HRANA U ZDRAVLJU I BOLESTI
FOOD IN HEALTH AND DISEASE

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THE INFLUENCE OF PHYSICOCHEMICAL CHARACTERISTICS OF THE SOIL ON THE DISTRIBUTION OF HEAVY METALS IN PLANT CULTURES

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Summary

Heavy metals are natural ingredients of many plant cultures, and they are increasingly found in the soil as the main group of inorganic contaminants. The consumption of food products, either fresh or processed, with an increased concentration of heavy metals leads to the deposition of these metals in the body, which further leads to a series of consequences in the form of cardiovascular, respiratory, and neurological problems. The behavior of heavy metals in soil and their accumulation by plants depends on many factors, such as soil structure, soil pH value, organic matter content, adsorption complex and other factors. Therefore, the aim of this work is to present the influence of physical and chemical soil parameters on the distribution of heavy metals (Pb(II) and Cd(II)) in plant cultures and to assess the health risk pollution of the mentioned metals in plant cultures (cucumber, pepper, tomato, eggplant).

Keywords: heavy metals, physicochemical characteristic, soil, plant cultures, health risk

Introduction

Heavy metals, as the main group of inorganic contaminants, are the source of pollution of relatively large areas of the soil, and due to their increased release into the environment, they are receiving increasing attention (Yang et al., 2005). In recent years, pollution by cadmium, one of the most dangerous heavy metals in the environment, is growing rapidly (Jakovljević and et al., 2017). Since many studies increasingly indicate the toxic effect of Cd(II) and Pb(II) on the biological system, soil contamination with these metals has become a major global health and environmental problem.

Cadmium is coming the body through food, drink and inhalation and has a negative effect on the kidneys, liver, bones, and the cardiovascular and respiratory systems (WHO, 1972). Cadmium causes acute and chronic changes in the body and they act cumulatively. When it enters the body, cadmium accumulates in the liver and kidneys, because its biological half-life in the human body is 20 to 30 years. Long-term intake of small concentrations of cadmium can have a major negative effect on human health such as neurotoxicity, respiratory changes. It is important to mention the connection between cadmium and prostate cancer, which IARC classifies as the first cancer group (US.EPA, 1985).

In numerous plant species, the intensity of cadmium transport in the aboveground organs is correlated with its concentration in the nutrient medium. Cadmium adopted from the nutrient medium is mostly retained in the roots, and the share of this element in the stem and leaves of plants is approximately the same or lower than its concentration in the underground part of the plant.

Like cadmium, increasing attention is being paid to lead, which is considered a toxic element for humans and plants. Lead enters the body through food and drink, as well as by inhaling small particles. Ingested lead is mainly deposited in bones, CNS and kidneys. The effects of even a small dose can be seen months and years later. The accumulation of lead in the surface part of the soil is also of great ecological importance since lead has a great influence on the biological activity of the soil. Since lead is generally well bound in all soil types, phytoextraction is limited. When a plant takes up lead, its translocation to higher parts of the plant is very weak. Most of the absorbed lead is concentrated in the roots (Kabata-Pendias, 2001). The intensity of lead contamination of plants decreases with their distance from major roads. Plants poorly absorb lead in inorganic form and transfer it to above-ground organs, except on acidic soils. Organic lead compounds are very quickly taken up and transported to the aerial parts of plants. Lead deposition in most plants is more intense in the roots than in the aerial parts. The great power of the roots in the accumulation of lead could also represent a form of protection of the above-ground part of the plant, since lead in higher concentrations inhibits the elongation of roots and the growth of leaves, inhibits the process of photosynthesis, and affects the morphological and anatomical structure of the plant.

The behavior of heavy metals in the soil is determined by numerous factors that can affect their mobility and accumulation by plants, the most

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important of which are soil structure, soil pH reaction, organic matter content (Pelivanoska et al., 2011). In addition to these, other factors can affect their mobility and harmful effects, such as humidity, calcium carbonate content, hydrated iron and aluminum oxides, cation exchange capacity, redox potential, groundwater level, etc. (Kastori et al., 2003). The characteristic of most toxic elements is that they react with various organic compounds creating stable complexes with ligands containing oxygen, sulfur or nitrogen as electron donors. The toxic effect is based on their irreversible binding to metabolically active groups in amino acids, polypeptides and proteins (Mihaljev et al., 2008).
Therefore, the aim of this work was to examine the physical and chemical characteristics of the soil, determine the concentration of heavy metals (Cd(II) and Pb(II)), and examine the influence of physical and chemical characteristics on the distribution of heavy metals in the most commonly grown vegetable (cucumber, pepper, tomato and eggplant). Correlation analysis determined the degree of soil pollution with heavy metals, determined the connection between the content of heavy metals in plant material and some properties of the soil, as well as the yield of plants, determined the transfer coefficient of the mentioned heavy metals from the soil to the plant, and calculated the risk index in order to assess the impact of heavy metals on human health.

Materials and methods

Materials

The experimental part was carried out at two locations marked as S (Srebrenik) and C (Campus in University of Tuzla) on a total land area of 500 m^2. Soil sampling for analysis was performed according to the instructions of Pernar (Pernar, 2012). In the month of March, an average sample was taken from the mentioned areas at a depth of 0-15 cm. The collected sample was air-dried, and then in an oven at 105 ºC for 3 hours. Along with the soil samples, an analysis of cement dust was performed, which was used for application on the surface of the land, after which plowing was performed in order to also examine the possibility of using cement dust as a substrate for growing vegetable (cucumber, eggplant, pepper, tomato). After 30 days, four plant cultures were planted on both sites with and without added substrate (cement dust), where growth and development were monitored in the March-July period, and after ripening, the fruits were delivered to the laboratory and prepared for analysis on the appropriate parameters.

Methods

The total content of heavy metals (Cd(II) and Pb(II)) in the soil, cement dust and vegetables was determined by the ICP-OES method on the "Perkin Elmer Optima 2100 DV" device. Digestion of samples (soil, dust and plant cultures) weighing 3.0000 g was carried out with 7 ml of concentrated HNO_3 and 4 ml of concentrated H_2O_2 at a temperature of 80 °C, for a total duration of 183 min, in an ultrasonic bath. After digestion, the samples were cooled for 10-15 minutes, and then the flask was topped up to 50 ml (Odobašić et al., 2017). The composition of cement dust was determined by XRD and XRF analysis. The content of humus in the soil and cement dust was determined by the Tjurin method and calculated according to the following equation:

$$\%\ humusa = \frac{(a-b)\cdot 0{,}0005172\cdot 100}{m} \qquad (1)$$

where is:
a-volume of Mohr's salt (ml) used for blank x0.1x 10;
b-volume of Mohr's salt (ml) used for the soil sample x0.1x10;
m-mass of the sample.

Calcium carbonate content by volumetric method BAS ISO 10693:2015 and calculated according to the following relationship:

$$\%\ CaCO_3 = \frac{B-S\cdot C_{NaOH}\cdot E.j._{CaCO_3}\cdot 20}{m} x100 \qquad (2)$$

where is:
B-volume of NaOH used for the titration of the blank sample (ml);
S-volume of NaOH used for soil sample titration (ml);
E.j.($CaCO_3$)-50.04 (mg/mmol)=50.04 (g/mol);
m-mass of the sample (g).

Results and discussion

Physical and chemical characteristics of the soil and cement dust

Table 1. Physical and chemical characteristics of the soil

Parameters	Soil	
	Location C	**Location S**
pH (H_2O)	6.92	5.19
pH (KCl)	5.71	3.93
Content of organic matter (%)	1.189	0.982
Chloride content (mg/g)	4.88	12.2
Electrical conductivity (µS/cm)	34	14.93
TDS (Total dissolved salt, mg/l)	21.76	9.55

Table 2. Physical and chemical characteristics of the cement dust

Parameters	Cement dust
pH (H_2O)	12.76
pH (KCl)	12.7
Content of organic matter (%)	1.01716
Chloride content (mg/g)	12.203
Carbonate content (%)	76.0608
Electrical conductivity (mS/cm)	5.31
TDS (Total dissolved salt, mg/l)	3398.4

As part of the physical and chemical analysis of soil and cement dust, the pH value (in distilled water and KCl), organic matter content, chloride content, electrical conductivity, carbonate content and the total amount of dissolved salts were determined. The pH analysis in KCl showed that it is a slightly acidic soil at locality C (pH=5.71), and a very acidic soil at locality S (pH=3.93). Using the Tjurin method, the humus content was determined to be 1.189% at location C, and 0.982% at location S, which according to Gračanin's classification indicates that the soil is poor in humus (Table 3.), and that the soil is covered with sediment of different granularity. The electrical conductivity value of a soil sample is proportional to the content of readily soluble salts and exchangeable cations present in the soil. (Pavelić T., 2019) By their dissociation or release in water suspension saturates the soil with nutrients and increases electrical conductivity. Increasing electrical conductivity also increases the concentration of total dissolved salts (TDS). The electrical conductivity value determined by conductometry method was 34 µS/cm for the C location and 14.93 µS/cm for the S location, while the amount of total dissolved salts was 21.76 mg/l for the C location and 9.55 mg/l for location S. Since the XRF and XRD analysis showed that the cement dust is rich in minerals and oxides, the expected result of the high value of electrical conductivity and the content of total soluble salts in the cement dust was also confirmed ie 5.31 mS/cm or 3398.4 mg/l.

Table 3. Limit values for humus content in soil (Method by Gračanin)

Soil supply	Humus %
Very poor in humus soil	<1
Poor in humus soil	1-3
Enough humus soil	3-5
Rich in humus soil	5-10
Extremly rich in humus soil	>10

Based on the obtained results shown in Table 2., it can be concluded that cement dust has an extremely basic reaction (pH=12.70), high conductivity (5.31 µS/cm) and high carbonate content (76.0608%), and that as such it is favorable for application to the soil surface, especially on soil with lower pH values. XRD analysis shows that the cement dust is rich in ferrite (25%) and lime (24%), and to a lesser extent maenite, sylvite, etc. (Fig. 1) XRF analysis shows that cement dust is very rich in CaO (68%), followed by K_2O (9%), SiO_2 (7%), Al_2O3 (4%), Fe_2O_3 and Cl (3%), MgO and Na_2O (1%) (Fig.2).

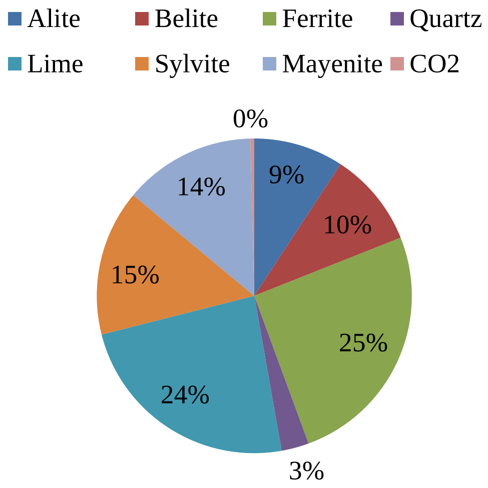

Fig 1. Minerals content in cement dust determined by XRD analysis

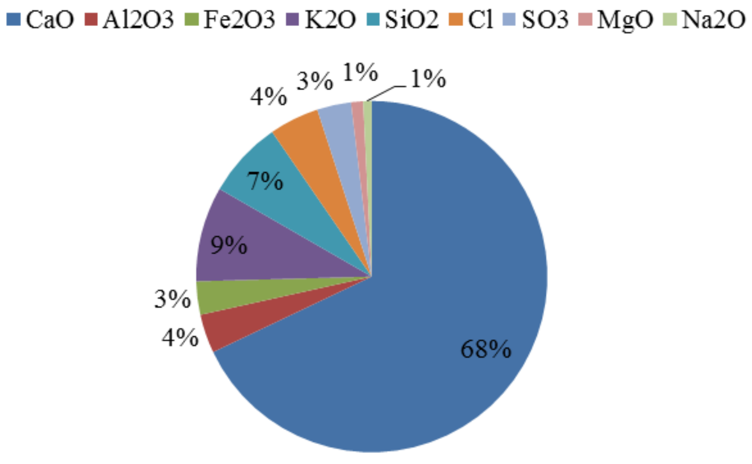

Fig 2. Oxides content in cement dust determined by XRF analysis

The maximum allowed amount (MDK) of dangerous and harmful substances in the soil can damage or change the productive capacity of agricultural land (Jakšić, 2013). Exceeding these values usually come from discharges from factories, spills from landfills, improper use of mineral fertilizers and plant protection products, and as such are defined by the Rulebook on permitted amounts of hazardous and harmful substances in the soil and their testing methods (Official Gazette 52/09). If the land contains a larger amount than the maximum allowed, it is not recommended for agricultural production because it represents a potential danger to human health.

Table 4. MDK Pb and Cd according to the Rulebook on permitted amounts of hazardous and harmful substances in the soil and their testing methods

Heavy metals	MDK for soil (mg/kg)	MDK for cement dust (mg/kg)
Pb	80	500
Cd	1	5

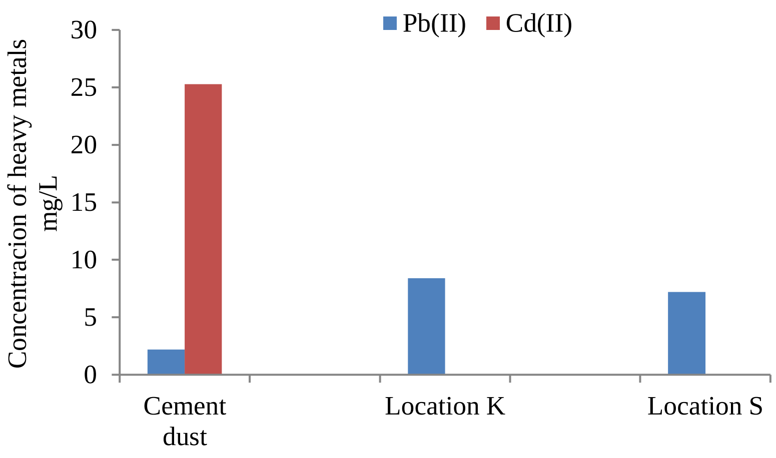

Fig 3. Content of heavy metals in soil and cement dust

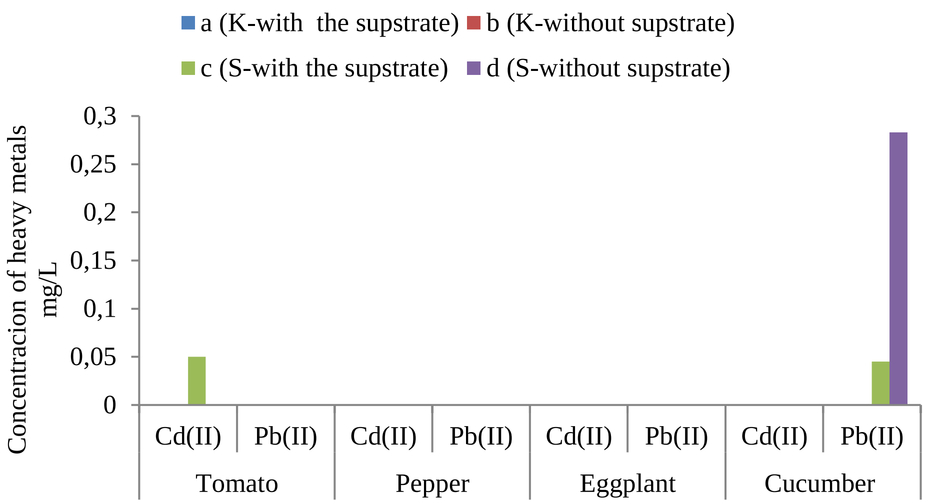

Fig 4. Content of heavy metals in plant cultures (tomato, pepper, eggplant, cucumber)

Determining the content of heavy metals in the soil is a basic indicator for determining the degree of pollution and suitability of the soil for plant production. Based on the presented results, it can be concluded that the soil at location C contains 8.4 mg/kg Pb(II) and 0.05 mg/kg Cd(II), while the analysis of location S showed a Pb(II) content of 7.2 mg/kg, while the Cd(II) content at this location not detected. The stated values do not exceed the permitted values of heavy metals in the soil defined by the Rulebook.

By comparing the obtained values of the content of heavy metals in cement dust with the permitted ones, it can be concluded that the content of the tested heavy metals also does not exceed the permitted values (Regulation on determination of permitted amounts of harmful and waste substances in the soil and methods of their testing, where Article 12 of the above was used of the Rulebook relating to MDK of heavy metals in sludges that are added to the soil in the same way as it was done with cement dust).

The analysis of heavy metals in plant cultures showed that the content of Cd(II) is present in tomatoes at the location S(II) in the sample with the substrate in the amount of 0.05 mg/kg, and Pb(II) in the cucumber in the location S in the sample with the substrate in the amount of 0.045 mg/kg and 0.283 mg/kg in the sample without substrate. Based on the average values of the analyzed elements in the tested samples, their content was in accordance with the Rulebook on maximum allowed concentrations in vegetables (Regulation and maximum allowed amounts for certain contaminants in food Sl.glasnik BIH no. 68). The obtained values of Cd(II) and Pb(II) content show that cement dust is safe for use in agriculture, because research was carried out on several of the most sensitive plant cultures (cucumber, pepper, tomato, eggplant). Also, during the growth and development of the tested cultures on the soil with the addition of cement dust, not a single type of chlorosis or necrosis was observed, and the plant cultures had a normal color and normal growth, which is another indicator of the safe use of cement dust in the soil for the cultivation of the mentioned plants culture.

The degree of soil pollution

Determining the degree of soil pollution is one of the proven ways of determining soil quality, where the necessary remediation measures are provided based on the established actual soil condition (Ahmetović et al., 2020). In many European countries, studies are conducted on the content of toxic metals in the soil, with the aim of managing its flow through the ecosystem and applying protective measures (Lado, 2007). For land used for agricultural purposes, it is necessary to determine the degree of pollution (PD) in order to categorize the land present (Wu, 2010). The degree of pollution is calculated from the following relation:

$$PD\ (\%) = \frac{Cm_{sample}}{C_{lim.value}} x100 \qquad (3)$$

where is:

C_m of the sample - metal concentration in the sample (mg/kg);

$C_{lim.value}$ – limit value of metals in the sample (mg/kg).

Based on the calculated values, the land is classified into one of the categories indicated in Table 5.

Table 5. Classification of soli for agricultural production

Class	Class definition
I	PD<25 %, clean soil, suitable for agricultural production
II	PD=25-50 % soil of increased pollution, cultivation of plants with the necessary protection against the heavy metals imission
III	PD=50-100 % soil of high pollution, cultivation of plants with increased protection measures against heavy metals imission
IV	PD=100-200% polluted soil and unsuitable for cultivation of plants, necessary remedial measures
V	PD>220% extremely polluted soil, ban on the cultivation of plants for human and animal use, necessary to perform comprehensive remediation and recultivation measures.

Table 6. Degree of soil pollution

Heavy metal (mg/kg)	Degree of pollution (PD) %	
	C locality	S locality
Pb	10,5	9
Cd	5	0

Soil pollution is defined as the accumulation of persistent toxic compounds, chemicals, salts, radioactive materials or disease-causing agents in the soil, which have harmful effects on plant growth and animal health (Okrent, 1999). Based on the calculated values, it was determined that the degree of contamination (PD) with lead is 10.5% at location C, and 9% at location S, while the degree of contamination with cadmium at location C is 5%. (Table 6.) Since Cd was not detected in the soil at location S, the level of pollution with this metal is not present either. According to the criteria from Table 5, the land at these locations belongs to Class I, the class of clean land suitable for agricultural production.
In order to determine the phytoremediation potential, the bioaccumulation and translocation factors were determined (Wu et al., 2010). The ability of the plant to accumulate heavy metals is shown as a bioaccumulation factor, which is calculated as the ratio of the metal concentration in the plant to the metal concentration in the soil (Cools, 2010):

$$BF = \frac{C_{plant}}{C_{soil}} \quad (4)$$

where is:
C_{plant}- metal concentration in the plant;
C_{soil}- metal concentration in the soil.

In order to determine the impact of heavy metals on human health, a risk index and risk assessment on human health were determined. International organizations (FAO/IAEA/WHO, JECFA, US EPA) have defined the maximum permissible concentrations of lead and cadmium based on the weekly metal intake of the average weight of an adult, and they amount to Pb-25 μg/kg b.w./week, Cd 7 μg/kg b.w. /weekly. (JECFA, 1993; FAO/WHO Food Standards Programme, 2002; FAO/WHO Standards Programme, 1998) The risk indices were calculated on the basis of metal concentrations obtained from the analysis of plant cultures and calculated according to the following relationship:

$$Risk\ indices = \frac{weekly\ intake\ of\ metals\ through\ vegetable\ consumption}{PTWI\ metals} x\ 100 \quad (5)$$

where:
PTWI is the conditionally acceptable weekly metal intake for adults.

Based on the risk index, an assessment of the impact on human health was calculated. Risk assessment is a conceptual framework that provides a mechanism for reviewing information to assess health or environmental outcomes. (National Research Council, 1983). For this purpose, the risk assessment is carried out using the Fine-Kinney method. (Havula, J. et al., 2011) The principle of this method is based on the assessment of metal exposure (E), consequences (C) and event probability (P) and is calculated according to the following relationship:

$$R = E \cdot P \cdot C \quad (6)$$

where is :
R-risk;
E-exposure;
P-probability of events;
C-consequences.

