ÉLELMISZERVIZSGÁLATI KÖZLEMÉNYEK

JOURNAL OF FOOD INVESTIGATION

SCIENCE – LIFE – QUALITY – SAFETY

SPECIAL EDITION I. – 2020 TUDOMÁNY-ÉLET-MINŐSÉG-BIZTONSÁG OCTOBER 2020

RESEARCH NETWORK FOR SAFER FOOD AND HEALTHIER CONSUMERS

EFOP-3.6.2-16 - 2017-00012
Development of a functional, healthy and safe food product range-model based on the principle of Farm-to-Fork in a thematic research network

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HU ISSN 2676-8704

Dear Readers,

This issue is the first one of special edition of Journal of Food Investigations (JFI – ÉVIK) since the editorial activity was moved to the WESSLING Nonprofit ltd. Budapest Hungary. The Editorial Staff were happy to get request from the University of Veterinary Medicine (Budapest, Hungary) to issue a special edition on the topic of food science and the related agricultural aspects. So, we are issuing six scientific papers in open access electronic version in English, containing different topics from farm to the fork.

Marcello and his collaborating authors are writing about the biochemical effects of fructose feeding of mammalian living being. The short paper says, in young rats the obligate fructose feeding the brain – the most "energy hungriest organ" – can cause several metabolic perturbations in their neural system and hippocampus. In adult mouse the pure fructose supplementation decreases the adult neurogenesis, worsens the hippocampus-associated learning memory.

Lányi et al was investigate an, effect on the cheese producing process a residue of a wide range used antibiotic, cephalexin. They stated, the carry-over of residues of cephalexin in cheese more less, the in the whey. Despite of it, the residues of these antibiotic, may influence of the quality of the dairy product. The authors gave a sketch of HPLC/MS/MS technique to investigate the residues of cephalexin from dairy products.

The topic of article of **Szabó and Ózsvári** related to the year-by-year most significant health problem, to the intolerance of lactose. They work was a survey of consumer awareness to lactose intolerance. The investigation based on filling out in a questionnaire. Az the results of their survey, the knowledge of the consumers involved in lactose intolerance should be enhanced to make influence on choice of safety product.

The topic of article of **Hejel et al** is the redox status of young veal. The reactive free oxygen derivatives are important on the process of phagocytosis, normal apoptosis and the maturation, but the excessive amount of oxygen free radicals can destroy the significant structures of living cells. The feeding containing several antioxidant feed supplements modifying of redox status of the young calves can be effective on the health of the growing young animals, via the reduce of oxidative stress processes in their metabolism, so may increase the productivity of dairy farms.

