008) observed no significant differences in bone mineral density (BMD), obtained by dual-energy absorptiometry (DXA) in two groups, whereas Lau, Kwok, Woo, and Ho (1998) showed that BMD at the spine was similar between vegetarians and non-vegetarians, but significantly lower at the some sites of the hip in vegetarians. Another study based on QUS also confirmed the lack of differences in bone parameters between vegetarians and non-vegetarians (Sambol et al., 2009). On the contrary, Fontana, Shew, Holloszy, and Villareal (2005) found low bone mass at clinically important skeletal regions in raw food vegetarians. Moreover, there are differences in bone density loss between vegetarians and non-vegetarians depending on the gender where bone mineral loss was observed in females and not in males (Marsh, Sanchez, Chaffee, Mayor, & Mickelsen, 1983). These contradictory results could imply a complex relationship between the intake of various nutrients and bone density according to dietary habits that could also vary between males and females. Ho-Pham, Nguyen, and Nguyen (2009) using a meta-analytic approach suggested that vegetarian diets, particularly vegan diets, are associated with lower BMD, but the magnitude of the association is clinically insignificant. Moreover, despite their lower protein and calcium intake, the bone health of vegetarians and vegans is protected by the low acid load of their diet. Only vegans may have a decreased BMD and an increased fracture risk due to low calcium consumption (Burckhardt, 2016). Altogether, according to the available literature, a well-planned and balanced vegetarian diet, accompanied by avoiding risk factors does not result in significant abnormalities in bone status parameters, which was also the case in our study.

5. Conclusions

The obtained results suggest that, although there was no difference in most of the health-related biomarkers in the two observed groups, in this particular study, which had a limited number of volunteers and used only a non-quantitative food frequency questionnaire, the patterns of biomarkers were in favour of the consumption of both plant and animal derived food. It seems that meat consumption did not affect the inflammatory and oxidative status or genome damage in non-vegetarians. Because of the evidence of a lower nutritional status of some micronutrients accompanied with a lower antioxidant defence system and higher genome damage frequency in vegetarians, supplementation of animal derived nutrients to this particular dietary group seems to be beneficial for improving some of the measured health-related biomarkers. It has to be stressed that our population, although belonging to the northern part of Croatia, tended to practice Mediterranean diet patterns, which is visible from our questionnaire. A lower level of meat consumption and preferential consumption of white meat, moderate consumption of fish and sea food accompanied with high consumption of fruits, vegetables and olive oil on a regular basis by non-vegetarians in our study demonstrated that a well-balanced diet including both animal and plant derived foods could be the healthiest choice with regard to the above assessed health-related biomarkers. It has to be pointed out that we also need to define other factors such as epigenetic factors and gene-environment interactions and provide a better definition of the optimal diet and regular physical exercise when assessing health-related dietary differences among the population. Moreover, understanding the influence of nutrition on different metabolic pathways and homeostatic control, as well as the mechanism of its regulation, could lead to evidence-based dietary intervention strategies aimed at restoring health and fitness in order to prevent diet-related diseases which could directly benefit clinicians and nutritionists in counselling regarding nutrition and dietetics. The major limitation of the study is a relatively small study group and the presented multi-biomarker approach indicates the necessity of evaluation of a large number of different parameters if compared with different dietary habits. Further research on a larger sample, especially among people from different geographic areas practicing various diets are warranted in order to facilitate public health programs devoted to the reduction of health risks due to nutritional factors.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics statement

On behalf of, and having obtained permission from all the authors, Goran Gajski declares that the material is not been published elsewhere, the paper is not currently being considered for publication elsewhere, all authors have been personally and actively involved in substantive work leading to the report and will hold themselves responsible for its content. Goran Gajski also declares that there are no potential conflicts of interest related neither to individual authors' commitments, project support nor to commitments of editors, journal staff, or reviewers.

Acknowledgments

The authors would like to thank all of the volunteers who participated in the study. We would also like to thank Ms. Maja Nikolić, Ms. Lea Stančin, Ms. Jasna Mileković and Mr. Mladen Komesar for excellent technical assistance and Mr. Makso Herman for language editing. This study received funding from the Ministry of Science, Education and Sports of the Republic of Croatia (Grant Nos. 022-0222148-2125, 045-1191348-0139, 098-0982913-2768, 0022-0222148-2142, 022-0222148-2135, 022-0222411-2409 and 108-1080316-0300), and was supported by the European Action for Science and Technology (CA COST Actions CA15132 – hCOMET, CA16112 – NutRedOx and CA16113 – *CliniMARK*).

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