Table 7. Scoring of exposure and risk assessment to heavy metals

Exposition	Score
Permanently	5
Often	4
Random	3
Rare	2
Very rare	1
Possiblity	**Score**
Frequent	5
Probably	4
Random	3
Not significant	2
Without any significance	1
Consequences	**Score**
Catastrophic	5
Very serious	4
Serious	3
Important	2
Not significant	1
Total risk assessment	
Scores	**Situation**
More than od 100	Very high risk
70-100	High risk
40-70	Important risk
20-40	Possible risk
Below 20	Acceptable risk
Risk assessment to heavy metals	**Score**
Pb	
Exposure	4
Possibility	4
Consequences	4
Total risk assessment for Pb	**64**
Cd	
Exposure	4
Possibility	3
Consequences	4
Total risk assessment for Cd	**48**

Exposure assessment (E) aims to determine the extent and nature of contact with substances in different mouths. It is valued from 1 to 5. When it comes to exposure to heavy metals (Cd(II) and Pb(II)) that can enter the human body daily through food and drink as well as by inhalation, risk assessment of exposure to these metals is common (4).

An assessment of the probability of an event (P) with respect to exposure aims to show the likelihood and predicted consequences. It is valued from 1 to 5. The probability of an event related to Cd (II) exposure to human health is estimated as random (3), since cadmium enters the soil through wastewater, phosphate-based fertilizers, sludge, etc. The probability of an event related to human health exposure to Pb (II) is estimated as probable (4) because large amounts of lead are taken up by plants from the air.

Consequences (C) describe possible side effects that may occur. They are rated from 1 to 5. The consequences of exposure to Cd (II) and Pb (II) are assessed as very serious (4) because the intake of any of these metals leads to neurotoxic, respiratory and kidney problems.

Based on the calculated exposure assessment parameters, it can be concluded that there is a important risk of heavy metals (Cd, Pb) to human health. Table 8. shows the value of the risk index for the used plant cultures and the bioaccumulation factor.

Table 8. Values of the bioaccumulation factor and risk index for the used plant cultures

Bioaccumulation factor	**Tomato**				**Papper**			
	1a	1b	1c	1d	2a	2b	2c	2d
Pb	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Cd	N.D.	N.D.	-	N.D.	N.D.	N.D.	N.D	N.D.
	Eggplant				**Cucumber**			
	3a	3b	3c	3d	4a	4b	4c	4d
Pb	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0,00625	0,0393056
Cd	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Risk indices								
	Tomato				**Papper**			
	1a	1b	1c	1d	2a	2b	2c	2d
Pb	-	-	-	-	-	-	-	-
Cd	-	-	0,238	-	-	-	-	-
	Eggplant				**Cucumber**			
	3a	3b	3c	3d	4a	4b	4c	4d
Pb	-	-	-	-	-	-	0,06	0,377
Cd	-	-	-	-	-	-	-	-

*N.D.-not detected

The risk index was determined in tomatoes and cucumbers from location S, while for other plants the risk index was not determined because the concentration of Pb(II) and Cd(II) in peppers, eggplants, tomatoes and cucumbers from location C was not determined. The risk index for tomato at location S with the addition of substrate was 0.238 % for Pb(II); 0.06% for Pb(II) determined in cucumber at location S with addition of substrate, and 0.377% for Pb(II) determined at location S without addition of substrate. As vegetables are often grown near roads, exposure to lead is a common occurrence, so the impact of the risk is also very high. The present lead is incorporated into the plant structure and reaches its edible parts, which is why the consequences of intake of this heavy metal are very high.

Conclusions

The analysis of the soil of localities S and C showed that it is a slightly acidic soil at location C, and a very acidic soil at location S (pH values determined in KCl). The analysis also showed that the mentioned locations are very poor in humus, and that the values of Pb(II) and Cd(II) are in accordance with the limit values determined according to the Rulebook. The analysis of the cement dust that was used as a substrate showed that it is suitable and safe for use in agriculture production.

The analysis of heavy metals in plant cultures showed the content of Cd(II) present in tomatoes at the location S in the sample with the substrate in the amount of 0.05 mg/kg, and Pb(II) in the cucumber in the location S in the sample with the substrate in the amount of 0.045 mg/kg and 0.283 mg/kg in the sample without substrate. The values found are the result of mostly lower soil pH, which can significantly affect the mobility of heavy metals, which is very important from the safety point of view. Based on the calculated values, it was determined that the degree of lead pollution (PD) is 10.5% at location C, and 9% at location S, while the degree of cadmium pollution at location C is 5%, and that the land at both sites corresponds to class I, suitable for agricultural production.

The risk index for tomatoes at location S with the addition of substrate was 0.238 % for Pb(II); 0.06% for Pb(II) determined in cucumber at location S with addition of substrate, and 0.377% for Pb(II) determined at location S without addition of substrate. As vegetables are often grown near roads, exposure to lead is a common occurrence, so the impact of the risk is also very high.

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USE OF COCOA SHELL IN INSTANT COCOA POWDERS – RESEARCH OF DIFFERENT HYDROCOLLOID ADDITIONS AND DRYING TECHNIQUES

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Summary

The aim of this research was to examine the influence of the addition of cocoa shell, xanthan gum, guar gum, gum arabic, and carboxymethylcellulose (CMC), as well as drying procedures on the properties of instant cocoa beverages. The cocoa shell was separated from the cocoa bean after roasting, milled, and after milling a fraction smaller than 71 µm was separated by sieving, and used in production. Instant powders were produced by agglomeration and drying (freeze-drying and oven-drying at 40 °C). The results showed that higher content of cocoa shell in instant cocoa beverages increased the wetting time, while the colour of the oven-dried powders was lighter compared to the freeze-dried samples. The bulk density of the powder was higher in samples with a higher content of shell. In samples with a higher amount of cocoa shell, the content of total polyphenols decreased compared to the samples without cocoa shell.

Keywords: cocoa beverage, cocoa shell, xanthan gum, guar gum, gum arabic, CMC, oven-drying, freeze-drying

Introduction

Instant cocoa powder is produced from cocoa powder, sugar and emulsifier as main raw materials, to which milk powder and hydrocolloids may be added as well, depending on the recipe. It is easily reconstituted in water or milk and has a long storage period. The production process includes agglomeration to increase dispersibility of the powder in milk or water. The agglomeration is a process in which cocoa powder is mixed with sugar and stabilizers with the addition of water to produce larger conglomerates, which are afterwards dried (Benković et al., 2015). Prolonged drying at elevated temperatures may cause loss of vitamins, minerals and other bioactive compounds, as well as influence colour and aroma of the final product. In addition, sugar crystallizes at relatively low temperatures and may cause problems during drying (Sedlar and Pervan, 2010; Hardy and Jideani, 2017). The alternative for conventional drying would be freeze-drying. The advantages of this process are the application of low temperatures and preservation of thermolabile compounds, better rehydration properties, and better efficiency of production. However, it requires higher energy input, both for freezing and freeze-drying, and demands moisture- and air-tight proof packaging (Hardy and Jideani, 2017).

As the demand for cocoa is increasing, while cocoa supply remains limited, it is justified to seek an alternative for cocoa powders in instant cocoa beverages. Cocoa shell is a by-product of cocoa industry with characteristic cocoa aroma, and is also rich in fibres, proteins and bioactive compounds. Use of cocoa shell in processing of cocoa products would reduce negative ecological and environmental effects of cocoa industry, and increase the production efficiency (Okiyama et al., 2017).

In our previous researches (Trgovac et al., 2022; Barišić et al., 2022; Barišić et al., 2021), we have successfully implemented cocoa shell in production of chocolate and chocolate fillings, and the aim of this research was to explore potential of using cocoa shell in production of instant cocoa beverages. Therefore, we investigated different proportions of cocoa shell along with the addition of different hydrocolloids (xanthan gum, guar gum, gum acacia and carboxymethyl cellulose (CMC)) as stabilizers, and compared the influence of oven-drying and freeze-drying.

Materials and methods

Samples

Cocoa shell was separated from the beans after the roasting, milled and sieved to obtain particles smaller than 71 µm.

Instant cocoa powders (Figure 1) were prepared according to recipes shown in Table 1, using cocoa

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powder (Kandit d.d. Osijek, Croatia), cocoa shell (substituting cocoa powder in proportions 5, 10 and 15 %), 69.5% sugar (Viro d.d. Virovitica, Croatia) and 0.4% lecithin (ACEF; Italy). Hydrocolloids: xsanthan gum, guar gum, gum arabic, powdered and liquid CMC were added in 0.1%. Instant powders were produced by agglomeration and drying in oven at 40 °C or by freeze-drying at the pressure of 0.25 mbar. Before freeze-drying, samples were frozen in ultra-freezer at –80 °C.

Table 1. Recipes of instant cocoa powders

SAMPLE*	COCOA POWDER (%)	COCOA SHELL (%)	XANTHAN GUM (%)	GUAR GUM (%)	GUM ARABIC (%)	CMC POWDER (%)	CMC LIQUID (%)
KG	30	0	0.1				
GG	30	0		0.1			
GA	30	0			0.1		
CP	30	0				0.1	
CT	30	0					0.1
KG-LJ5	25	5	0.1				
GG-LJ5	25	5		0.1			
GA-LJ5	25	5			0.1		
CP-LJ5	25	5				0.1	
CT-LJ5	25	5					0.1
KG-LJ10	20	10	0.1				
GG-LJ10	20	10		0.1			
GA-LJ10	20	10			0.1		
CP-LJ10	20	10				0.1	
CT-LJ10	20	10					0.1
KG-LJ15	15	15	0.1				
GG-LJ15	15	15		0.1			
GA-LJ15	15	15			0.1		
CP-LJ15	15	15				0.1	
CT-LJ15	15	15					0.1

*All samples contained 69.5% sucrose and 0.4% lecithin.

Figure 1. Samples of instant cocoa powder with powdered CMC before drying (left to right: sample without cocoa shell, sample with 5% cocoa shell, sample with 10% cocoa shell and sample with 15% cocoa shell)

Colour

Colour was measured in CIEL*a*b* and LCh° systems, using chromameter (Konica Minolta CR-400, Tokio, Japan) using extension for powdered samples. Five measurements were done for each sample, and an average and standard deviation were calculated for the expression of the results.

Parameters are as follows:
L* - lightness (0 – black, 100 – white)
a* - negative values are green domain, positive ones are in red
b* - negative values are in blue domain, positive in yellow
C – chroma, the distance from the lightness axis, starts with 0 in the center
h° – hue, starts in red axis: 0 ° is +a*, 90 °C is +b*

Total colour difference was calculated in relation to the sample with xanthan gum, according to Equation 1:

$$E = \sqrt{(L^* - L_0^*)^2 + (b^* - b_0^*)^2 + (a^* - a_0^*)^2} \quad (1)$$

where: L^*_0, b^*_0, a^*_0 represent values for sample with xanthan gum.

Wettability

Wettability of instant cocoa powders was determined according to Schubert (1980), and the obtained results are expressed as the time (in seconds, s) needed for all the powder (2 g) to wet and penetrate through the surface of the distilled water (20 mL) at ambient temperature. All analyses were done in triplicates.

Bulk density

Bulk density was determined according to Escalada Pla et al. (2012). Each sample was weighed at the analytical balance into the cup to fill the pre-determined volume. All analyses were done in triplicates.

Total phenolic compounds

The extraction of phenolic compounds was done according to Adamson et al. (1999). Instant cocoa powders were defatted with *n*-hexane, where 2 g of sample was extracted with 10 mL of *n*-hexane three times and air-dried for 24 h. Defatted samples were extracted three times with 5 mL of 70% methanol, using ultrasound for 30 min. Samples were centrifuged at 3000 rpm for 10 min and supernatants were collected in 10 mL-measuring flask and filled up with 70% methanol.
Modified Folin-Ciocalteu method, in acidic conditions (without Na_2CO_3) was used for measurement of total phenolic compounds (Vinson et al., 2001) to avoid interference of sugars. To 100 μL of the sample, 1 mL of 10% Folin-Ciocalteu reagent was added. After 2 min, the mixture was incubated for 20 min at ambient temperature in dark. Absorbance was measured at 750 nm, and quantification was done using gallic acid as the standard (concentration range 0.14 – 0.70 mg/mL). The results are expressed as mg of gallic acid equivalent per g of defatted sample (mg GAE/g).

Results and discussion

Samples of instant cocoa powder were prepared according to 20 recipes shown in Table 1. A part of each sample was oven-dried at 40 °C, and the other part was freeze-dried, so in total 40 samples were prepared. Colour parameters of the samples are shown in the Table 2. In the oven-dried samples, although in majority of cases cocoa shell added in 5% caused a slight decline of L* values, the increase of its proportion in the powder caused an increase of L* values. In freeze-dried samples this trend was not so pronounced, although it is visible that increase of cocoa shell contents generally increases L* values. Additionally, when compared to oven-dried counterpart, freeze-dried samples had lower values of L*, indicating that they were darker. The differences between a* and b* values between oven- and freeze-dried samples were less pronounced, also with lower values detected in freeze-dried samples. When comparing hydrocolloids, CMC resulted in higher values of measured colour parameters than other three. However, combined effect of all colour parameters are brown in all samples, with small differences in the shade. This is supported by calculated ΔE values shown in Figure 2. All ΔE values are below 4, what shows that colour differences are perceptible by human eye only by close observation. Chroma values and hue were also higher for oven-dried samples. All these imply that consumers would prefer oven-dried samples due to more pronounced and “clearer” brown, which is associated with higher content of cocoa (Folkenberg et al., 2007). Redgwell et al. (2003) reported higher proportions of fibers, mono- and polysaccharides in cocoa shell in relation to cocoa bean. Fibres are good water adsorbents and could contribute to darkening, along with Maillard reactions products and protein-tanin complexes which are usually generated during the thermal processing.

Table 2. Colour parameters of instant cocoa powders measured by chromameter

SAMPLE	L*	a*	b*	C	h°
OVEN DRYING					
KG	40.90 ± 0.04	10.51 ± 0.05	14.49 ± 0.04	17.90 ± 0.02	54.04 ± 0.19
GG	39.74 ± 0.02	10.88 ± 0.08	14.39 ± 0.06	18.04 ± 0.10	52.91 ± 0.12
GA	41.75 ± 0.02	9.88 ± 0.06	14.17 ± 0.07	17.28 ± 0.05	55.10 ± 0.26
CP	40.26 ± 0.02	10.69 ± 0.07	14.54 ± 0.04	18.05 ± 0.05	53.68 ± 0.19
CT	42.89 ± 0.02	10.83 ± 0.05	15.67 ± 0.05	19.05 ± 0.04	55.34 ± 0.17
KG-LJ5	39.42 ± 0.05	9.69 ± 0.04	13.34 ± 0.05	16.49 ± 0.03	54.01 ± 0.25
GG-LJ5	38.63 ± 0.02	9.80 ± 0.06	13.17 ± 0.02	16.42 ± 0.04	53.31 ± 0.21
GA-LJ5	38.04 ± 0.05	9.57 ± 0.06	12.96 ± 0.04	16.12 ± 0.05	53.75 ± 0.38
CP-LJ5	41.40 ± 0.46	9.60 ± 0.06	13.59 ± 0.03	16.64 ± 0.03	54.78 ± 0.20
CT-LJ5	41.13 ± 0.01	9.92 ± 0.01	13.85 ± 0.16	17.08 ± 0.04	54.51 ± 0.09
KG-LJ10	42.77 ± 0.01	9.50 ± 0.03	14.69 ± 0.13	17.48 ± 0.08	57.18 ± 0.15
GG-LJ10	40.96 ± 0.03	10.05 ± 0.06	14.68 ± 0.01	17.79 ± 0.03	55.61 ± 0.16
GA-LJ10	41.73 ± 0.02	10.26 ± 0.02	15.23 ± 0.03	18.37 ± 0.04	56.02 ± 0.04
CP-LJ10	43.13 ± 0.04	10.11 ± 0.06	15.46 ± 0.04	18.47 ± 0.03	56.82 ± 0.20
CT-LJ10	42.49 ± 0.02	9.62 ± 0.05	14.78 ± 0.03	17.63 ± 0.02	56.93 ± 0.16
KG-LJ15	44.61 ± 0.01	9.78 ± 0.03	16.06 ± 0.03	18.80 ± 0.01	58.65 ± 0.13
GG-LJ15	44.06 ± 0.05	9.71 ± 0.04	15.65 ± 0.03	18.42 ± 0.03	58.17 ± 0.15
GA-LJ15	42.39 ± 0.01	9.77 ± 0.05	15.06 ± 0.04	17.95 ± 0.02	57.03 ± 0.18
CP-LJ15	44.55 ± 0.04	9.55 ± 0.07	15.58 ± 0.06	18.27 ± 0.03	58.49 ± 0.26
CT-LJ15	44.46 ± 0.02	9.60 ± 0.05	15.63 ± 0.04	18.35 ± 0.02	58.43 ± 0.18
FREEZE-DRYING					
KG	37.42 ± 0.02	10.12 ± 0.04	12.55 ± 0.03	16.12 ± 0.01	51.13 ± 0.16
GG	38.81 ± 0.02	10.51 ± 0.04	13.64 ± 0.03	17.22 ± 0.01	52.41 ± 0.15
GA	36.46 ± 0.02	10.96 ± 0.03	12.70 ± 0.03	16.78 ± 0.03	49.18 ± 0.11
CP	38.03 ± 0.01	10.51 ± 0.03	13.23 ± 0.02	16.90 ± 0.01	51.55 ± 0.13
CT	39.54 ± 0.01	9.58 ± 0.05	11.89 ± 0.04	15.27 ± 0.02	51.14 ± 0.25
KG-LJ5	38.04 ± 0.05	9.57 ± 0.06	12.96 ± 0.04	16.12 ± 0.05	53.75 ± 0.38
GG-LJ5	37.59 ± 0.07	9.41 ± 0.05	12.50 ± 0.05	15.65 ± 0.02	53.03 ± 0.26
GA-LJ5	37.30 ± 0.03	9.68 ± 0.04	12.71 ± 0.04	15.98 ± 0.04	52.72 ± 0.13
CP-LJ5	37.39 ± 0.03	10.13 ± 0.06	13.20 ± 0.03	16.65 ± 0.03	52.50 ± 0.22
CT-LJ5	35.92 ± 0.02	10.01 ± 0.04	12.64 ± 0.06	16.12 ± 0.04	51.61 ± 0.20
KG-LJ10	39.71 ± 0.03	9.18 ± 0.07	12.98 ± 0.05	15.90 ± 0.03	54.73 ± 0.27
GG-LJ10	40.10 ± 0.03	9.22 ± 0.01	13.30 ± 0.05	16.18 ± 0.05	55.26 ± 0.08
GA-LJ10	34.97 ± 0.02	9.28 ± 0.04	11.36 ± 0.05	14.50 ± 0.30	50.46 ± 0.23
CP-LJ10	39.46 ± 0.38	9.39 ± 0.12	13.48 ± 0.09	16.43 ± 0.10	55.13 ± 0.38
CT-LJ10	38.36 ± 0.02	9.42 ± 0.04	13.31 ± 0.03	16.30 ± 0.03	54.71 ± 0.15
KG-LJ15	39.49 ± 0.03	9.25 ± 0.05	13.53 ± 0.03	16.39 ± 0.02	55.64 ± 0.20
GG-LJ15	37.16 ± 0.04	9.08 ± 0.03	12.03 ± 0.05	15.28 ± 0.03	52.95 ± 0.19
GA-LJ15	35.69 ± 0.02	9.09 ± 0.04	11.72 ± 0.03	14.84 ± 0.02	52.21 ± 0.19
CP-LJ15	38.57 ± 0.03	9.07 ± 0.05	12.95 ± 0.04	15.82 ± 0.05	55.00 ± 0.16
CT-LJ15	38.99 ± 0.06	8.89 ± 0.06	13.06 ± 0.03	15.80 ± 0.04	55.76 ± 0.21

KG – xanthan gum, GG - guar gum, GA – gum arabic, CP – powdered CMC, CT – liquid CMC, LJ5 – 5% cocoa shell, LJ10 – 10% cocoa shell, LJ15 – 15% cocoa shell; all samples contained 69.5% sugar and 0.4% lecithin

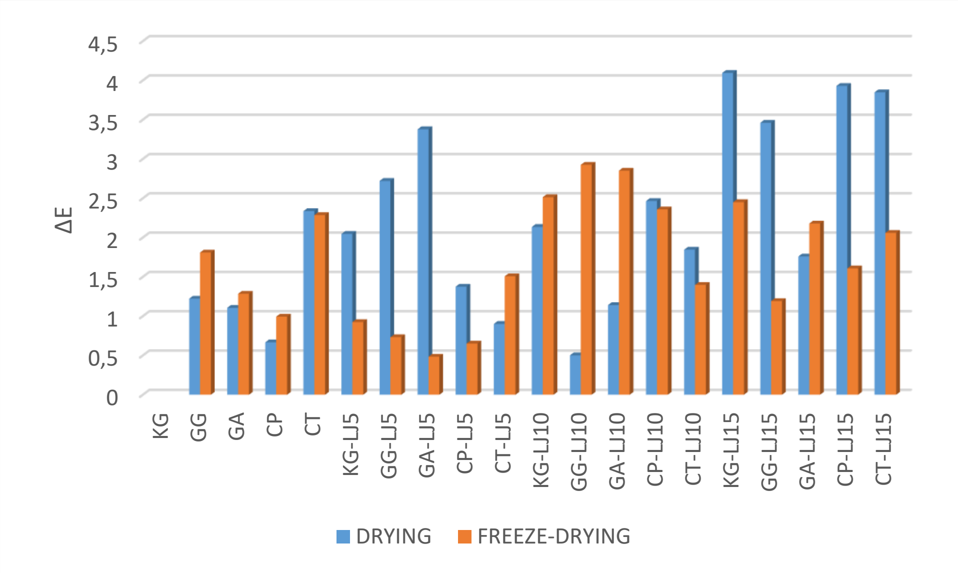

Figure 2. Total colour difference (ΔE) between instant cocoa powder samples dried in certain regime. Sample with xanthan gum was taken as a reference
(KG – xanthan gum, GG - guar gum, GA – gum arabic, CP – powdered CMC, CT – liquid CMC, LJ5 – 5% cocoa shell, LJ10 – 10% cocoa shell, LJ15 – 15% cocoa shell; all samples contained 69.5% sugar and 0.4% lecithin)

Wettability was better in the samples without added cocoa shell (Figure 3). The time needed for all the powder to penetrate through the surface increased with the higher content of cocoa shell. Oven-dried samples with 15% of cocoa shell needed significantly longer time to wet than freeze-dried counterparts, unlike the samples with 5 and 10% of cocoa shell, where better wettability was achieved by oven-drying. Wettability of these samples (app. 10 – 40 s) was in the range reported by Buljat et al. (2019) for samples of foam mat dried cocoa powders. Gum arabic significantly prolonged wetting time when instant cocoa powders were prepared without and with 5% cocoa shell, while samples with higher amounts of shell showed this effect in cases when guar and xanthan were used.. This points out that selection of hydrocolloids should largely depend on the raw materials and their proportions in the formulations. Shittu and Lawal (2005) reported that particle size, fat contents and bulk density influence wettability, while Cvitanović et al. (2010) reported that wettability may be improved by the addition of sugar (it has the highest solubility), agglomeration, addition of lecithin or by their combination.