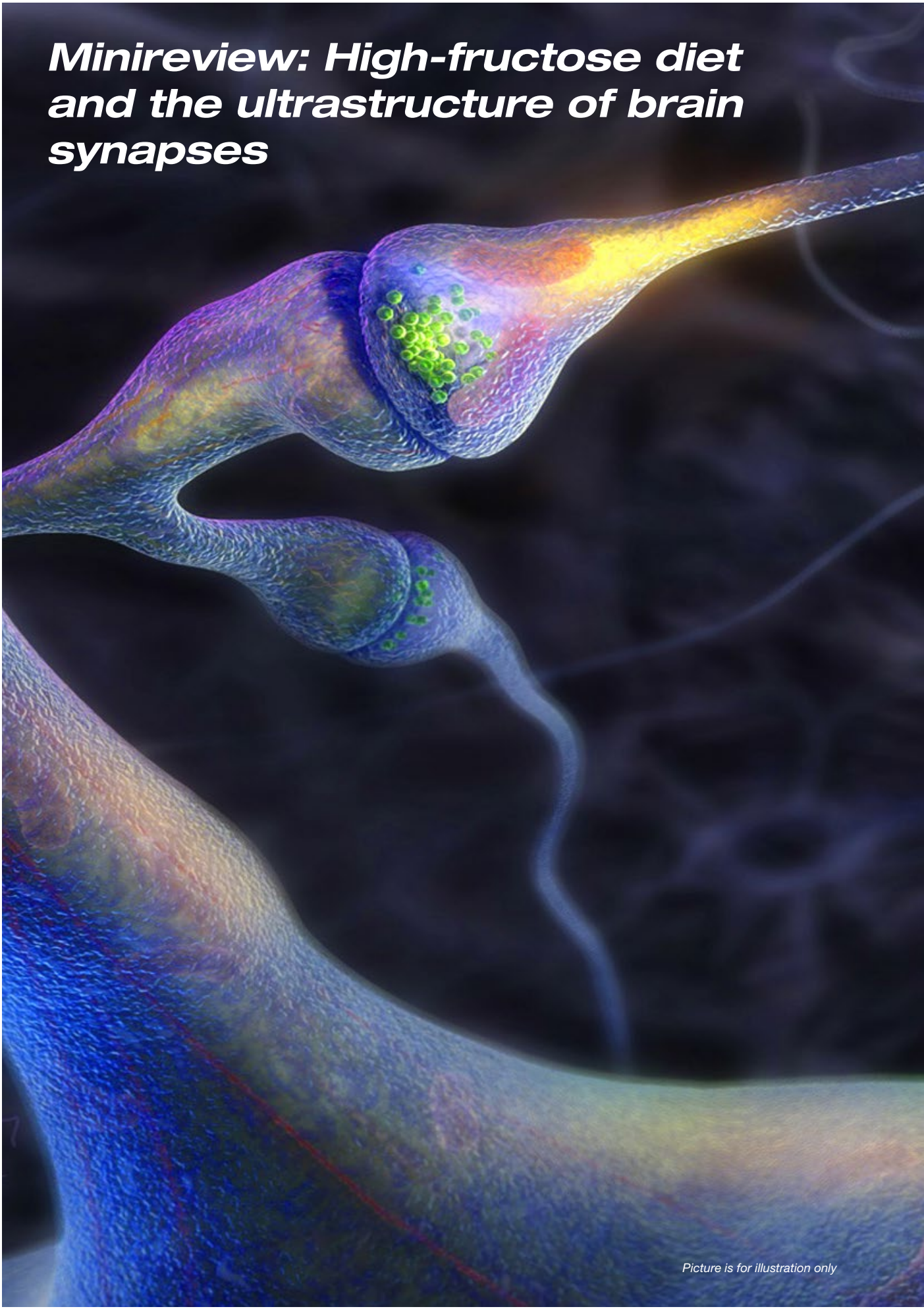
The topic of functional foods is frequented amongst the food scientific literature. **Király et al** refers about investigation of health beneficial effects of Hungarian apple varieties. Several Hungarian apple varieties have considerable antioxidant components, so these varieties can be used even as functional foods. The analysis of several sugar content was carried out using HPLC/VIS system, the antioxidant capacity was measures by modified FRAP method. In the text of the paper we will use the Hungarian names of the apple varieties, but in order to explain the understanding the meaning of the names, we give a raw translation in the end of the article.

To provide the food quality is starting either in the field or in the stable. The work of **Hornung et al** is about the orcharding of strawberry plantation. The authors have investigated the composition of soil nematode communities. The nematodes can cause the infection of fruits but may contribute to form the optimal soil conditions to get more healthy and good quality fruits. In the experiment it was found, the covering of soil helps to maintain the taxonomic diversity of soil invertebrates, causing an advantageous effect on fruit production.

These papers was written in the framework of the application of University of Veterinary Medicine (Budapest, Hungary), EFOP-3.6.2-16 - 2017-00012 Development of a functional, healthy and safe food product range-model based on the principle of Farm-to-Fork in a thematic research network. I wish a good reading and good health for our Readers:

Dr. Tamás János SZIGETI
Editor-in-cheef

Minireview: High-fructose diet and the ultrastructure of brain synapses



Picture is for illustration only

G. Mark Marcello[1], Péter Sótonyi[1], Miklós Süth[1], Bence Rácz[1]*

Minireview: High-fructose diet and the ultrastructure of brain synapses

Keywords: fructose, sucrose, synaptic plasticity, ultrastructure, brain

1. SUMMARY

The brain is the hungriest organ. With this great energy demand, the brain's function may depend on what it is being fed to a greater extent than previously appreciated. Consumption of fructose and glucose in sustained high quantities in industrially processed foods and beverages in the modern Western diet raises complex questions of metabolic, and neurological well-being. Here, we review the effects of sugar on hippocampus associated short term memory. The following work on rodent models, and human clinical trials expound the influence of increased fructose versus glucose, or starch intake on the synaptic organization of a brain region extensively involved in cognitive functions. Through the well supported structure-function relationship of dendritic synapse profiles, synaptic functionality effects of increased fructose versus glucose consumption on hippocampal synapse ultrastructure may be seen. Together with behavioral, and functional findings, ultrastructural data demonstrate potential changes in hippocampal associated cognitive processes directly related to either elevated fructose or glucose intake.

2. Abbreviations used in this paper:

HFCS: High fructose corn syrup

HFHS: High fat high sugar

LTD: Long-term depression

LTP: Long-term potentiation

MSB: Multi-synaptic bouton

PSD: Postsynaptic density

3. Effect of fructose on the neural system

Function and structure must be considered concurrently to untangle the neurological effects of persistent increased sugar intake. In processed foods and drinks 'sugar' is added primarily as either sucrose (the disaccharide of covalently bonded fructose and glucose) or high fructose corn syrup (HFCS). HFCS may contain a higher percentage of glucose in certain applications but the commonly used HFCS-55 solution (55% fructose and 45% glucose) is most commonly used in rodent model studies. **[1]** Sugar supplemented industrially processed foods are a notable component of the Western diet.