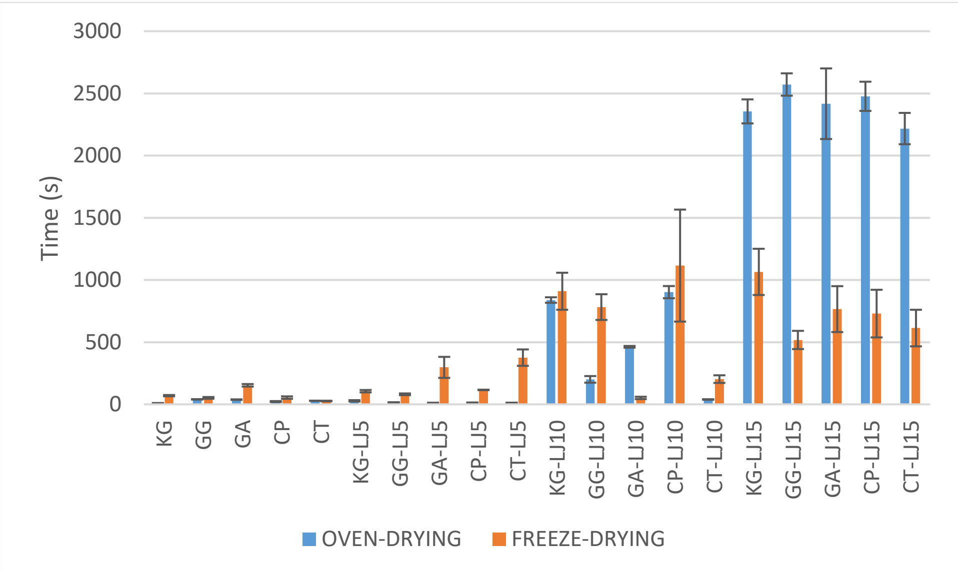

Figure 3. Wettability of instant cocoa powder samples
(KG – xanthan gum, GG - guar gum, GA – gum arabic, CP – powdered CMC, CT – liquid CMC, LJ5 – 5% cocoa shell, LJ10 – 10% cocoa shell, LJ15 – 15% cocoa shell; all samples contained 69.5% sugar and 0.4% lecithin)

Bulk density of the samples ranged 0.95 – 1.09 g/cm^3 for oven-dried, and 0.93 – 1.06 g/cm^3 for freeze-dried samples (Figure 4). Freeze-dried samples had smaller bulk density than their oven-dried counterparts, with the exception of the samples with 10% of cocoa shell. During freeze-drying, water sublimates, while during oven-drying water evaporates. Ice particles take more space than liquid water, therefore larger porosity is a common result of freeze-drying (Buljat et al., 2019). Chaloeichitratham et al. (2018) reported lower bulk density of freeze-dried curry compared to oven-dried samples.

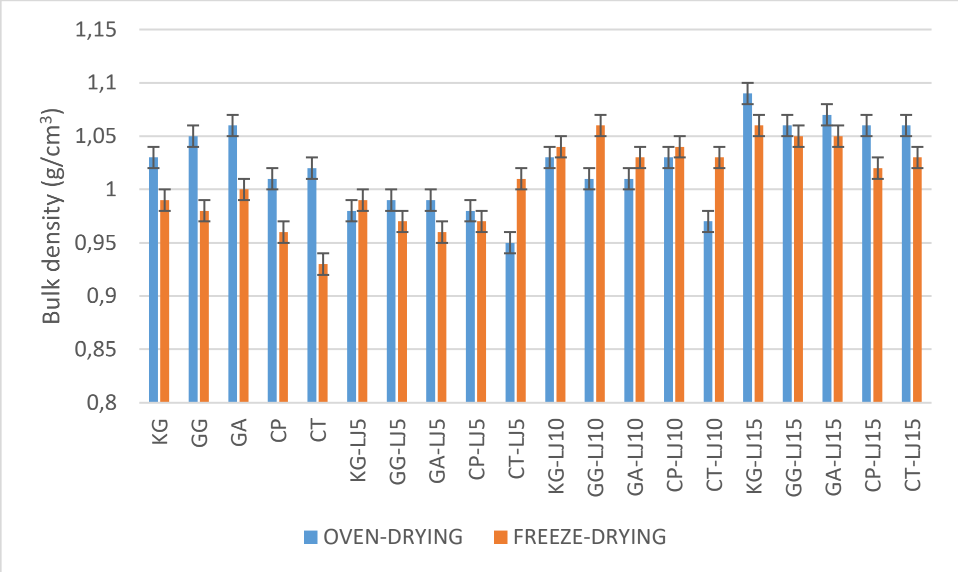

Figure 4. Bulk density of instant cocoa powder samples
(KG – xanthan gum, GG - guar gum, GA – gum arabic, CP – powdered CMC, CT – liquid CMC, LJ5 – 5% cocoa shell, LJ10 – 10% cocoa shell, LJ15 – 15% cocoa shell; all samples contained 69.5% sugar and 0.4% lecithin)

According to literature data, content of phenolics in cocoa powder is 48 ± 2.1 mg/g (Crozier et al., 2011). Contents of total phenolic compounds (TPC) in this research ranged from 18.41 mg GAE/g of defatted sample for the freeze-dried sample with xanthan gum and 15% of cocoa shell to 32.88 mg GAE/g of defatted sample for oven-dried sample with powdered CMC and 5% of cocoa shell (Figure 5). All oven-dried samples except the sample with guar gum and without cocoa shell had higher TPC content than their freeze-dried counterparts, and increase of cocoa shell contents decreased TPC content what was already observed in our previous research on chocolate (Barišić et al., 2020), and in Karim et al. (2014) who reported lower TPC in cocoa shell in relation to cocoa bean. Although freeze-drying preserves thermolabile compounds and higher content of TPC has been reported for freeze-dried cocoa beans (Majid and Rining, 2018) and freeze-dried jujube (Wojdylo et al., 2019), the results of this research may be explained by formation of coloured and reducing compounds during oven-drying (Platzer et al., 2021; Everette et al., 2010).

The influence of hydrocolloids on preservation of phenolic compounds did not follow any trend and it depended both on drying process and the proportion of cocoa shell in the sample.

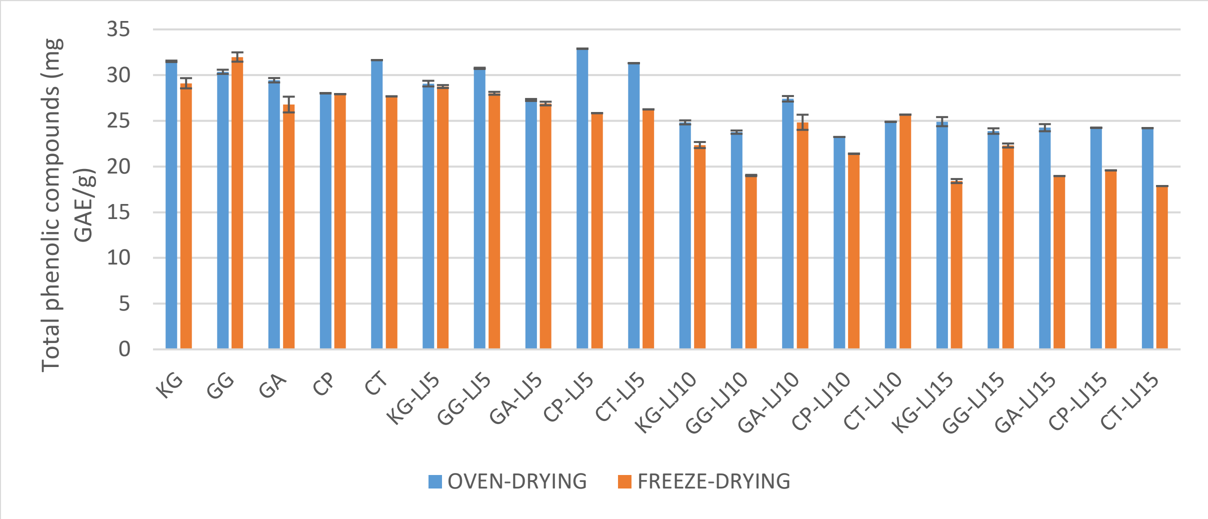

Figure 5. Content of total phenolic compounds of instant cocoa powder samples
(GAE- gallic acid equivalents, KG – xanthan gum, GG - guar gum, GA – gum arabic, CP – powdered CMC, CT – liquid CMC, LJ5 – 5% cocoa shell, LJ10 – 10% cocoa shell, LJ15 – 15% cocoa shell; all samples contained 69.5% sugar and 0.4% lecithin)

Conclusion

The results showed that increasing the content of cocoa shell in instant cocoa beverages increased the wetting time, and the colour of convection-dried powders was lighter compared to the freeze-dried samples. The bulk density of the powders was higher in samples with a higher content of shell. In samples with a higher proportion of cocoa shell, the content of total phenolics decreased compared to the samples without cocoa shell.
Although none of the hydrocolloids used in the research stand out as the best one, additional research on other properties (e.g. microbial quality, particle size, polyphenolic profile, fiber content etc.) and sensory analysis could reveal this issue.

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NUTRITION AS A SCIENTIFIC FIELD IN CROATIA: BIBLIOMETRIC ANALYSIS OF THE PERIOD 2010 – 2020

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Summary

The scientific community’s interest in nutrition, i.e., the interaction of human and food consumption, is constantly rising worldwide and in Croatia. The published research results deepen our understanding of the processes involved in the interplay between food consumption and health, as well as different diseases and malfunctions in the human body. This research provides insight into scientific themes and focuses on nutrition research in Croatia.
The study aims to provide an overview of the development of nutrition as a scientific field in Croatia from 2010 to 2020 using descriptive bibliometric analysis and science mapping. The analysis was made on a sample of 1173 articles indexed in the Web of Science Core Collection (WoS CC) database.
In the analyzed period, nutrition and its impact on people's health, especially on children's health, are the focus of Croatian scientists' research. Furthermore, it can be concluded that the same research trends continue in future research.

Keywords: nutrition, Croatia, bibliometric analysis, science mapping

Introduction

Nutrition or food science is a scientific discipline, with intensive growth in the world in the last 150 years. Kenneth J. Carpenter reviewed the development of nutrition in his articles, clearly showing that scientists have recognised the importance of nutrition both for the individuals and for society (Carpenter, 2003a; 2003b; 2003c; 2003d). Understanding food properties and eating habits are fundamental because they are the basis for nutritional improvements which directly influence human health (Ulfa et al., 2022). National legislation regarding food (National Gazette 81/13, 14/14, 30/15, 115/18), food hygiene and microbiological criteria for food (National Gazette 81/13, 115/18) in Croatia was adopted in 2013. According to the Agency for Science and Higher Education and Database of Study Programmes (MOZVAG), there are different nutrition-related studies in Croatia (Table 1). Faculty of Food Technology and Biotechnology, University of Zagreb offers under- and graduate programs in Nutrition, as well as postgraduate university PhD study programs in Biotechnology and Bioprocess Engineering, Food Technology and Nutrition. In contrast, Faculty of Food Technology Osijek, Josip Juraj Strossmayer University of Osijek (PTFOS) offers postgraduate university PhD programs in Food Technology and Nutrition, in addition to postgraduate specialist study in Nutrition (MOZVAG Database – selection of scientific field 2022). Food Science and Nutrition graduate study is also offered at PTFOS, but this programme is categorized in the field food technology in the MOZVAG database (MOZVAG Database – food technology 2022).

Table 1. Study programs in the scientific field of Nutrition, grouped by degree holder (source: MOZVAG Database – selection of scientific field, 2022)

Title of the study programme	Holder*	Type of study	Duration	Qualification
Nutrition	PBFZG	undergraduate university study	3	EQF6 Bachelor
Nutrition	PBFZG	Graduate university study	2	EQF7 Master
Biotechnology and Bioprocess Engineering, Food Technology and Nutrition	PBFZG	Postgraduate university (PhD) study	3	EQF8 PhD
Food Technology and Nutrition	PTFOS	Postgraduate university (PhD) study	3	EQF8 PhD
Nutrition	PTFOS	Postgraduate university (specialist) study	1	specialist master, university specialist

*PBFZG, Faculty of Food Technology and Biotechnology Zagreb; PTFOS, Faculty of Food Technology Osijek

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In his model of knowledge accumulation, Price asserted in 1963 that scientific progress is achieved by the researchers with local, national and international characteristics who study scientific themes by relying on work of other scientists (Price, 1963). Bibliometrics is a method of quantitative study of the presentation of knowledge through the investigation of the properties and behaviour of bibliographic records, independent of the content of scientific literature ("Bibliometrija", 2021). Bibliometric indicators have proven to be useful tools for evaluating research results, provided that they have a sufficient level of sophistication, that their pitfalls are taken into account, and that they are used in combination with other, more qualitative knowledge about the units to be evaluated (Moed, 2000). Scholarly communication is constantly growing, making being up-to-date with published works very hard in any scientific field, including nutrition. Consequently, the number of systematic reviews of the literature demonstrating dynamics of the scientific development in particular scientific fields through the establishment of knowledge bases, research focal points, and research fronts over a time period and indicating the current themes in scientific field is also increasing. This is also the case in the realm of nutrition (Figure 1), where a significant increase after 2018 may be observed.

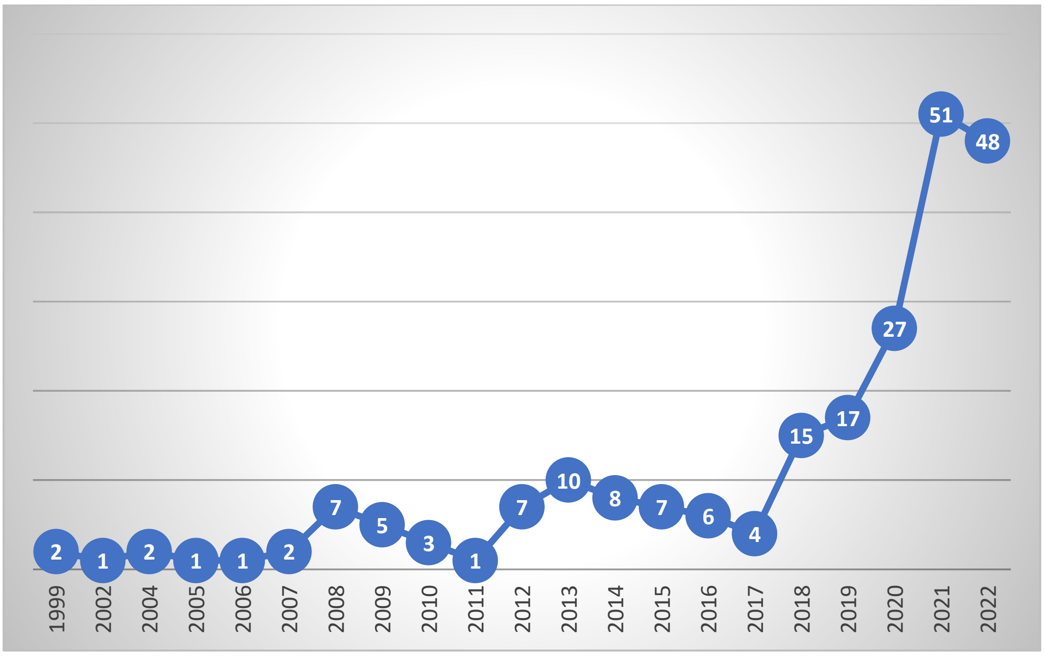


Figure 1. The number of published systematic reviews in the field of nutrition (*source: Web of Science Core Collection*)

Descriptive research is research that studies the number of publications in certain fields in a way that compares research patterns in the analysed period, either for a particular scientific field, institution or country (Pehar, 2010). In numerous nutrition-related literature reviews, descriptive bibliometric analysis has already been conducted. Smith (1999) identified the leading journals in dietetics and established the indexing scope. van Raan and van Leeuwen (2002) reviewed the research undertaken at the Institute for research of food and nutrition in Netherlands, as well as its influence and interdisciplinarity, along with methodological discussion of bibliometric indicators applied in the analysis. There have also been reviews of the scientific activities concerning flavonoids in food, the influence of various plants as suitable food supplements, and phytochemicals and their effects on human body (Perez-Vizcaino and Fraga, 2018; Chhicara et al., 2021; Venthodika et al., 2021). Different authors have also published systematic literature reviews of different commercially available foods and food components utilizing descriptive bibliometric methods and science mapping. Kamdem et al. (2019) provided a bibliometric overview of the most significant scientific achievements published in the journal Food Chemistry since its first issue (1976). Skaf et al. (2020) conducted a literature review on food safety using social networks analysis. Shen, Wei and Sheng (2021) determined the current characteristics and key points of food safety management, envisaging future research trends. Sendhil et al. (2022) provided bibliometric trends of

the literature addresing consumer preferences towards genetically modified food, whereas Young et al. (2019) reviewed literature on resveratrol. Mbao et al. (2022) analysed trends in the usage of diatoms in water quality monitoring and bioassessment in Africa.
This study aims to examine the field of nutrition in the Republic of Croatia from 2010 to 2020 through descriptive bibliometric analysis and science mapping.

Material and methods

Different bibliometric determinants targeting measurement and evaluation of activities and influences of authors, journals and institutions were included in the descriptive bibliometric analysis, and scientific mapping was used to visualize the dynamics of the development of scientific research in the field of nutrition in Croatia. The scientific collaboration was analysed using affiliations stated in the articles (Supplementary material 1), where articles with only Croatian affiliations were separated from those with affiliations in other countries. These provided answers to questions regarding the formation of knowledge bases, the evolution of research focal points and research fronts across time, and potential future research directions. The research is designed to encompass bibliometric elements of nutrition research and present them using the different visualisation methods and techniques.

Data source and literature search strategy

The Web of Science Core Collection (WoS CC) citation indexes were used as a starting point for identifying and acquiring relevant scientific publications on nutrition published by authors with Croatian affiliations. The search was conducted using the terms “nutrition” in *All fields* and “Croatia” in *Address*, with the Boolean operator AND connecting the terms. This search strategy resulted in a total of 2522 publications, which were later limited to the period 2010 – 2020 (1620 results). Abstracts (97), proceedings papers (50), editorials (28), book chapters (28), corrections (16), letters (16), notes (4), publications with expression of concerns (1), retracted publication (1) were excluded from the sample because of their minor relevance for the purpose of this study. In the last round, 1171 journal articles and 210 reviews were selected.
Articles on animal feed, plant nutrition, history, medical research of influence of medicaments on human body, medicinal care, aquaculture, food industry by-products, genetic analyses, morphometry (non-invasive method for determination of size, proportion and composition of human body) were excluded from the subsequent analysis based on an additional title, keyword and abstract screening (208). As a consequence, 1173 articles were selected as a representative sample for bibliometric analysis.

Bibliometrics analysis and science mapping

Data from WoS CC were exported into spreadsheet and tabular formats for further bibliometric analysis and scientific mapping. Visualisation mapping was performed using CiteSpace II. The algorithm of spectral grouping (tf*idf), log-likelihood ratio tests, and mutual information were used to classify the data in CiteSpace II. Clusters were assigned based on the size of the data (the largest is always #0). Modularity and pondered mean silhouette are metrics that indicate the quality of grouping data into clusters and are important parameters for data analysis and interpretation. The higher the modularity and silhouette, the better the results (Chen and Song, 2019). According to Chen, Ibekwe-SanJuan and Hou (2010), focal points of research and research trends can be revealed by citing different types of nodes, such as keywords, authors, institutions, countries and references, as well as concurrent phenomena.

Results and discussion

The language of published articles

The research includes articles and reviews published in scholarly journals. The majority of the 1173 articles are written in English (96.5%), with 29 articles (3%) written in Croatian and three articles (0.29%) in German, one in Czech and one in Italian (Figure 2).

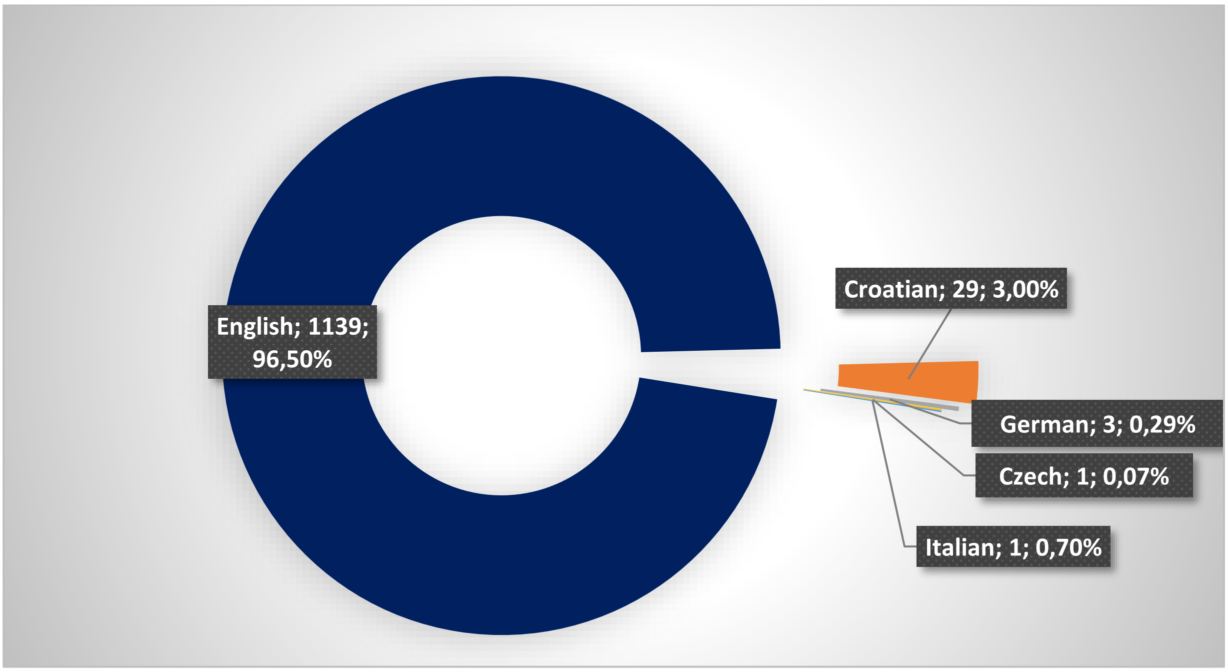


Figure 2. Language of articles published by Croatian scientists in WoS CC-indexed journals between 2010 and 2020

Annual growth of nutrition-related journal articles in Croatia

The analysis of the number of published articles indexed in WoS CC indicates a steady increase in nutrition research by Croatian scientists. During the period covered by this study, the number of publications tripled (Figure 3), suggesting that Croatian researchers are following the global trend of increasing interest in this scientific topic. Since 2017, the number of articles published in nutrition has increased significantly.

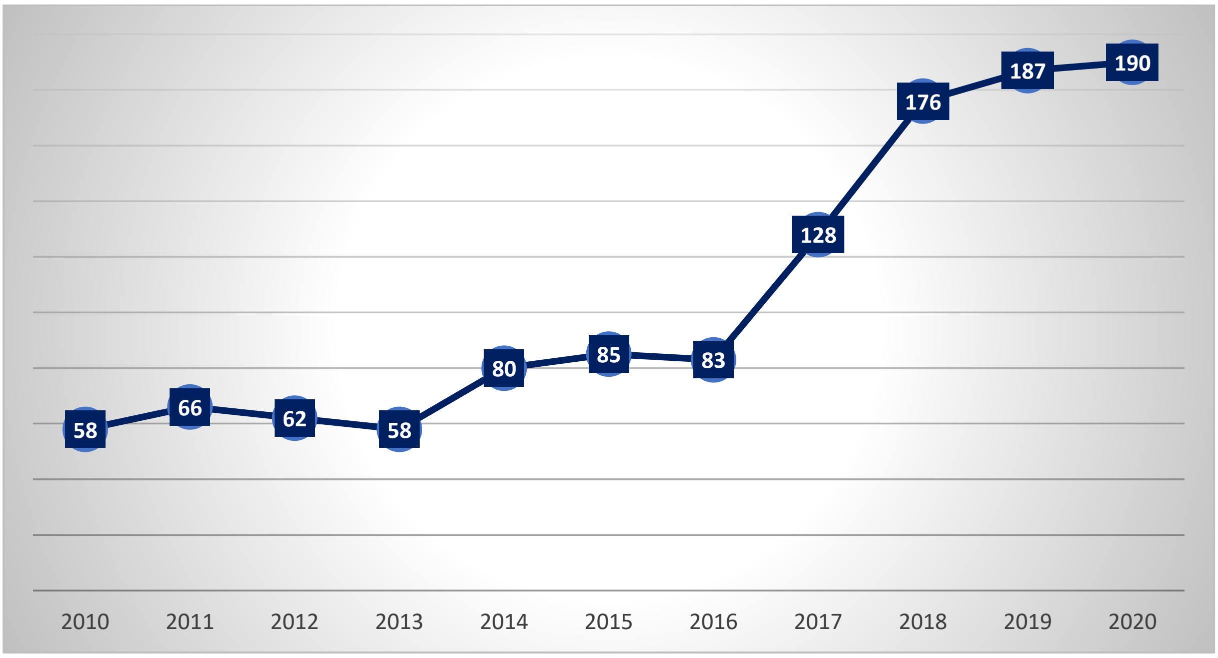


Figure 3. The number of scientific articles published by Croatian authors and indexed in WoS CC

Most important source journals

The bibliometric analysis of the journals in which Croatian authors publish nutrition-related articles revealed that the top ten articles are: *Journal of Pediatric Gastroenterology and Nutrition*, *Clinical Nutrition*, *Nature Genetics*, *Collegium Antropologicum*, *Nutrients* and *Journal of Food and Nutrition Research* (Table 2). According to Journal Citation Reports, impact factor (IF) of these journals in 2021 ranged from 0.609 to 202.731. Moreover, *Lancet*, *Food Chemistry*, *Nutrients*, *Clinical Nutrition* and *Nature Journal of Pediatric Gastroenterology and Nutrition* had very high citation counts (Figure 4).

Table 2. Top ten journals ranked by the number of articles (co)authored by Croatian researchers indexed in WoS CC, in the scientific field nutrition in the period 2010 – 2020

Journal	Number of articles published by Croatian authors	WoS Category	IF 2021.	Quartile
Journal of Pediatric Gastroenterology and Nutrition	56	Pediatrics – SCIE; Nutrition & Dietetics – SCIE; Gastroenterology & Hepatology - SCIE	3,355	Q3; Q3; Q2
Clinical Nutrition	37	Nutrition & Dietetics – SCIE	7,643	Q1
Nature Genetics	33	Genetics & Heredity - SCIE	41,376	Q1
Collegium Antropologicum	26	Anthropology - SSCI	0,609	Q3
Nutrients	24	Nutrition & Dietetics – SCIE	6,706	Q1
Journal of Food and Nutrition Research	23	Food Science & Technology - SCIE	1,138	Q4
Lancet	19	Medicine, General & Internal - SCIE	202,731	Q1
Food Chemistry	16	Nutrition & Dietetics – SCIE; Chemistry, Applied – SCI; Food Science & Technology - SCIE	9,231	Q1; Q1; Q1
Nature	14	Multidisciplinary Sciences - SCIE	69,504	Q1
Acta Clinica Croatica	13	Medicine, General & Internal - SCIE	0,932	Q4

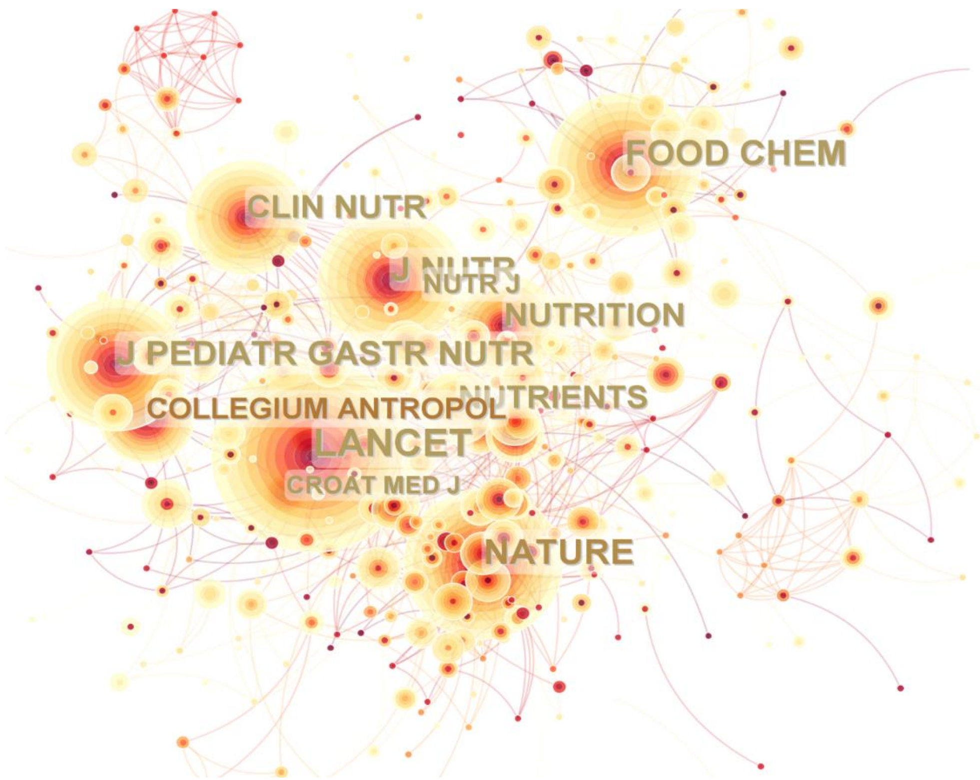


Figure 4. Journals indexed in WoS CC, in which Croatian authors published articles in the scientific field nutrition in the period 2010 - 2020, with more than 150 citations

Publication models

In the analysed sample, 46% of articles are published based on the traditional subscription or pay-per-view model, where there is typically no publication fee, although some publishers require payment of author processing charges (Baffy et al., 2020), and 54% of the articles are published using the open access model (gold, diamond, green) (Figure 5).

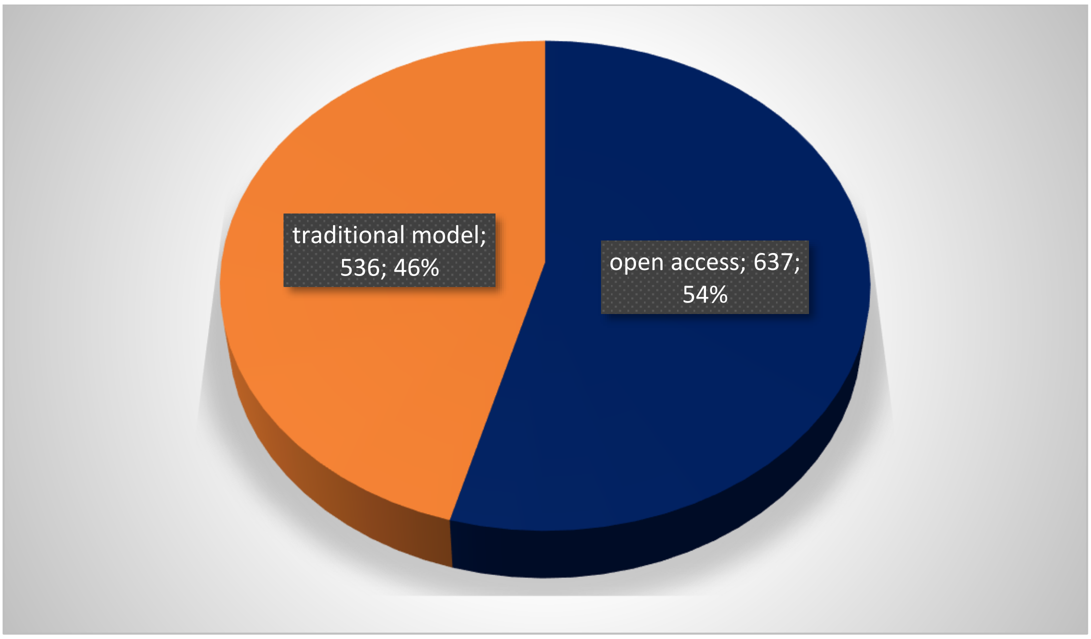


Figure 5. Publication model for articles written by Croatian authors, published in the scientific field of nutrition between 2010 and 2020

Since 2017 (Figure 6), the number of open access articles has been increased, which correlates with total publication activity of Croatian scientist in the field (Figure 3).

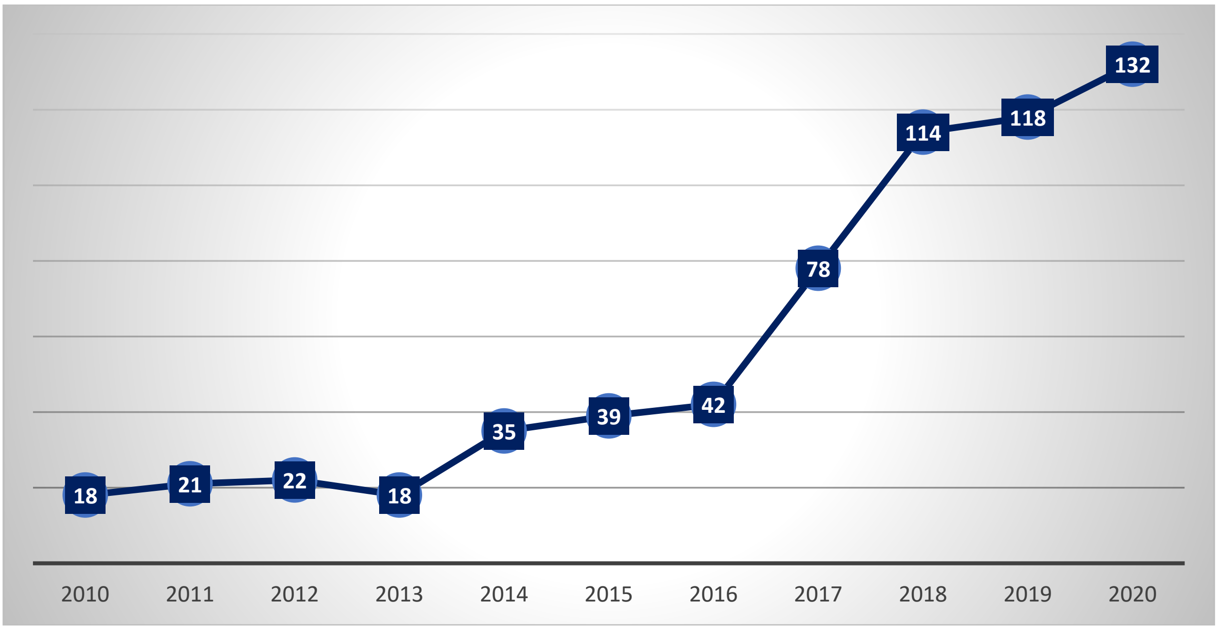


Figure 6. Annual growth in the number of open access articles (co)authored by Croatian authors in the scientific field nutrition between 2010 and 2020

WoS Research Areas

Figure 7 depicts WoS research areas with ten or more nutrion-related papers published by Croatian scientists. This elucidates the interdisciplinary nature of the research field. The majority of articles is categorized under Nutrition Dietetics (276) and Food Technology (219), but the importance of nutrition in other scientific disciplines, such as medicine, biochemistry and molecular biology, analytical chemistry etc. is also identified.

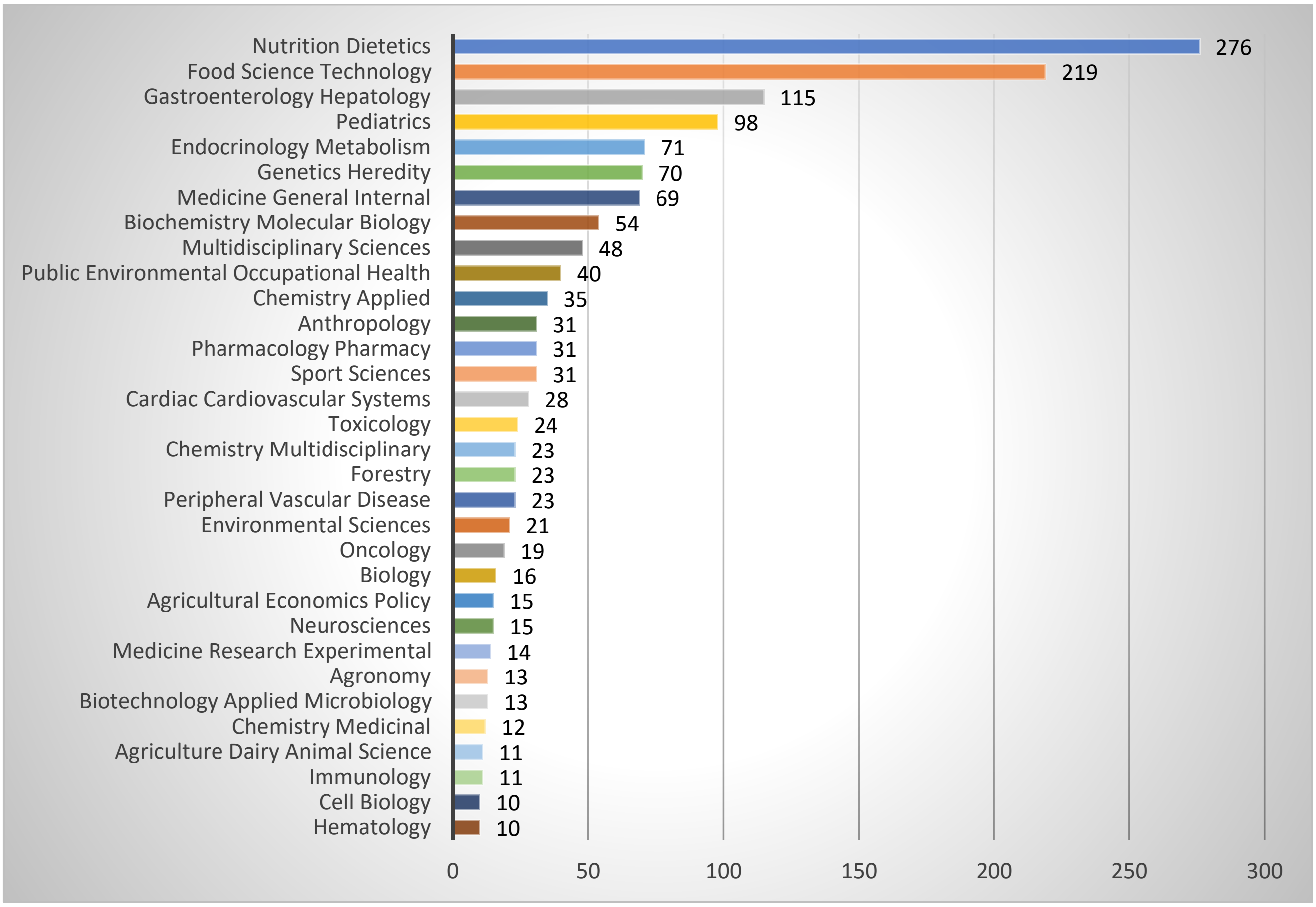


Figure 7. WoS subject categories with more than 10 nutrion-related articles published by Croatian authors between 2010 and 2020

Scientific collaboration

Researchers from the following institutions collaborated on the 316 papers published by the national cooperation (Figure 7): University of Zagreb, Josip Juraj Strossmayer University of Osijek, University of Rijeka, University of Split, Institute for Medical Research and Occupational Health, Croatian Veterinary Institute Zagreb, Institute Ruđer Bošković, University Hospital Dubrava, Croatian Forest Research Institute, Faculty of Kinesiology Zagreb, Institute of Agriculture and Tourism Poreč, Croatian Agency for Agriculture and Food, University Hospital Sestre milosrdnice, University Hospital Merkur, University Hospital Osijek, Croatian Institute for Transfusion Medicine, Croatian Institute of Public Health, General Hospital Dr. Josip Benčević Slavonski Brod, Institute for Adriatic Crops and Karst Reclamation, Children Hospital Srebrnjak, Children Hospital Zagreb, Agricultural Institute Osijek.

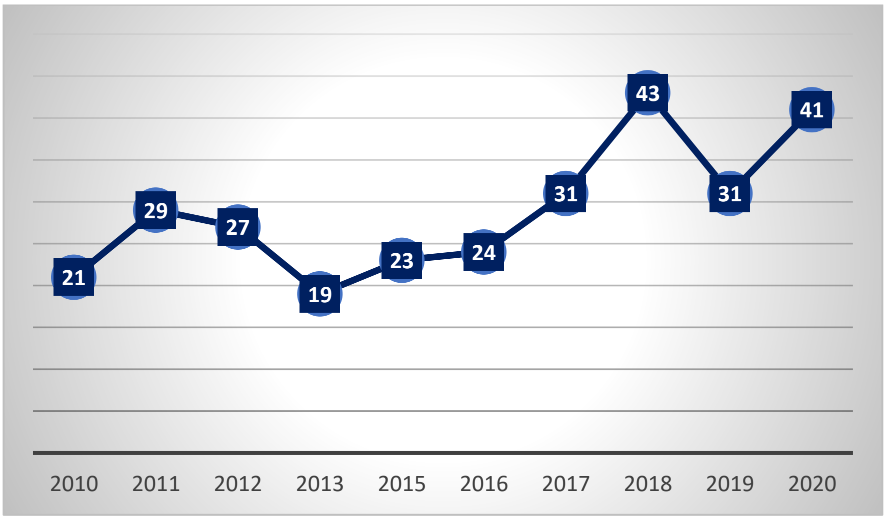


Figure 8. The number of articles published in the scientific field of nutrition by Croatian authors as part of the national collaboration between 2010 and 2020

University of Zagreb has the most intensive national collaboration (58%), followed by Josip Juraj Strossmayer University of Osijek (18%), University of Rijeka (13%), University of Split (5%) and Institute for Medical Research and Occupational Medicine and Croatian Veterinary Institute (each with 3%) (Figure 9). However, these numbers cannot be taken at face value; one must additionally consider the number of scientists per institution performing research on the topic of nutrition (which is not shown in this article).

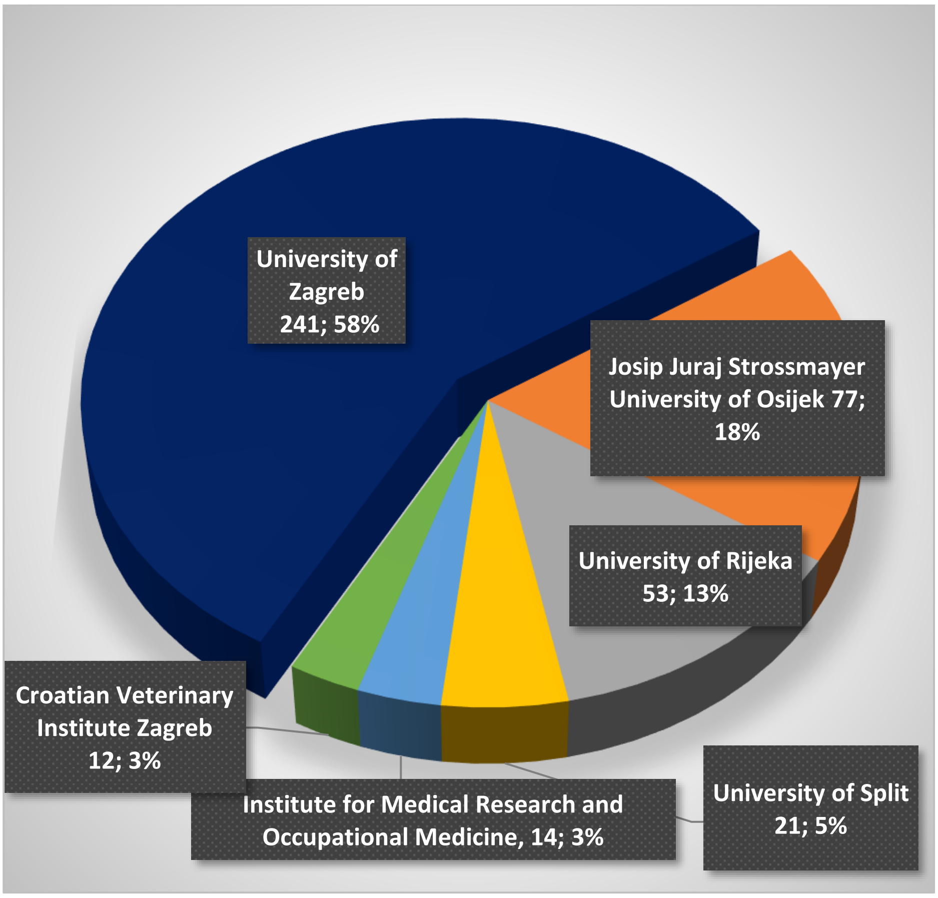


Figure 9. Dominant institutions in the articles published by Croatian authors in the scientific field nutrition in national collaboration in the period 2010 – 2020 (Institutions with more than 10 published articles were taken into metrics)

Croatian scientists have collaborated with scientists from 161 countries (Supplement Material 1). For visualization, only countries with 100 articles and more were considered (Figure 10). Cluster density denotes dependence of different collaborations on the themes, whereas circle size suggests scientists' and countries' centrality. A modularity value of 0.3898 indicates that network components are tightly coupled, while a mean silhouette value of 0.8073 suggests clustering configurations are of good quality. According to the frequency with which their names appear in the articles, the following countries are the most tightly coupled: Italy (367), Germany (334), Spain (329), England (329), USA (311), Netherlands (284), France (270), Poland (215), Sweden (211), Denmark (208), Switzerland (199), Canada (195), Scotland (190), Belgium (185), Australia (176), Norway (169), Finland (166), Israel (164), Greece (164), Czech Republic (145), Austria (144), Portugal (139), China (134), Slovenia (134), Hungary (127), Romania (116), Estonia (114), Brazil (108), Serbia (106), Iran (102).

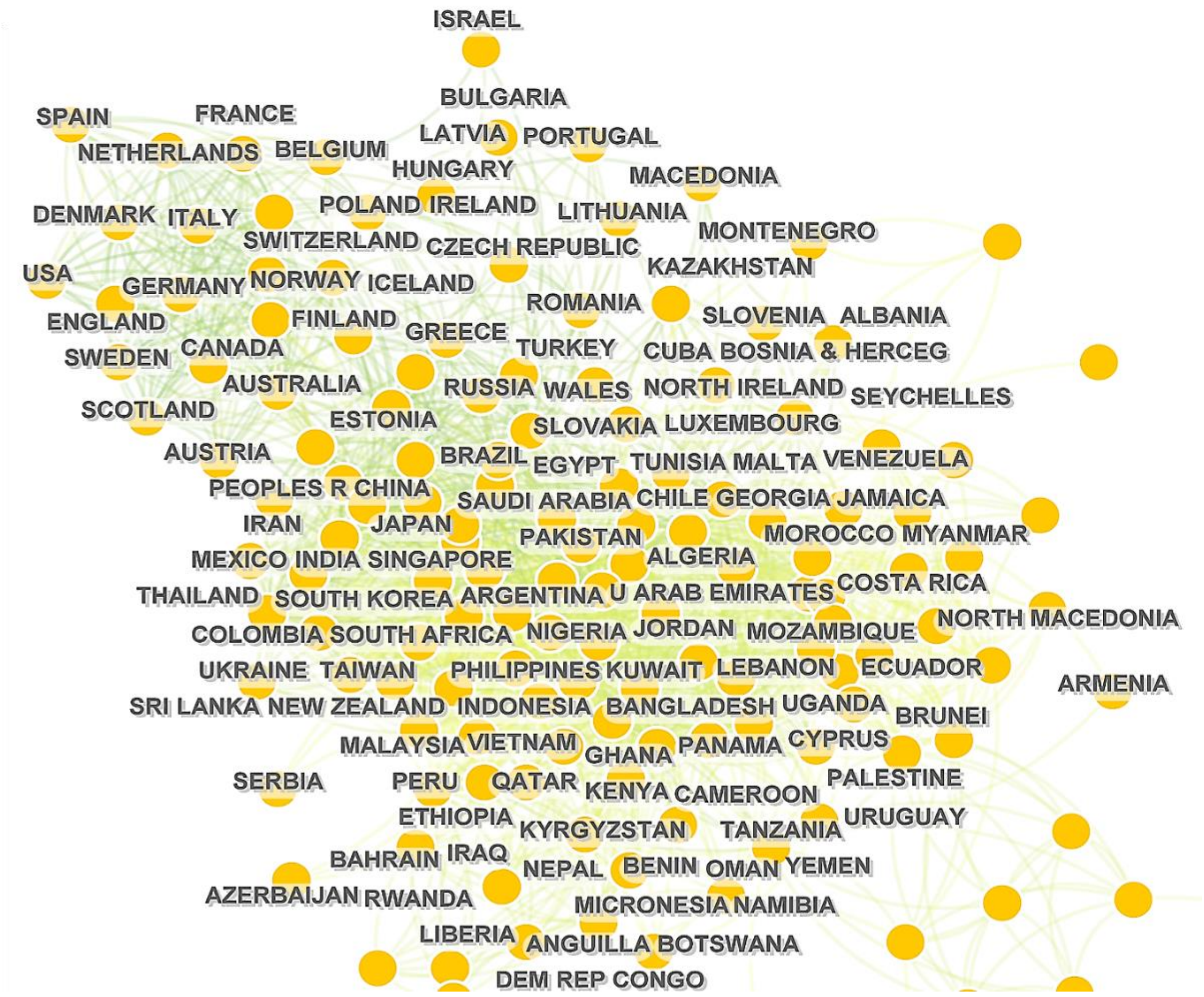


Figure 10. Visualisation of international collaboration of Croatian authors in the scientific field nutrition between 2010 and 2020

Total of 14 clusters were formed in CiteSpace, however only 4 with tight inner connections are shown in Figure 11. Other countries have low connection (<10), which is indicator of a limited international collaboration. Different themes were covered by the most active collaboration, which is shown in Figure 11, through network of keyword clusters (Table 3).

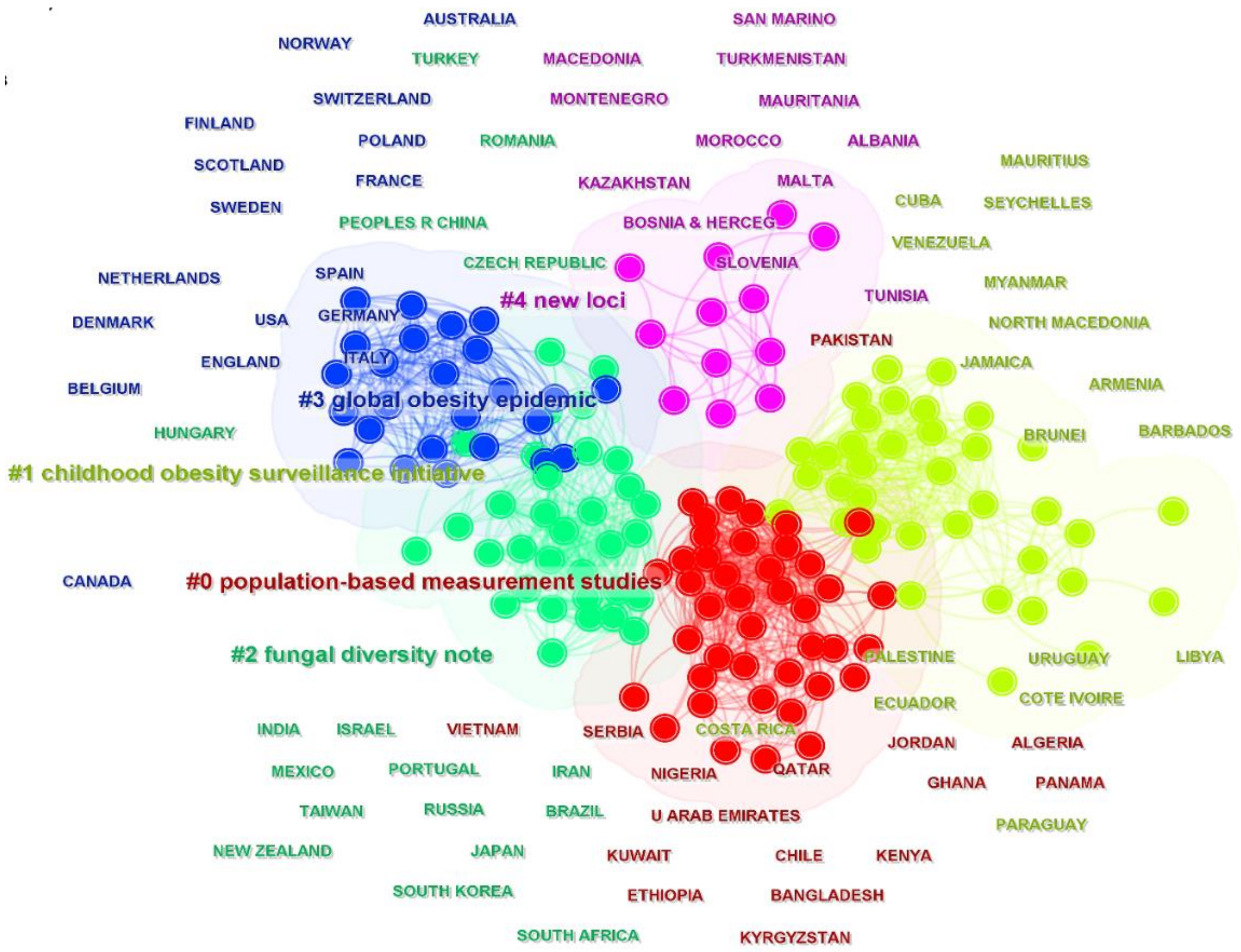


Figure 11. Main clusters within international collaboration of Croatian authors in the scientific field nutrition between 2010 and 2020

The largest cluster of (#0) population-based measurement studies began before 2010, peaked in 2015 and has been declining since 2019. These studies are the outcome of international scientific cooperation (Luxembourg, Qatar, Chile, Nigeria, Jordan, Pakistan, Tanzania, Cameroon, Serbia, …). Child obesity (#1) has been important subject since 2016 and is occupying scientists ever since. Nutraceuticals and their influence on health are clustered in group #2, with 2011 – 2015 being the peak of the research activity. The global obesity epidemic (#3) has been intensively studied until 2013 by Croatian scientists in collaboration of partners from England, Germany, Italy, and then Saudi Arabia. Food and loci interactions with various diseases (#4) have been widely researched up until 2019.

Table 3. Network of key words within international collaboration of Croatian authors in the scientific field nutrition between 2010 and 2020 in shown clusters (Source: CiteSpace II)

Cluster ID	Size	Silhouette	Mean (Year)	Label (LSI)	Label (LLR)	Label (MI)
0	41	0.757	2015	descriptive epidemiology; statistical issues; global health; blood pressure; cardiovascular disease | stress; vacuole; autolysosome; flux; lysosome	descriptive epidemiology (7.02, 0.01); burden of disease (4.64, 0.05); familial hypercholesterolaemia (3.49, 0.1); microsporidia (2.3, 0.5); neopereziida ord. nov. (2.3, 0.5)	arterial stiffness (0.15); outcomes research (0
1	37	0.79	2016	blood pressure; global health; population health; non-communicable disease; hospital malnutrition | hospital malnutrition; health insurance; nutritional management; global health; blood pressure	health insurance (4.84, 0.05); nutritional management (4.84, 0.05); subsidy (4.84, 0.05); hospital malnutrition (4.84, 0.05); child (3.83, 0.1)	arterial stiffness (0.05); outcomes research (0
2	33	0.794	2011	cardiovascular disease; familial hypercholesterolaemia; study design; arterial stiffness; health insurance | diabetes mellitus; heart failure; chronic kidney diseases; arterial stiffness; health insurance	recommendations (4.09, 0.05); pezizomycetes (4.09, 0.05); position paper (4.09, 0.05); lecanoromycetes (4.09, 0.05); nutraceuticals (4.09, 0.05)	arterial stiffness (1.78); outcomes research (1
3	25	0.904	2010	parenteral nutrition; fat emulsions; fatty acids; oropharynx cancer; tissue plasminogen activator | diabetes mellitus; temporal change; cyclical variation; oropharynx cancer; tissue plasminogen activator	body mass index (5.34, 0.05); parenteral nutrition (5.08, 0.05); nutrition (4.18, 0.05); infusions (3.56, 0.1); non-hdl cholesterol (3.56, 0.1)	nutrition assessment (2.1); parenteral (2.1); bath
4	12	0.868	2014	temporal change; diabetes mellitus; cyclical variation; familial hypercholesterolaemia; cardiovascular disease | autophagosome; flux; phagophore; stress; vacuole	temporal change (7.15, 0.01); incidence (4.04, 0.05); mortality (3.99, 0.05); bcaa (3.76, 0.1); world gastroenterology organization (3.76, 0.1)	bcaa (0.61); world gastroenterology organization (

Keyword analysis

Keywords in the articles were used to analyse research themes and focal points. Cite Space analysis revealed 1294 keywords based on the following criteria: g-index (k=25); LRF = 3.0, LBY = 5; e = 1.0. Size and connections between clusters distinguish topics that are more tightly coupled. Risk, prevalence, disease, children, health, cardiovascular diseases, meta-analysis, nutrition, risk factor are the most often used keywords in papers analysed. Figure 12 illustrates how visualisation of keywords corresponds to their frequency in articles, with colours representing the research subjects.

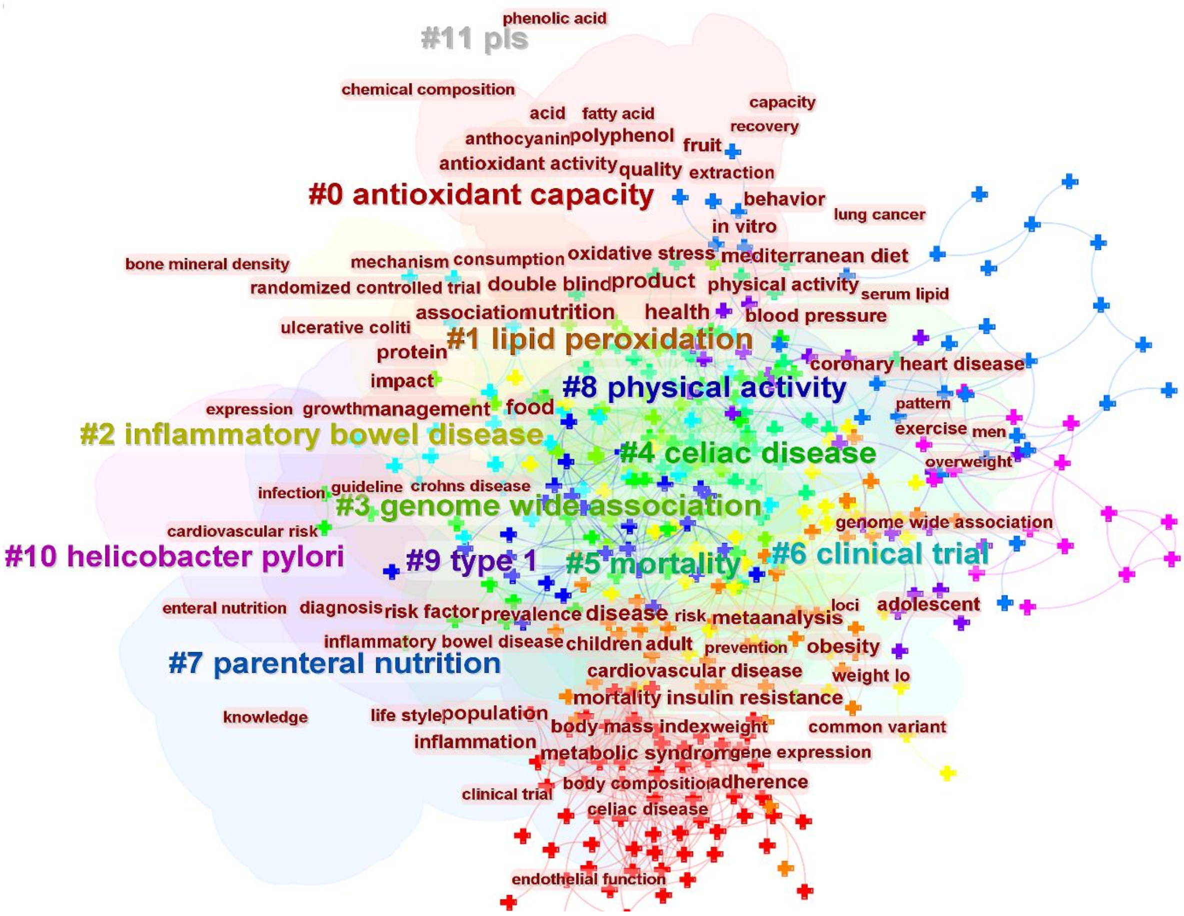


Figure 12. Bibliographic coupling of keywords in the articles published in the scientific field nutrition between 2010 and 2020 and their clusters

The bibliographic coupling of keywords resulted in 14 clusters, 11 of which had tough coupling of documents within clusters. These clusters are shown on a timeline (Figure 13). The cluster #0 has 92 documents with a silhouette of 0.778. Since 2014, the highest activity has been noticed in antioxidant capacity (25.99 1.0E-4); followed by antioxidant activity (29.72 1.0E-4), flavonoids (22.27 1.0E-4), polyphenols (18.54 1.0E-4) and children (15.14 1.0E-4).

Cluster #1 lipid peroxidation contains 68 documents with the lowest coupling between documents (S = 0.66). Documents are linked by keywords within research of diseases, pediatric patients and diet that continue to occupy the scientific community.

Cluster #2, inflammatory bowel diseases, is composed of 64 documents (S = 0.712) coupled through influence of diet on different bowel diseases.

Cluster #3 genome wide association has 52 tightly coupled documents (S = 0.741) regarding meta-anaylses of obesity and its link to genomic inheritance.

Cluster #4 peaked in 2016. Coeliac disease and influence of diet on its progress are theme in 47 documents coupled within the cluster (S = 0.695).

Cluster #5 morbidity is composed of 14 documents with tight coupling (S = 0.929), covering themes such as nursing, breast milk, physical properties, nutritive status, colorectal carcinoma, hygiene quality, therapeutical properties, chemical composition and nutritive status of breast milk.

Cluster #6 clinical trial covers 14 tightly coupled documents (S = 0.923) dealing with nutritive rehabilitation in parenteral diet at home, while cluster #7 (12 documents, S = 0.902) focuses on parenteral diet and lung diseases.

12 tightly coupled (S = 0.923) documents are clustered in #8, with systemic analyses of physical activity, hygiene, child growth, water quality and global load in Africa, and 8 documents regarding macro- and micronutrients in pregnant and breastfeeding women are coupled in cluster #9 (S = 0.963).

Cluster #10 is composed of only 5 documents (S = 0.982) regarding functional properties of different foods and their link to child obesity in children with *Helicobacter pylori*.

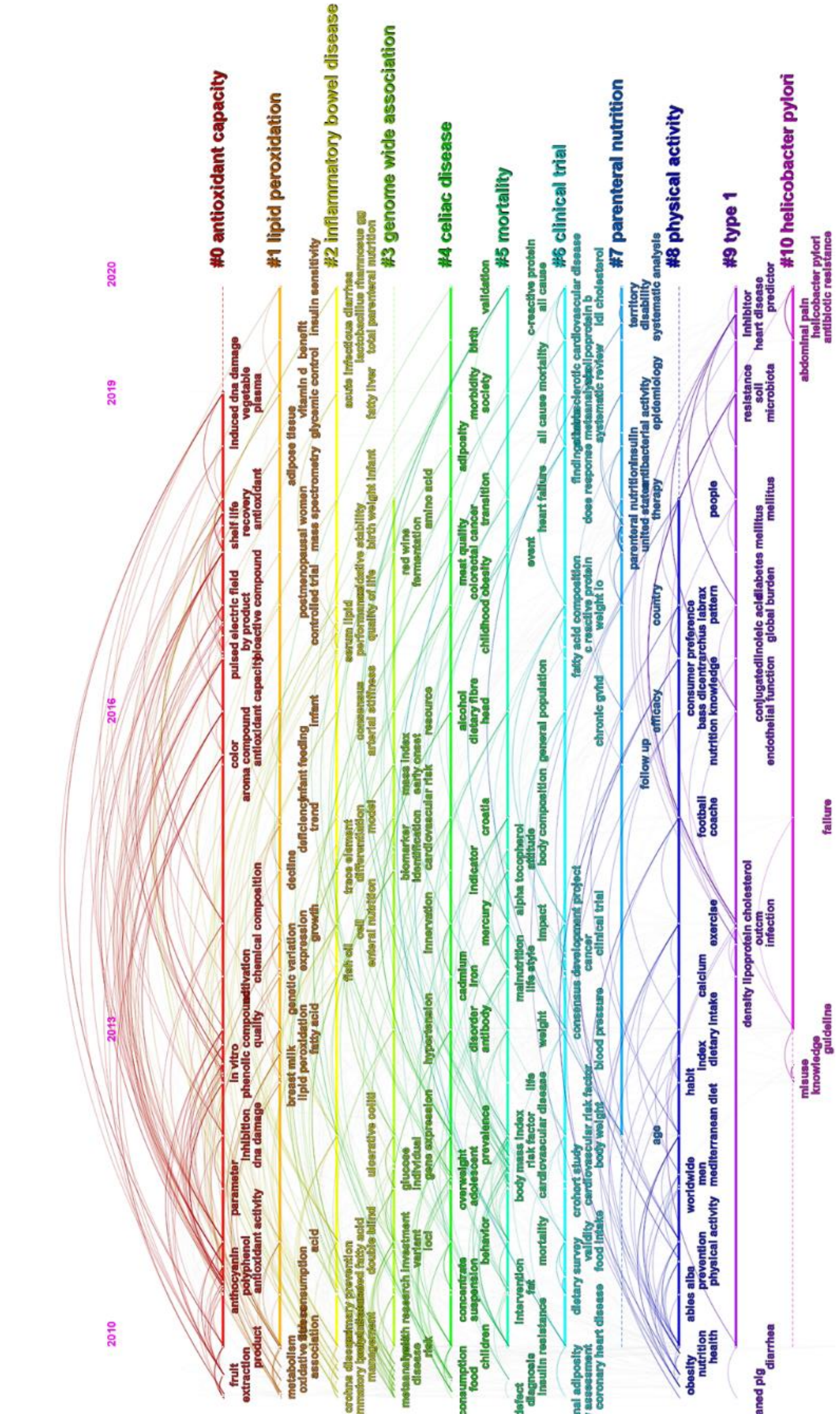


Figure 13. Timeline of bibliographic coupling of keywords in the articles published in the scientific field nutrition in the period 2010 – 2020 and their clusters

Cited reference analysis

Cluster analysis established focal points of research in the field of nutrition, with cited references as nodes. Criteria for selection were as follows: g-index = 25; LRF = 3.0, L/N = 10, LBY = 5; e = 1.0. Figure 15 represents results of visualisation of network of references, with N = 606, E = 2092, density = 0.0114, modularity Q = 0.8634 (>0.3), mean silhouette S = 0.6629 (>0.4) showing tight coupling of documents within each cluster.

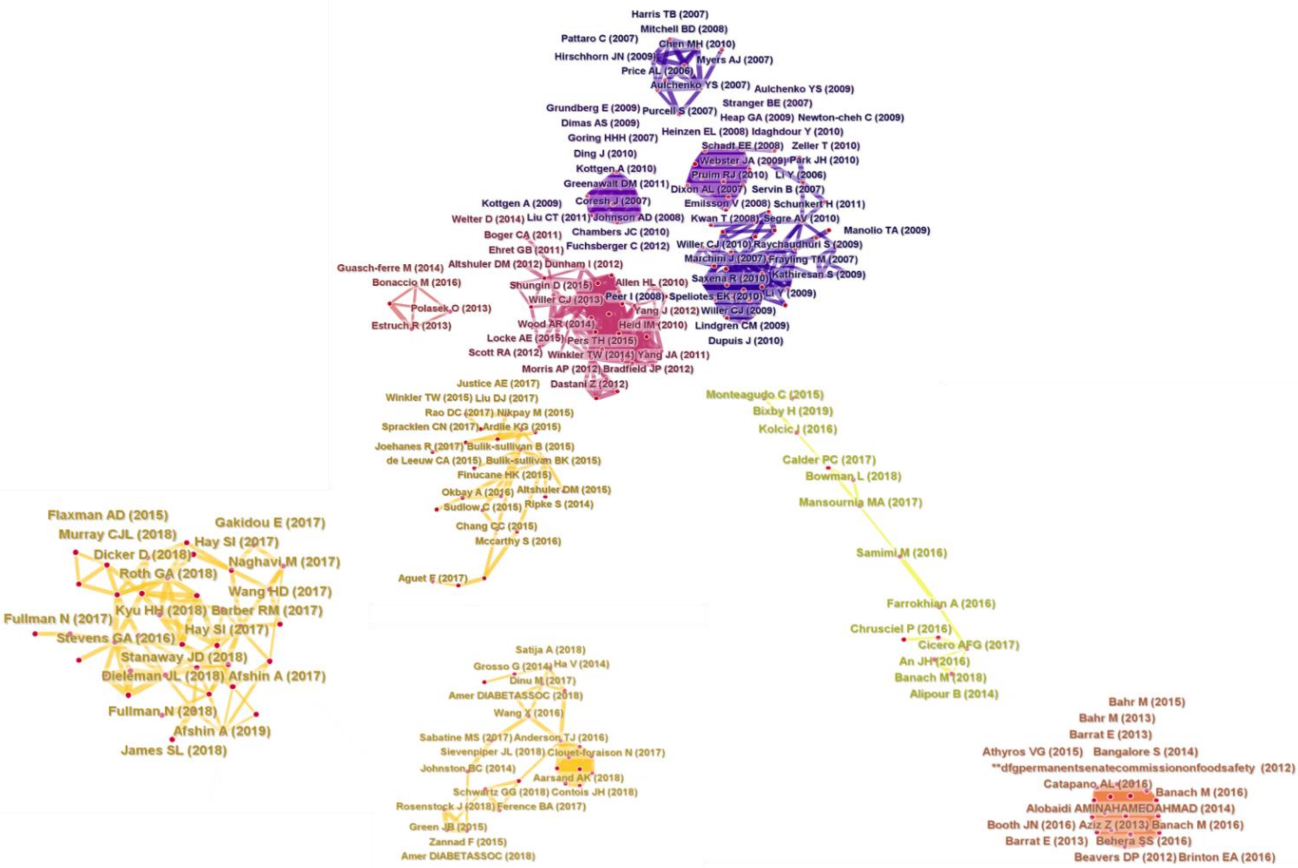
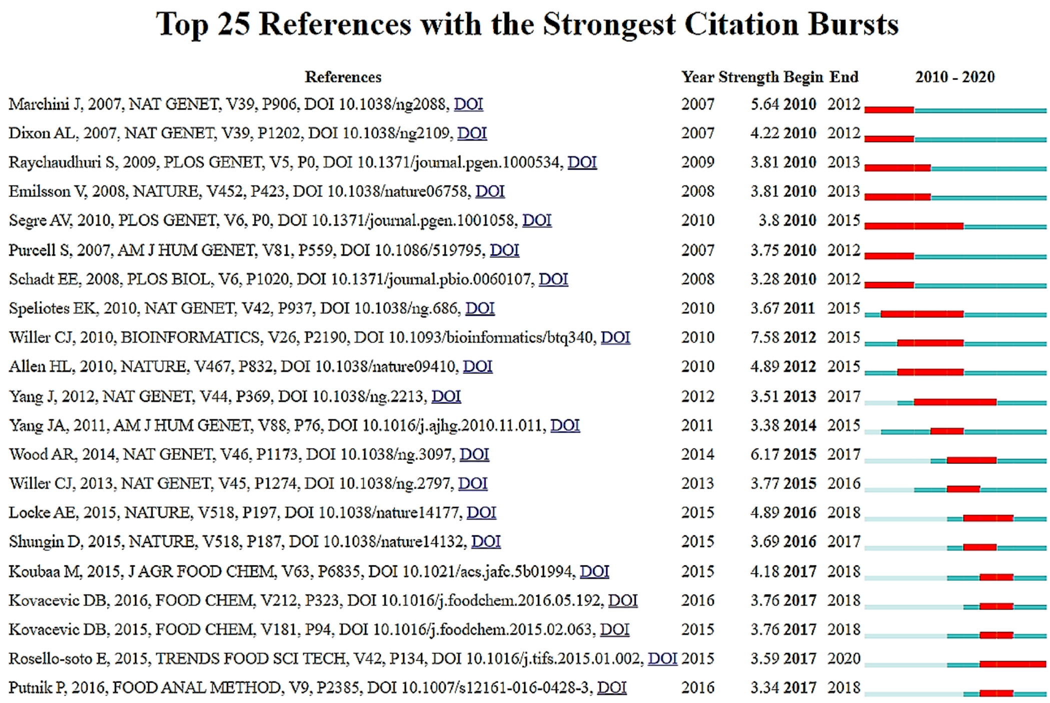


Figure 14. Visualisation of the network of cited references and their clusters in articles published between 2010 and 2020 in the scientific field nutrition

Analysis of co-citation references was used to determine focal points of research. Figure 15 displays the 25 top cited publications, as determined by their burst values. The red colour marks indicate periods when these publications were cited most frequently. Following articles written by Croatian authors are within top 25:

1. Danijela Bursać Kovačević, Jasenka Gajdoš Kljusurić, Predrag Putnik, Tomislava Vukušić, Zoran Herceg, Verica Dragović-Uzelac. (2016) Stability of polyphenols in chokeberry juice treated with gas phase plasma. Food Chemistry, 212, 323-331
2. Danijela Bursać Kovačević, Predrag Putnik, Verica Dragović-Uzelac, NadaVahčić, Martina Skendrović Babojelić, Branka Levaj. (2015.) Influences of Organically and Conventionally Grown Strawberry Cultivars on Anthocyanins Content and Color in Purees and Low-sugar Jams. Food Chemistry, 181, 94-100
3. Predrag Putnik, Danijela Bursać Kovačević, Marija Penić, Maja Fegeš, Verica Dragović-Uzelac. (2016) Microwave-Assisted Extraction (MAE) of Dalmatian Sage Leaves for the Optimal Yield of Polyphenols: HPLC-DAD Identification and Quantification. Food Analytical Methods, 9, 2385-2394
4. Predrag Putnik, Francisco J. Barba, Ivana Španić, Zoran Zorić, Verica Dragović-Uzelac, Danijela Bursać Kovačević. (2017) Green extraction approach for the recovery of polyphenols from Croatian olive leaves (Olea europea). Food and Bioproducts Processing, 106, 19-28



Top 25 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	2010 - 2020
Marchini J, 2007, NAT GENET, V39, P906, DOI 10.1038/ng2088, DOI	2007	5.64	2010	2012	
Dixon AL, 2007, NAT GENET, V39, P1202, DOI 10.1038/ng2109, DOI	2007	4.22	2010	2012	
Raychaudhuri S, 2009, PLOS GENET, V5, P0, DOI 10.1371/journal.pgen.1000534, DOI	2009	3.81	2010	2013	
Emilsson V, 2008, NATURE, V452, P423, DOI 10.1038/nature06758, DOI	2008	3.81	2010	2013	
Segre AV, 2010, PLOS GENET, V6, P0, DOI 10.1371/journal.pgen.1001058, DOI	2010	3.8	2010	2015	
Purcell S, 2007, AM J HUM GENET, V81, P559, DOI 10.1086/519795, DOI	2007	3.75	2010	2012	
Schadt EE, 2008, PLOS BIOL, V6, P1020, DOI 10.1371/journal.pbio.0060107, DOI	2008	3.28	2010	2012	
Speliotes EK, 2010, NAT GENET, V42, P937, DOI 10.1038/ng.686, DOI	2010	3.67	2011	2015	
Willer CJ, 2010, BIOINFORMATICS, V26, P2190, DOI 10.1093/bioinformatics/btq340, DOI	2010	7.58	2012	2015	
Allen HL, 2010, NATURE, V467, P832, DOI 10.1038/nature09410, DOI	2010	4.89	2012	2015	
Yang J, 2012, NAT GENET, V44, P369, DOI 10.1038/ng.2213, DOI	2012	3.51	2013	2017	
Yang JA, 2011, AM J HUM GENET, V88, P76, DOI 10.1016/j.ajhg.2010.11.011, DOI	2011	3.38	2014	2015	
Wood AR, 2014, NAT GENET, V46, P1173, DOI 10.1038/ng.3097, DOI	2014	6.17	2015	2017	
Willer CJ, 2013, NAT GENET, V45, P1274, DOI 10.1038/ng.2797, DOI	2013	3.77	2015	2016	
Locke AE, 2015, NATURE, V518, P197, DOI 10.1038/nature14177, DOI	2015	4.89	2016	2018	
Shungin D, 2015, NATURE, V518, P187, DOI 10.1038/nature14132, DOI	2015	3.69	2016	2017	
Koubaa M, 2015, J AGR FOOD CHEM, V63, P6835, DOI 10.1021/acs.jafc.5b01994, DOI	2015	4.18	2017	2018	
Kovacevic DB, 2016, FOOD CHEM, V212, P323, DOI 10.1016/j.foodchem.2016.05.192, DOI	2016	3.76	2017	2018	
Kovacevic DB, 2015, FOOD CHEM, V181, P94, DOI 10.1016/j.foodchem.2015.02.063, DOI	2015	3.76	2017	2018	
Rosello-soto E, 2015, TRENDS FOOD SCI TECH, V42, P134, DOI 10.1016/j.tifs.2015.01.002, DOI	2015	3.59	2017	2020	
Putnik P, 2016, FOOD ANAL METHOD, V9, P2385, DOI 10.1007/s12161-016-0428-3, DOI	2016	3.34	2017	2018	

Figure 15. The top 25 cited references, and their clusters with the strongest citation bursts within the articles published in the scientific field nutrition from 2010 to 2020. Red colour indicates the periods with highest number of citations.

Conclusion

This study examined articles in the field of nutrition authored by at least one Croatian author and indexed WoS CC database. The results show that publication trend is continually increasing, with the highest growth since 2016. Croatian scientists have good collaboration both nationally and internationally, with the most intensive collaboration with EU countries. The research in the field of nutrition is interdisciplinary, covering nutrition, food technology, medical sciences, molecular biology, chemistry, and other sciences, which is proved through journals in which results have been published. During the analysed period (2010 – 2020), the research focused on diet and its interaction with adult and child health, which is continuing trending.

However, this study does have a few limitations, the most notable of which are the following: the keywords that were chosen do not necessarily contain all of the articles that are connected to nutrition, and the data that is presented here only includes articles that are indexed in WoS CC.

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Supplement Material 1. Number of articles published in collaboration with scientists from abroad

Country	Nr of articles
Croatia	1173
Italy	375
Germany	340
England	336
Spain	336
Usa	318
Netherlands	290
France	273
Poland	221
Sweden	215
Denmark	211
Canada	201
Switzerland	201
Scotland	194
Belgium	188
Australia	179
Norway	174
Greece	169
Finland	168
Israel	165
Czech Republic	147
Austria	145
Portugal	145
Peoples R China	137
Slovenia	137
Hungary	129
Romania	121
Estonia	116
Brazil	112
Serbia	111
Iran	109
Russia	102
Japan	101
Iceland	100
India	99
Ireland	96
Singapore	90
Turkey	87
Saudi Arabia	85
Mexico	81
South Africa	81
South Korea	81
Taiwan	74
Argentina	69
New Zealand	69
Malaysia	66
Pakistan	65
Philippines	64
Colombia	61
Slovakia	60
Egypt	59
Nigeria	58
Indonesia	51
Peru	50

Country	Nr of articles
Ukraine	50
Vietnam	48
Lithuania	47
Qatar	47
Sri Lanka	45
U Arab Emirates	45
Kuwait	44
Jordan	43
Bulgaria	42
Ethiopia	42
Bangladesh	41
Chile	41
Wales	41
Ghana	39
Kenya	39
Thailand	39
Kyrgyzstan	38
Algeria	37
Bosnia Herceg	35
Iraq	35
Latvia	35
Panama	35
Nepal	34
Cyprus	33
North Ireland	33
Tunisia	31
Lebanon	30
Luxembourg	29
Cameroon	27
Benin	26
Bahrain	25
Kazakhstan	25
Georgia	23
Malta	23
Morocco	23
Tanzania	23
Mozambique	22
Palestine	20
Uganda	20
Yemen	20
Costa Rica	19
Ecuador	19
Jamaica	19
Oman	19
Micronesia	18
Rwanda	17
Anguilla	15
Montenegro	15
North Macedonia	15
Brunei	14
Venezuela	14
Dem Rep Congo	13
Botswana	12
Macedonia	12

Country	Nr of articles
Albania	11
Armenia	11
Azerbaijan	11
Cuba	11
Liberia	11
Myanmar	11
Zambia	11
Namibia	10
Paraguay	10
Seychelles	10
Uruguay	10
Cote Ivoire	9
Mauritius	8
Barbados	6
Libya	6
Belarus	5
Bhutan	5
Kosovo	5
Trinidad Tobago	5
Fiji	4
Greenland	4
Malawi	4
Moldova	4
Solomon Islands	4
Sudan	4
Tajikistan	4
Tonga	4
Turkmenistan	4
Uzbekistan	4
Burundi	3
Dominican Rep	3
Gambia	3
Mauritania	3
New Caledonia	3
San Marino	3
Togo	3
Burkina Faso	2
Cape Verde	2
Chad	2
El Salvador	2
Faroe Islands	2
Guyana	2
Liechtenstein	2
Mongolia	2
Papua N Guinea	2
Senegal	2
Suriname	2
Bolivia	1
Cambodia	1
Madagascar	1
Nicaragua	1
Serbia Monteneg	1
Sierra Leone	1
Zimbabwe	1

MICROBIOLOGICAL CONTAMINATION OF CONFECTIONARY CAKES IN CROATIA

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original scientific paper

Summary

Confectionery cakes are products obtained by mixing, shaping, baking, or other suitable processing procedures of two or more ingredients, giving the characteristic sensory properties of the product. They can be filled or topped with fruit, chocolate and other creams or toppings. The chemical composition and high water and sugar content make confectionery cakes suitable for the growth and multiplication of various microorganisms. Since contamination can occur at all stages of the production process, conducting good hygiene practices is necessary to obtain a product safe for consumption.
The aim of this study was to provide insight into the contamination of confectionery cakes at the market in the Republic of Croatia by potentially pathogenic microorganisms and microorganisms as indicators of hygienic production. The cakes were sampled during a one-year period in 12 cities in Croatia. Samples were analysed according to the microbiological criteria prescribed by Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs and as recommended by the Guideline of Microbiological Criteria (Ministry of Agriculture, 2011).
Results of the study showed that no pathogenic bacteria whose presence could have adverse health effects, were identified in confectionery cakes.

Keywords: confectionery cakes, microbiological contamination, food safety

Introduction

Some bacteria that contaminate food during its production are potential causes of diseases in humans. In accordance with the general requirements for food safety, which are prescribed by Article 14 of Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, food should not be placed on the market if it is not safe. Food safety is ensured by a preventive approach, such as implementing good hygiene practice (GHP) and controlling hazards and critical control points (HACCP). In terms of the presence of certain microorganisms in food, the microbiological food safety criteria are related to pathogenic bacteria, and the process hygiene criteria to those that are not, but their presence must still be monitored and in the case of unsatisfactory test results certain measures have to be undertaken (Regulation 2073/2005). The whole production process acceptability is estimated based on the process hygiene criteria (Regulation 2073/2005).

Due to their chemical composition and high-water content, confectionary cakes are suitable for the growth and reproduction of various microorganisms. Contamination of confectionary cakes can occur at all stages of the production process: due to the use of contaminated raw materials, especially if the cakes are not heat treated before consumption, after thermal processing, during inadequate storage and/or transportation and due to non-hygienic handling (Kumar et al., 2011; Shahbaz et al., 2013; Chaudhari et al., 2017; El-Kadi et al., 2018). Higher moisture content products are more likely to cause foodborne illness because they promote the growth of a wide range of bacteria, yeast, and other fungus species. Bakery products with high moisture content or low acid content provide an ideal habitat for pathogenic bacteria to thrive. The growth of spoilage organisms such as osmophilic yeasts and mould is usually limited to intermediate moisture products (Gill et al., 2020).
According to the Official Gazette on cereal and cereal products (OG 81/2016), cakes are products obtained by mixing, shaping, baking or other appropriate processing procedures of the mixture of two or more ingredients, thus achieving the characteristic sensory properties of the product. They can be stuffed or overflowing with fruit, chocolate and other fillers or dressings.

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The study was conducted in order to determine the occurrence of potentially pathogenic microorganisms and microorganisms' indicators of process hygiene in confectionery cakes in the Republic of Croatia.

Materials and methods

The research was conducted over the one-year period in 12 cities in the Republic of Croatia. 230 individual samples of confectionary cakes of different types (with fruit and/or creams) that were offered in catering facilities, either for consumption on the spot or for sale, were sampled.

Samples were taken by certificated employees of the Teaching Institute for Public Health "Dr. Andrija Štampar" and the Food Control Center of the Faculty of Food Technology and Biotechnology of the University of Zagreb. Each sample was taken as one unit in an amount from 200 to 500 g. The sample was put in a PVC container (bag) containing information on the place and time of sampling, the product's name, and the product's temperature value while sampling. The sample was put in the portable cooler and within not more than two hours delivered to the laboratory for further analysis.

Microbiological analyses were performed using internationally recognized ISO methods as listed in Table 1.

Table 1. ISO methods used in microbiological analyses

Microorganisms	Method
Aerobic mesophilic bacteria	HRN EN ISO 4833-1:2013 or HRN EN ISO 4833-2:2013
Enterobacteriaceae	HRN ISO 21528-2:2017
***Salmonella* spp.**	HRN EN ISO 6579-1:2017
Listeria monocytogenes	HRN ISO 11290-2:2017
Staphylococcus aureus	HRN EN ISO 6888-1:2004
Moulds	HRN ISO 21527-1:2012 and HRN ISO 21527-2:2012

Microbiological criteria prescribed by Regulation 2073/2005 were applied to the obtained results of the sampled cakes and were interpreted in accordance with the recommendations (Table 2) from the Guide on Microbiological Criteria (Ministry of Agriculture, 2011).

Table 2. Guide on Microbiological Criteria – criterion related to confectionary cakes

Food	**Microorganisms/their toxins and metabolites**	**Sampling plan**		**Criterion**
Pastries (pastry cakes) with fillings and ready-made creams	**Recommendation**			
	Aerobic mesophilic bacteria	5	2	m = 10^4 cfu/g M = 10^5 cfu/g
	Salmonella spp.	5	0	n.n. 25 g
	Enterobacteriaceae	5	2	m = 10^2 cfu/g M = 10^3 cfu/g
	Coagulase positive staphylococci /*Staphylococcus aureus*	5	1	m = 10 cfu/g M = 10^2 cfu/g
	Moulds	5	1	m = 10 cfu/g M = 10^2 cfu/g

m= minimal value for given criterion; M=maximal value for given criterion; cfu=colony forming units

Since only one sampling unit, instead of prescribed five, was taken, in case that the result for particular microorganism from Table 2 was above or equal to "m", between "m" an "M" or above "M", the sample was declared as "unacceptable".

Statistical analyses were done for minimum, maximum and average values of microorganism contamination, using Microsoft Excel.

Results and discussion

Out of a total of 230 samples of confectioner's cakes, no pathogenic microorganisms were detected in a quantity that poses a risk to human health.

Salmonella spp. was not detected in 25 g in any sample, which corresponds to the results of a three-year study conducted in Canada (Canadian Food Inspection Agency, 2016-2018) and in Ireland (FSAI, 2018). *Listeria (L.) monocytogenes* was not detected in an amount above 100 cfu/g, nor in the Irish study. However, *L. monocytogenes* was previously found in cakes and pastry products collected from different hotels, restaurants and pastry shops in Croatia in 12 samples (4.27%) out of 283, with the result of 10^6-10^7 cfu/g (Uhitil et al., 2004). In the Canadian study, it was pointed out that *L. monocytogenes* was found in only one case below 10^2 cfu/g. However, given that the sources of contamination for *L. monocytogenes* are numerous, in the case of poor cleaning and sanitation, all of them, i.e. the product itself, the personnel who handle food, the environment (utensils and equipment) and the consumers themselves can be the source or carriers of pathogens. microorganisms (Lianou and Sofos, 2007). In the Canadian study in only one sample *S. aureus* was found for the criteria $10^2<X<10^4$ cfu/g. In our research, *S. aureus* was found in 86.52% (199) cases up to 10 cfu/g, while in 23 cases it was found 10^2, in 7 cases 10^3 and in one case 10^4 cfu/g (Table 2). In the Irish research, they found coagulase-positive staphylococci, which they found to be acceptable ($10^2< X <10^4$ cfu/g) in 1.5% and unacceptable in 0.3% of cases. ($\geq 10^4$ cfu/g). Given that all the samples that were declared as unacceptable were kept at a temperature of 5-10 °C, they concluded that in addition to proper handling, a very important factor is the appropriate temperature of cake storage that limits the growth of bacteria, and consequently the risk of gastrointestinal diseases. Their recommendation is that cakes should not be kept at a temperature above 5 °C (FSAI, 2018). Staphylococcal toxin production is expected in cases where *S. aureus* is present in 10^5 cfu/g, or higher (HPA, 2009), therefore, despite the presence of this bacteria found in our research, there will be no harmful effects on human health.

Aerobic mesophilic bacteria (AMB), *Enterobacteriaceae* and moulds are microbiological parameters used as indicators of production hygiene. AMB were determined in an amount greater than 10^4 in 35% of cases, to which fruit cakes (43%) contributed more than other cakes (32%). In cases where cfu/g was greater than 10^4, for fruit cakes it ranged from 1.9×10^4 to 10^8 cfu/g, with a mean value of 5.1×10^6 cfu/g. In the other cakes, they were present in the range of 1.2×10^4-10^8 cfu/g, and the mean value was 3.1×10^6 cfu/g, so slightly lower values than in fruit cakes. Our results are worse than the result of Meldrum et al. (2005), who found aerobic colony counts in 15% (121/808), of the samples of custard slices surveyed but similar to Kumar et al., (2011) who found aerobic colony counts, in contamination of bacteria ranging 1.37×10^6 cfu/g to 11.27×10^6 cfu/g. Kotzekidou (2013) reported on 13.5% of desserts oven baked with $>10^5$ cfu/g AMB, and in desserts with dairy cream AMB count ranged from 10^3 to 10^9 cfu/g, obtained from a 10-year inspection survey.

A number of *Enterobacteriaceae* above the value of 10^2 cfu/g were found in 35% of cakes, which was contributed more by fruit cakes where they were found in 39% of cases, than other cakes where they were found in 30% of cases. In cases where cfu/g was greater than 10^2 cfu/g, for fruit cakes it ranged from 5×10^2 to 10^5 cfu/g, with a mean value of 2.9×10^4 cfu/g. In the other cakes, they were present in the range of 3×10^2-5×10^6 cfu/g, and the mean value was 2×10^5 cfu/g. Kotzekidou (2013) found *Enterobacteriaceae* in 38.5% oven-baked desserts samples and highest *Enterobacteriaceae* contamination in desserts with dairy cream (76.7%) with distribution in the range of 10^3 to $< 10^7$ cfu/g.

Table 3. Absolute and relative number of cakes contaminated with microorganisms in total, and divided in creamy/chocolate and fruit cakes

Microbiological parameters	Total		Creamy/chocolate cakes		Fruit cakes	
cfu/g	N	%	N	%	N	%
AMB* <= 10000	149	65	110	68	39	57
AMB*>10000	81	35	51	32	30	43
Enterobacteriaceae <=100	150	65	113	70	42	61
Enterobacteriaceae >100	80	35	48	30	27	39
Moulds<=10	211	92	146	91	65	94
Moulds >10	19	8	15	9	4	6
Staphylococcus aureus <=10	199	87	143	89	56	81
Staphylococcus aureus >10	31	13	18	11	13	19

**AMB= Aerobic mesophilic bacteria*

Moulds above 10 cfu/g was found in a total of 8% (19), i.e. in 6% of fruit cakes and 9% of other cakes. In cases where cfu/g was greater than 10, for fruit cakes, it ranged from 2×10^2 to 8×10^3 cfu/g, with a mean value of 2.9×10^3 cfu/g. In the other cakes, they were present in the range of 10^2-3.1×10^3 cfu/g, and the mean value was 8×10^2 cfu/g, thus lower than that found in the fruit cakes. Moulds and yeasts, can cause food spoilage. Mould spores are killed due to baking process, and contamination (O'Brien, 2004) is a consequence of cakes exposure to air and surfaces (Gill et al., 2020), but it can also occur, in production steps such as cooling, slicing, transporting, packing and storage.

Hassanzadazar et al. (2018) reported on 48,39% contamination of cream filled pastries with moulds and yeasts. They concluded that the cream-filled pastries containing fruit or nuts usually have higher mould contamination because of microbial contamination of used raw materials. The presence of mould in confectionary cakes was determined by Sharifzadeh et al. (2016) in 27.5% of samples, Pajohi-Alamoti et al. (2016) in 45% puff pastry and 30% jelly roll samples, and Kumar et al. (2011) reported on yeast and mould counts ranging from $1.33x10^5$cfu/g to $92.5x10^5$cfu/g, which suggests the conclusion that mould contamination is a ubiquitous problem and that not enough attention was paid to its prevention. Water activity plays important element for microbiological contamination, which survive or reproduce when water activity is high like in cakes/creamy or fruity based products (Abdullah et al., 2000; Syamaladevi et al., 2016). Particularly moulds but also some species of yeasts and bacteria may infect cakes because of increased sugar content which leads to cake spoilage (Gill et al., 2020).

Conclusions

Cakes as RTE foods, can be contaminated with pathogens during production, handling, packaging and distribution. Since these products will not undergo any procedure that can inactivate bacterial pathogens their presence represents a potential risk to consumer for foodborne illnesses. However, in our research no foodborne bacterial pathogen was find in cakes samples, and for this type of food we can conclude that they are safe for consumption.

On the other hand, cakes were contaminated with microorganisms which represent hygiene parameters indicator and it is recommended for producers, retailers and consumer as well, to maintain safe and good hygienic practice while handling. Requirement for maintenance of the cold chain is mandatory prescribed in Commission Regulation (EC) No. 852/2004, and recommendation is to provide storage temperature from 0 °C to 5 °C (FSAI, 2018).

However, microorganism's indicator of good hygiene practice contamination number, no matter if higher than recommended, cannot be related with possible pathogen contamination. Reliable result indicating presence or absent of pathogen bacteria, can be only obtained with specific microbiological analytical method.

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PHYTOSTEROLS AS A MEAN TO ALTER CHOLESTEROL LEVELS

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review paper

Summary

Cholesterol is a molecule wich represents the basic building part of every cell. Its homeostasis is crucial for proper cellular and systemic functions and it is very important to maintain it within reference values. Altered cholesterol balance causes cardiovascular diseases as well as other diseases, like neurodegenerative and cancer. High level of cholesterol can be aleviated with medical therapy but also with a diet rich in phytosterols. The main sources of phytosterols are vegetable oils, nuts, and legumes, but positive effect on lipid profile have fruits and vegetables, and some medicinal plants. Additionally, positive effect was found for combination of phytosterols with read yeast rice and in combination with probiotic bacteria. The most important property of phytosterols is reduced absorption of endogenous and exogenous cholesterol in the body. According to a number of studies their sufficient intake can significantly alter absorption of cholesterol from foods in the small intestine.
Conclusion: Daily diet that includes foods containing phytosterols can maintain normal blood cholesterol levels and prevent diseases due to hypercholesterolemia, which are still te main cause of death in Croatia and surrounding countries.

Keywords: cholesterol, phytosterols, hypercholesterolemia

Introduction

Cholesterol is considered as one of the most important structural components of cell membranes and is the basic building part of every cell (Cerqueira et al., 2016). In certain organs cholesterol has specific roles such as: synthesis of bile acids in hepatocytes, synthesis of steroid hormones in the cortex of adrenal glands and gonads, transport of liposoluble (A, D, E and K) vitamins (Lemke et al., 2007). It is a chemically rigid and almost planar molecule with a steroid skeleton of four fused rings, three six-membraned and one five-membraned (Carqueira et al., 2016). Cholesterol is the most widespread sterol in the human body, and as far as its origin is concerned, it can be endogenous and egzogenous . Most cells in human body have the ability to synthesize cholesterol on their own , which is called endogenous cholesterol, while its other source can be food through which cholesterol is introduced externally, so called exogenous cholesterol. Given the ability of the body to create it in the large quantities, it is sufficient to consume 150-300 mg of cholesterol daily from outside. Children need a proportionally larger amount, since it plays a significant role as a structural element of all cellular and intracellular membranes (Voet, 2005).
Most of the cholesterol is produced in the liver, while its synthesis can also occur in the intestinal mucosa and adrenal gland, from where it is transported by bloodstream to the cells of the body. Like other lipids cholesterol is also insoluble in water. Cholesterol is transported in the bloodstream by binding proteins, thus building lipoproteins. There are several types of lipoproteins and they are divided by density, so we have: chylomicrons which are the largest in diameter and have the lowest density, very low density lipoprotein (VLDL), intermediate (transitional) density lipoprotein (IDL), low density lipoprotein (LDL) and large lipoprotein density (HDL). Lipoproteins which contain more lipids in their composition have a lower density. Cholesterol is present in the blood in free and esterified form, when it is bound with one fatty acid molecule. Cholesterol esterification is carried out in plasma under the action of the enzyme lecithin-cholesterol-acetyltransferaze (LCAT), which is found in blood plasma. In plasma, approximately 75% of cholesterol is esterified, most often with a polyunsaturated fatty acid, namely linoleic acid. Cholesterol is eliminated from the body through bile, conversion into cholic acids, peeling of the skin, a small amount is lost through urine, while lactation women lose a certain amount throuhg milk (Parket et al., 2006).
The harmful effect of cholesterol is manifested when it is present in blood in significantly higher than normal concentrations. Increased food intake increases its concentration in the blood. It has been proven that with every 100 mg of increased dietary cholesterol intake, the cholesterol value in the blood of adults increases by 0.25 mmol/l. Other factors such as genetic, endrocine and some others can influence the

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increase of its concentration in the blood (AJCN, 1997).
Hypercholesterolemia is a condition that indicates the presence of high levels of cholesterol in the blood. It is also a form of hyperlipidemia (high level of lipids in the blood), hyperlipoproteinemia (high level of lipoproteins in the blood) and dyslipidemia (any abnormality of lipids and lipoproteins in the blood). Elevated levels of LDL in the blood can be due to diet, obesity, genetic disease (such as LDL receptor mutations in familial hypercholesterolemia), or the presence of other diseases such as type 2 diabetes mellitus and an underactive thyroid gland (Durrington, 2003).
All animal cells produce cholesterol and use it at the same time to build their membranes, while plant cells produce phytosterols in smaller amounts, which are similar to cholesterol (Behrman et al., 2005). All lipoproteins carry cholesterol, but elevated levels of all lipoproteins except HDL are associated with an increased risk of atherosclerosis and coronary heart disease, especially elevated levels of LDL (Carmena et al., 2004). On the other hand an elevated level of HDL has a protective effect (Kontush et al., 2006). In most European cantries, cholesterol is measured in mmol/l. For healty adults, the recommended upper limit for total cholesterol is 5.2 mmol/l, of which the upper limit for LDL is 3.3 mmol/l (MC, 2022). Increased intake of soluble fiber has been shown to lower LDL levels, with each additional gram of soluble fiber lowering LDL by an average of 2.2 mg/dl (0.057 mmol/l) (Brown, 1999). Increased consumption of whole grains also reduces blood LDL levels, with whole grain oats being particulary effective (Hoolaender, 2015). Phytosterols which encourage from plants also showed great importance in reducing LDL levels (Ito et al., 2011). A meta-analysis of randomized controlled trials in adults was preformed to establish a continuous dose-response relationship that would allow prediction LDL-lowering efficacy at different doses of phytosterols. As a result of the study, it was found that phytosterol at an average dose of 2.15 g/day reduces LDL by an average of 8,8% (Demonti et al., 2009).
Sitosterolemia is a hereditary lipid disorder manifested by elevated serum sitosterol and may result in an increased risk of premature cardiovascular disease. Since sitosretol cannot be accurately measured by routine diagnostic tests, this means that the diagnosis of sitosterolemia can often be difficult, especially with many clinical features that overlap with familial hypercholesterolemia. Lipid testing including sitosterol is recommended in children, especially those with uncontrolled hyprecholesterolemia, to identify potential patients with sitosterolemia who would otherwise be overlooked (Lee et al., 2020).

Phytosterols

Phytosterols are a group of steroid alcohols that occur naturally in plants (Ramamutrthi et al., 1993). They are structurally and physiologically similar to cholesterol and represent a large group of steroid triterpens. They are necessary to maintain normal function in the cell membrane of the plant. Phytosterols can lower the intestinal absorption of cholesterol and on that way reduce the serum level od LDL and the risk of atherosclerosis (Wang, 2011). Phytosterols have been observed to displace cholesterol from micelles by dynamic competiton where unabsorbed cholesterol is eliminated in the stool, in which case its absorption and blood levels are reducing (AbuMweis et al., 2014). The most common phytosterols in the human diet are sitosterols, stigmasterols and campesterols and can be found in free form, as a fatty/cinnamic acid esters or as glycosides processed by pancreatic enzymes. Accumulating evidence reveals that phytosterols and diets enriched in them can control glucose and lipid metabolism, as well as insulin resistence (Prasad et al., 2022).
Plant sterols have a potential preventive function in aterosclerosis due to their ability to lower cholesterol (Han et al., 2015). Experimental and clinical studies have demonstrated the effect of phytosterols on lowering blood cholesterol and plasma LDL, but the effects of plant sterols beyond cholesterol lowering are still questionable. Since inflammation and endothelial dysfunction are involved in the phatogenesis of atherosclerosis, studies aim to evaluate the effect of phytosterols on biomarkers involved in the progression of aterosclerosis and are this effects independed of changes in plasma LDL (Ilha, 2020). In one stady that aimed to evaluate the effect of low-fat products enriched with plant sterols along with the diet Step 1 of the National Cholesterol Education Program on serum lipids and lipoproteins. The study was a double-blind, randomized, placebo-controlled crossover design with a run-in period and 2 intervention periods, each lasting 4 weeks. A total of 46 subjects with mild hypercholerolemia completed the study. The tested products consised of 20 g of low-fat margarine and 250 ml of low-fat milk, which supplied a total of 2.3 g of plant sterols. As a result of the study, total serum cholesterol and LDL were significantly reduced, by 5.5% and 7.7% compared to placebo. Serum apolipoprotein B was significantly reduced, by 4.6%, and apolipoprotein B/apolipoprotein A-I by 3.4%. The conclusion is that

the combination of low-fat margarin and milk enriched with plant sterols significantly reduces total serum cholesterol, LDL, apolipoprotein B and the ratio of apolipoprotein B to apolipoprotein A-I in humans with mild hypercholesterolemia, but without affecting C-reactive protein (Madsen et al., 2007).

One systemic review analyzed the cholesterol-lowering efficacy of phytosterols/stanols in normocholesterolemic and hypercholesterolemic subjects. It is important to note that familial hypercholesterolemia is characterized by high concentrations of LDL and is considered a global public health problem due to the high frequency of premature coronary heart disease in these patients. Six of the 13 reviewed studies were of sufficient quality. Studies have used fat spreads as a carrier with doses ranging from 1.6-2.8 g/day phytosterols/stanol. The subjects were heterozygotes aged 2 to 69 years. The duration of the study was from 4 weeks to 3 months. Fat spreans enriched with 2.3 +/- 0.5 g of phytosterols/stanols per day significantly reduced triglycerides, an average reduction of 0.65 mmol/l and LDL od 10-15% with an average reduction of 0.64 mmol/l (Moruisi et al., 2006).

In order to charachterize the effect of plant sterols/stanols on serum lipids in people with hypercholesterolemia who are on statin therapy, a meta-analysis of randomized controlled trials was conduced. The analysis evaluetes the use of plant sterols/stanols in combination with statins i hypercholesterolemic subjects who reported efficacy data on total cholesterol , LDL, HDL or triglycerides. The results of 8 studies (n=306 patients) had satisfied the inclusion criteria. After a meta-analysis, it was concluded that the use of plant sterols/stanols in combination with statin therapy significantly lowers the values of total cholesterol and LDL, but not HDL or triglycerides (Scholle et al., 2009).

In a pilot study involving children and adolescents with hypercholesterolemia, the effects of a combination of plant sterols, fish oil and B vitamins on the levels of four independent risk factors for cardiovascular disease were investigated; LDL ccholesterol, triacylglycerol, C-reactive protein and homocysteine. In one study 25 participants with an average age of 16 years with BMI of 23 participated in the study and recived a daily emulsified preparation consisting of plant sterol esters (1300 mg), fish oil (providing 1000 mg of EPAplus DHA), vitamin B12 (50 μg), B6 (2.5mg), folic acid (800 μg) and coenzyme Q10 (3mg) for 16 weeks. Atherogenic and inflammatory risk factors, lipophilic vitamins in plasma, provitamins and fatty acids were measured at the beginning of the 8th and 16th week. The results showed that the levels of total serum cholesterol, LDL, VLDL, LDL-2 subfractions, IDL-1, IDL-2 and homocysteine in plasma were significantly reduced at the end of the intervention ($p<0.05$). Triacylglycerol levels were reduced by 17.6% but did not reach a significant value. No significant changes in high-sensivity C-reactive protein, HDL and apolipoprotein A-1 were observed during the study period. Based on the obtained results, it is concluded that the daily intake of a combination of plant sterols, fish oil and B vitamins can modulate the lipid profile of hyprecholesterolemic children and adolescents (Garaiova et al. 2013).

The efficacy of phytosterols and fish oil on a high-oleic sunflower oil diet was evaluated in growing hypercholesterolemic rats. During 8 weeks, the control group received a standard diet, while the experimental rats were fed an atherogenic diet for period of 3 weeks, after which they were devided into four groups fed a diet with monounsaturated fatty acids (MUFA) for 5 weeks. The diet did not change weight or growth, but rats showed a decrease in total cholesterol, non-HDL and triglycerides, and an increase in HDL levels (Alsina et al., 2016).

The main sources of phytosterols are vegetable oils (linseed, olive, soybean, sesame, wheat germ), nuts (walnut, almond, pistachio, cashew, Brazil nut, hazelnut, macadamia), legumes (bean, pea), fruit (apple, cranberry, blueberry, red grapes), vegetables (broccoli, cabbage, cauliflower, garlic, onion, tomato), plants (cocoa, artichoka). A beneficial effect of phytosterols in combination with red yeast rice and in combination with probiotic bacteria was also recorded.

Flaxseed oil

Flax seeds are one of the most important sources of dry oils, with an oil content od 31% to 43%. Flaxseed is a rich source of omega-3 fatty acids, namely α-linolenic acid (ALA), short-chain polyunsaturated fatty acids (PUFA), soluble and insoluble fiber, phytoestrogen ligands, proteins and oxidases (Ivanova et al., 2012; Singh et al., 2011; Oomah, 2001; Toure et al., 2010). Flaxseed oil contains 58% α-linolenic acid, which is a powerful antioxidant (Perumal et al., 2019). It also contains stearic, oleic, linoleic and palmitic acid and vitamin E (Moallen, 2018). Flaxseed oil protects the human body from cardiovascular diseases, inhibits pro-inflammatory mediators, reduces LDL, plays a significant role in bone health and reducing the risk of hormonally mediated cancers (Nelsor et al, 2019). A recent meta-analysis of 62 randomized controlled trials with a total of 3772 participants suggests that flaxseed supplementation can reduce total serum

cholesterol, triglycerides and LDL in individuals with high baseline blood lipids (Hadi et al., 2020).
The protein content of flaxseed varies from 20% to 30%, comprising approximately 80% globulin (lignin and kinlinin) and 20% glutelin (Hall et al., 2006). Active peptides with cholesterol-lowering properties have been isolated from flaxseed proteins, which are by-products of the industrial production of flaxseed oil, which significantly improved the economic and medical value of flaxseed proteins. The most effective isolated peptide Ile-Pro-Pro-Phe (IPPF) inhibits intestinal absorption of cholesterol by modulating the expression of cholesterol transporters and reduces cholesterol synthesis in the liver by inhibiting the mevalonate pathway. According to research, IPPF can be used as a new type of food-derived intestinal cholesterol absorption inhibitor to reduce dietary cholesterol absorption and cholesterol synthesis inhibitor and has the same pharmacological mechanism as statins (Bao et al., 2022).

Olive oil

The therapeutic effects of olive (*Olea europaea* L.) for a long time have been recognized; it leads to a decrease in blood sugar, cholesterol and uric acid levels (Hawkely et al., 2010). The most important nutritional elements of olives are MUFA an PUFA. Consumption of MUFAs such as oleic acid reduces several cardiovascular risk factors (Schwingshackl et al., 2012), while the presence of omega-3 and omega-6 fatty acids emphasizes the importance of olives as an important food source of essential fatty acids. In addition of fatty acids and phenolic compounds, the nutritional value of olives is also related to other lipids such as phytosterols and non-lipid compounds such as tocopherols and carotenoids (Boskou et al., 2015). Olive oil mainly consists of triacylglycerols, which represent a diverse group of glycerol esters with different fatty acids. The predominant fatty acid is monounsaturated oleic acid, up to 83%. Palmitic acid, linoleic acid, stearic acid and palmitoleic acid are also present (Boskou, 2009; Luchetti, 2002; Ramirez-Tortosa et al., 2006). Research results suggest that supplementing the diet with olive oil and plant sterols esterified to fatty acids of olive oil favorably alters the plasma lipid profile and may reduce the susceptibility of LDL to lipid peroxidation in individuals with hypercholesterolemia (Chen et al., 2007).

Soybean and soyabean products

Soy (*Glycine max*) is a plant species from the leguminosus group which is an important source of plant proteins, isoflavones, phytoestrogens and polyphenols that have ability to inhibit LDL oxidation (Maki et al., 2010). Raw soy contains 20% fat including saturated fat (3%), monounsaturated (4%) and polyunsaturated fat, mainly in the form of linoleic acid. Soybean oil is one of the main term by-product in the soybean processing process and is rich in phytosterols (Ramamurthi et al., 1993). Just like cholesterol in animals, phytosterols regulate the fluidity of plant cell membranes and characteristics in cell differentiation and proliferation (Piironen et al., 2000). The FDA has stated that daily intake of moderate amounts of phytosterols may reduce the risk of heart disease. Long-term intake of foods rich in phytosterols could effectively reduce plasma cholesterol levels and the risk of atherosclerosis (Ostlud et al., 2003). In a randomized, controlled, palallel trial, soy was shown to have a cholesterol-lowering effect in 65 men and women with moderate hypercholesterolemia who experienced a reduction in total LDL (Maki et al., 2010). In a randomized crossover study of 24 subjects , soy consumption reduced lipid peroxidation *in vivo* and increased LDL resistance to oxidation (Wiseman et al., 2000). In a meta-analysis of eight randomized studies, scientists found that participants who consumed high concentrations of isoflavones had LDL concentrations approximately 6mg/dl lower than participants who consumed the same amount of soy protein with low concentrations of isoflavones ($P<0.0001$). Similar effects were observed when patients who were normocholesterolemic were analyzed separately. Becouse soy is generally associated with reduced serum cholesterol concentrations (Belleville et al., 2002). In a 4-week double blind, placebo-controlled, cross-over study, 38 moderately hypercholesterolemic volunteers (58±12 years, LDL ≥ 130 mg/dl) participated and were randomly assigned to consume 400 ml/day of soy milk or soy milk + phytosterols (1,6 g/day). Blood samples were collected and lipid profile and biomarkers for inflammation and endothelial dysfunction were determined. The results showed that treatment with phytosterol reduced the concentration of endothelin-1 in plasma by 11% ($p=0.02$) independent of the variations in plasma LDL level. Phytosterol reduced the concentration of total cholesterol in plasma (-5,5%, $p < 0,001$), LDL (-6,4%, $p < 0,05$), without changing the concentration of HDL ($p > 0.05$). Therefore, it can be seen that supplementation with phytosterols effectively lowers endothelin-1 independently of the reduction of LDL levels in plasma, contributing to the understanding of the effect of plant sterols on endothelial function and prevention of cardiovascular disease (Ilha, 2020). The FDA has approved a daily intake of 25g of soy protein in a diet low in saturated fat and cholesterol as a means of reducing the risk of cardiovascular disease. Soy is also advocated in the treamtent of hypercholesterolemia (FDA, 1999).

Sesame oil

Sesame (Sesamum indicum) yields an oil rich in MUFA and PUFA. Many studies have revealed that sesame oil contains lignans such as sesamin, sesamolin and several antioxidant compounds such as sesaminol (Egbekun et al., 1997). Lignans are metabolites formed from two phenylpropanoid molecules. In sesame, lignan synthesis involves the fusion of oxopropane side chains of cinnamyl alcohol to the furan core, and these metabolites are designated as furofuran lignans (Wu et al., 2019). In addition to lignans, several bioactive compounds such as phenols, phytosterols, phytates and tocopherol have been identified in sesame (Pathak et al., 2017). Sesamin has been shown to reduce total serum cholesterol and LDL levels in patients with hypercholesterolemia (Hirata et al., 1996). A randomized, placebo-controlled study in overweight men and woman, given sesame seed equivalents containing 50 mg of sesamin per day, showed no reduction in blood lipids or blood pressure, and markers of systemic inflammation and lipid peroxidation were unaffected, although urine excretion confirmed that lignans were absorbed and metabolized (Penavlo et al., 2006). On the other hand, administration of sesame to postmenopausal women reduced their serum cholesterol and androgen precursor levels and increased the ratio of tocopherol (Wu et al., 2006).

Wheat germs

Wheat (*Triticum aestivum*) is one of the most widely consumed edible whole germs in the world. Wheat consist of abouth 80% endosperm, 15% bran and 5% germ (Savin, 2004). It is rich in starch, fiber, minerals, vitamins and phytochemicals such as phenolic compounds, phytosterols and swingolipids and most of them are concentrated in the outer layers (bran) of the grain (Cheng et al., 2021). Weath germ (embryo) is a concentrated source of antioxidans such as polyphenols, carotenoids and tocopherols (the most common natural sources of vitamin E) (Vaher et al., 2010; Zhu et al., 2011). Nomerous *in vivo* and *in vitro* studies have investigated various health aspects of weath germ, especially wheat germ oil (Arshad et al., 2013; Kherd et al., 2017) which can improve lipid metabolism (Khalil et al., 2010). In the conducted research, wheat germs with a high concent of phytosterols in relation to total fat were selected for one group as a trial food with a low fat content, while the other group of respondents used wheat germs without phytosterols. Absorption of cholesterol from meals containing wheat germ with phytosterols is significantly lower than absorption of cholesterol from foods with wheat germ that does not contain phytosterols. This suggests that endogenous phytosterols in wheat germ and possibly other low-fat vegetable foods may have important effects on cholesterol absorption and metabolism that are independent of major nutrients (Ostlud et al., 2003).

Nuts

Nuts are a food rich in energy and contain a complex matrix of beneficial nutrients and bioactive substances including MUFAs and PUFAs, high-quality proteins, fiber, non-sodium minerals, tocopherols, phytosterols and antioxidant phenols (Ros et al., 2021). They contain a number of phytochemicals including carotenoids, phenolic acid, phytosterols and polyphenolic compounds such as flavonoids, proanthocyanidins and stilbenes, as well as phytates, sphingolipids, lignans. The phytochemical content of nuts can very significantly depending on the type of nuts, genotype, pre-harvest and post-harvest conditions, as well as storage conditions. Genotype affects phenolic acid, flavonoids, stilbenes and phytosterols. Phytochemicals found in nuts are associated with antioxidant, anti-inflammatory, antiproliferative, antiviral, chemopreventive and hypocholesterolemic effects (Bolling et al., 2011). Phytosterols in nuts range from 95-280 mg/100 g. Walnuts are particulary rich in total phenols with 1625 mg gallic acid equivalents/100 g. Stilbene resveratrol is found in peanuts and pistachios in amounts of 84 and 115 μg/100 g. Proanthocyanins are found in almonds, cashews, hazelnuts, pistachios, peanuts and walnuts with concentrations varying from 9-494 mg/100 g (Chen et al., 2008). Randomized control trials consistently show that nuts lower cholesterol. It is also an interesting fact that although foods are rich in energy, they do not predispose to obesity, even on the contrary, they can help weight loss. Level 1 evidence from the PREDIMED trial showed that consumption of 30 g of nuts per day significantly reduced the risk of the composite endpoint of major adverse cardiovascular events (Ross et al., 2021). In the conducted study, the effects of nuts on risk factors for major cardiovascular diseasee were investigated. The effects of nuts (walnuts, pistachios, macadamias, cashews, almonds, hazelnuts and Brazil nuts) on blood lipids were investigated. The results showed that nut intake (portion/day) lowered total cholesterol (-4.7 mg/dl) and LDL (-4.8 mg/dl). The dose-response between nut intake and total cholesterol and LDL was non-linear (P-non-linearity <0.001each), with stronger effects observed for ≥60 g nuts/day. No significant heterogeneity was observed by nut type or other factors. It is concluded that the intake of nuts lowers total cholesterol and LDL. The main determinant of cholesterol lowering appears to be the dose of nuts rather than the type (Gobbo et al., 2015).

Leguminos (fabaceae)

Legumes are a good source of bioactive compounds such as polyphenols, phytosterols, indigestible carbohydrates and have an important physiological and metabolic role. Ferulic acid is the most abundant phenolic acid present in legumes, while flavonol glycosides, anthocyanins and tannins are responsable for the color of the seed coat. Sitosterol (which is also the most abundant), stigmasterol and compesterol are the main phytosterols present in legumes. Fiber, resistant starch and oligosaccharides function as probiotic and possess several other health benefits such as anti-inflammatory and antitumor effects and lowering blood glusose and lipid levels. Beans and peas contain higher amounts of oligosaccharides than other legumes (Singh et al., 2017). Consuming soluble fiber in the diet is associated with health benefits, including the reduction in lipid levels. By consuming water-soluble fibers that from viscosity, the level of total cholesterol and LDL can be reduced by about 5-10 %, and minimal changes in HDL have been observed. The cholesterol-lowering properties of soluble fibers depend on their physical and chemical properties, and medium to high molecular weight fibers are more effective in lowering lipid levels (Suramoudi et al., 2016).

Fruits (apple, cranberry, blueberry, grapes) as a source of phytochemicals

Epidemiological studies indicate that fertile consumption of foods rich flavonoids is associated with a reduced risk of cardiovascular diseases, which is probably the result of their antioxidant activity and ability to inhibit endogenous cholesterol synthesis (Kruger et al., 2014). Fruits and vegetables are a good source of flavonoids, so the recommended intake of five a day would have a positive effect on the level of lipids in the blood.

It was experimentally shown that in hamsters fed cholesterol-enriched food, the consumption of anthocyanin can reduce the concentration of LDL in the plasma and increase the concentration of HDL by manifesting antiatherogenic effects (Liang et al., 2013). Similar results were observed in humans after 3 months of anthocyanin supplementation (160 mg twice daily) in a double-blind, randomized, placebo-controlled tial of 120 subjects with dyslipidemia (Qin et al., 2009).

Quercetin is one of the main flavonoids found in food, and it is associated with the prevention of LDL oxidation and atherosclerosis by exhibiting anti-inflammatory, antiproliferative and antioxidative effects (Liang et al., 2013; Qin et al., 2009). In a double-blind cross-over study on 49 healthy male subjects, it was determined that quercetin intake (150 mg/day) reduced postprandial triglyceride concentrations and increased HDL concentrations (Pfeuffer et al., 2013). It was found that the consumption of a functional drink rich in fruit poyphenols (apple, cranberry, blueberry) for two week can significantly reduce the concentration of triglycerides, total cholesterol and cholesterol in the liver in experimental sponthaneously hypertensive rats fed an atherogenic diet (Gunathilake et al., 2013).

Cranberry (Vaccinium macrocarpon)

Cranberry as one of the main sources of polyphenols has found its place for widespread use due to its antioxidant properties. It contains a number of phytochemicals including three classes of flavonoids (flavonols, anthocyanins and proantocyanins), catechins and phenolic acids, substances associated with a wide range of biologocal effects, including antioxidant activity, madulation of enzyme activity and regulation of genetic expression (Neto, 2007). The nutritional attributes of cranberry make it one of the most important nutritional targets in the prevention of cardiovascular disease by having a beneficial effect on cardiovascular disease risk factors that include dyslipidemia, oxidative stresa, hypertension, inflammation and endothelial dysfunction. After long-term cranberry use, clinical studies have shown significant improvment in lipid profile, apoA-I and oxidative stress, and reduction in apoB, fasting plasma glucose and C-reaktive protein (Shidfar et al., 2012; Daffey et al., 2015).

Grapes (Vitis vinifera)

Grapes are a good source of polyphenol antioxidant. A 6-week randomized, double-blind, placebo-controlled study was conducted to evaluate the effects of integral grape extract on antioxidant status and lipid profile in 24 prehypertensive, obese, and/or prediabetic subjects. The results showed an increase in HDL, a decrease in the ratio of total cholesterol to LDL and improved antioxidant capacity (Evans et al., 2014).

Vegetables (cabbage, garlic, onion, tomato) as a source of phytochemicals

Cabbage (Cruciferae, Brassicaceae)

Plants which belongs to the Brassicaceae family (cabbage, brussels sprouts, broccoli and cauliflower) contain glucosinolates, polyphenols, carotenoids and phytosterols which are anti-inflammatory and antioxidant nature. In an intervention study, 38 healthy volunteers (23 woman and 15 man) were fed portion of 300 g/day of blach and red cabbage for a period of two weeks. The results showed a significant increase in plasma carotenoid concentration (lutein and β-carotene) and total antioxidant capacity. A decrease in blood glucose and an improved lipid profile were observed, along with a decrease in total an LDL cholesterol and LDL oxidation (Bacchetti et al., 2014).

Garlic (Allium sativum)

Bioactive compounds in garlic are enzymes (eg. allinase) and sulfur-rich compounds such as enzymatically produced allin and its compounds (eg. allicin). Allicin concentrations in garlic vary depending on the metod of its processing. Since allicin is an unstable compound it is quickly transformed into various chemicals. However, even in the abstance of allicin, garlic still retains its positive effects on the cardiovascular system. Clinically , the benefits of garlic have been reported to alleviate several conditions, including hypertension, hypercholesterolemia, diabetes and atherosclerosis. The possible antibacterial, antihypertenzive and antitrombotic properties of garlic also make it an important antiatherogenic agent (Majewski, 2014).

Onion (Allium cepa)

Onion take a high place among vegetables that are rich in flavonoids, mainly containing quercetin. Its most significant benefits associated with cardiovascular disease include lowering blood pressure and oxidized LDL, and acting as aninflammatory marker. However, the best effects od onion are observed when approximately 150 mg of quercetin is consumed, which corresponds to an intake of approximately 700 mg/day of onions (Toh et al., 2013).

Tomato (Lycopersicon esculentum)

Tomato is one of the most consumed vegetables in the world and is a rich source of carotenoids and flavonoids (Hanson et al., 2004). It is a key component of the Mediterranean diet and its consumption is directly related to the reduction of the risk of inflammatory processes, various chronic diseases and carcinogenesis, as well as the inhibition of LDL oxidation, which helps to lower blood cholesterol levels (Pinela et al., 2016). A randomized, single-blind, controlled clinical trial in humans showed that raw tomato consumption had a beneficial effect on HDL concentrations in overweight women (Cuevas-Ramos et al., 2013).

Plants (artichoke, cacao) as a source of phytosterols

Artichoke (Cynara scolyms)

Due to significant amount of polyphenols in its artichoke extracts, artichoka can exhibit a hypercholesterolemic effects (Arnaboldi et al., 2022). A trial was conducted to evaluate the effects of artichoke leaf extract on plasma lipid levels and general well-being in otherwise healthy adults with mild to moderate hypercholesterolemia. Number of 131 individuals were tested for total plasma cholesterol in range 6.0-8.0 mmol/l, with 75 appropiate volunteers randomized into the trial. Vounteers consumed 1280 mg of a standardized artichoke leaf extract per day or a matching placebo for 12 weeks. Total cholesterol in the plasma of the treated group decreased by an average of 4,2% (from 7.16 mmol/l to 6.86 mmol/l) and increased in the control group by an average of 1.9% (6.90 mmo/l to 7.03 mmol/l), where the difference between the groups is statistically significant ($p=0.025$). No significant differences were observed between groups for LDL, HDL or triglyceride levels. Consumption of artichoke leaf extract resulted in a modest but beneficial statistically significant difference in total cholesterol after 12 weeks (Bundy et al., 2008).

Cacao (Theobroma cacao)

Cacao contains polyphenols that reduce LDL oxidation, act as anti-inflammatory, antioxidant, moderate immune response, improve vascular function and reduce thrombocytes adhesion (Schinella et al., 2010). Antioxidants in cacao polyphenols change the glycemic response, change the lipid profile and reduce thrombocytes aggregation, inflammation and blood pressure, and can alleviate intestinal inflammation by reducing neutrophil infiltration and the production of proinflammatory enzymes and cytokines (Mohamed 2014). In a study in which 25 woman and 25 man participated, the effects of a three-week consumption of 50 g of flavonoid-rich dark chocolate on the oxidative stress of lipoproteins *in vivo* and *in vitro* were investigated, and the results were that the lipoprotein profile was improved with greater beneficial effects in women that in men (Nanetti et al., 2012).

Nutraceuticals with subchapters: red yeast rice and probiotic bacteria

RED YEAST RICE

Phytosterols and red yaest rice are very often studied nutraceuticals for lowering cholesterol, that is inhibiting intestinal absorption and hepatic synthesis of cholesterol (Cicero et al., 2017). The cholesterol-lowering effectiveness of red yeast rice is directly related to the amount of monacolin K in the extract (up to 10mg per day). Consuming macrolin K on a daily basis lowers LDL levels, where any reduction in LDL is accompanied by a similar raduction in total cholesterol. Although it has a mechanism of action that is similar to statins, daily consumption between 3 and 10 mg of macrolin K causes minimal risk, and mild myalgias are seen only in the weakest patients (Cicero et al., 2019). A study was conducted that aimed to examine the effects of phytosterols and red yeast rice on the lipid profile and their relationship. A double-blind, clinical trials was conducted randomizing 90 subjects with moderate hypercholesterolemia to treatment with phytosterols 800 mg (group 1), red yeast rice standardized to contain 5 mg macrolin from Monscus purpureus (group 2), or both in combination with nutraceuticals (group 3). The results obtained after 8 weeks of treatment in group 1 did not reveal significant variations in lipid parameters. In group 2 there was a significant raduction ($p < 0.001$) of LDL (-20.5% compared to the initial value) and apolipoprotein B (-14.4% compared to the initial value) as occurred in group 3 (LDL versus baseline: -27.0% apolipoprotein B versus baseline: -19.0%) ($p < 0.001$). Changes in LDL and apolipoprotein B were significantly different comparing group 2 with group 1 ($p < 0.05$). The change in LDL was also significantly higher in group 3 than in group 2 (p <005). The obtained results indicate that the combination of phytosterols and red yeast rice has an additive effect on lowering cholesterol, achieving a clinically significant reduction of LDL in patients with mild hypercholesterolemia (Cicero et al., 2017).

Probiotic bacteria

Phytosterols and probiotic bacteria are natural hypocholesterolemic agents with potential cardiovascular benefits. Accordingly, a stady was conducted to evaluate the effects of probiotic and phytosterols supplementation alone or in combination on serum and liver lipid profiles and thyroid hormones in hypercholesterolemic rats. The probiotic treatment consisted of 8 probiotic strains: 2 each of *Lactobacillus acidophilus*, *Lacrobacillus casei*, *Lactobacillus gasseri* and *Lactobacillus reuteri*. Rats were fed for 8 weeks and treatment with a high-cholesterol basal diet to induce hypercholesterolemia. The results of the study showed that supplementation significantly reduced serum total cholesterol, LDL, HDL and tryglicerides compared to control groups. The symbiotic treatment was more effective in lowering LDL, while the mix probiotic treatment was more effective in lowering serum total cholesterol and LDL than the treatment containing phytosterols. Treatment containing phytosterols induced increased thyroid activity, which was evident by increased levels of serum total thyroxine, total triiodothyronine and free triiodothyronine. Based on the obtained results, it can be concluded that the lipid profile can be effectively reduced in order to reduce the incidence of cardiovascular diseases by using a combination of *Lactobacillus*-based probiotics and phytosterols in functional food (Awaisheh et al., 2013).

Conclusion

Food that is rich in phytosterols show favorable effects on the lipid profile in the blood, and therefore food that containing phytosterols and/or nutritional supplements should be included in the daily diet. Such a diet would have far-reaching effects on the entire population, not only on people who have hypercholesterolemia, because cardiovascular deseases and strokes are the main cause of death in Croatia and the countries of the region.

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