An applicable simplification of the complex Western diet is the high fat high sugar (HFHS) diet. The sugar half of the HFHS diet is practically represented by sucrose or HFCS-55; in turn, roughly half of these sugars are fructose. **[2]**

To get a more precise understanding of what fructose does to the brain, studies focus on either pure fructose compared to glucose or look at the two sugars together in the form of sucrose or HFCS. In adult, and in young rats it was shown that short term pure fructose feeding may induce metabolic perturbations with markers of neuroinflammation in the hippocampus. **[3,4]** In an adult mouse model of metabolic syndrome, pure fructose supplementation in water was found to decrease adult neurogenesis alongside decreased performance in a paradigm of hippocampus-associated learning and memory **[5]**, showing decreased long-term potentiation (LTP), and decreased long-term depression (LTD) - both forms of memory-related synaptic plasticity. **[6]**

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Another mouse model study found that while high fructose diet decreases physical activity, it does not affect neurogenesis, and learning. **[7]**

Such contradicting findings may be seen throughout the current literature of neuroscience focused on fructose. It is clear that more studies are warranted in order to gain a more exact understanding of the impact of fructose on multiple aspects of brain function. Pairing functional with structural findings therefore seems essential. More precise quantification of synaptic ultrastructure is required to support findings of decreased functional synaptic plasticity, and decreased cognitive performance on hippocampus-associated spatial learning, and memory tasks. Most studies use biochemical methods with a molecular approach on brain homogenates to reveal potential effects of fructose on hippocampal cognitive function. Extrapolation of the effect of fructose supplementation on the fine structure of neuropil from hippocampal homogenates lends itself to questionable conclusions of synaptic structural plasticity.

The dendritic spine-synapses of hippocampal CA1 – the hippocampus' region of primary excitatory input **[8]** - fluctuate in size with different morphological characteristics in response to input from Schaffer collaterals of CA3 as well as afferent input from outside the hippocampus. The ultrastructural changes of dendritic spines in response to axonal input may be considered neuroanatomical indicators of synaptic efficacy. **[9]** The CA1 is the brain region with the most quantitative data available. **[10]** Dendritic spines enlarge following LTP, and decrease in size following LTD. **[10]** As such, LTP and LTD associated functional synaptic plasticity may be neuroanatomically quantified through measuring ultrastructural parameters of structural synaptic plasticity. In addition to ultrastructural measurements of dendritic spines, the neuropil may be investigated for specific markers of structural synaptic plasticity. A multi-synaptic bouton (MSB), a structure represented by one terminal bouton forming a synapse with two or more dendritic spines, and a perforated post-synaptic density (pPSD) are both structures indicating a robust synaptic connection. MSBs are markers of late phase LTP and indicate naturally forming complexity with maintained information specificity in a neural network. **[11,12]** MSBs indicate presynaptic functional strength while pPSDs are markers of powerful postsynaptic strength/excitability **[13,14]**. Therefore, finding MSBs, and pPSDs may be taken as indicators of structural synaptic plasticity as well as robust network functionality. As the flow of information transfer in neuronal circuitry dynamically changes so the underlying synaptic ultrastructure changes with it. A change in functionality in this case may be supported by observable, and quantifiable structural change.

Studying the effect of fructose supplementation on the extensively studied hippocampus is relevant in beginning to unravel how increased fructose intake may influence the brain's function, and structure. The hippocampus is more directly relevant however; it has been shown to have a significant higher order functional role in the complex circuitry of feed intake regulation. **[15]** The hippocampus is responsive to both leptin, and insulin; fascinatingly, double meal eating has been observed in amnesic people with hippocampal damage. **[15]** Studying the hippocampus may not only clarify the effect of fructose supplementation on spatial learning, and memory but may show changes in the feed intake regulation of fructose itself. To determine what effect increased fructose supplementation may have on memory it is important to clarify what question is being asked, and then tested. In rodent models: species, age, form and concentration of sugar, length of treatment, presence of body weight gain, and supplementation in water or in food are all factors which provide a specific trial environment which may only be accurately compared to analogous studies. Such likeness is essential for appropriate comparability between rodent model trials before we may begin to tease apart findings of fructose' cognitive influence in human clinical trials, and what true cognitive impact fructose may have epidemiologically in sustained lifelong Western diet.

4. Acknowledgement

The Project is supported by the European Union and co-financed by the European Social Fund (grant agreement no. EFOP-3.6.2- 16-2017- 00012). BR were supported by the János Bolyai Research Fellowship of the Hungarian Academy of Sciences.

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Effects of cephalexin residues on the starter culture's microbial activity during the fresh cheese making process

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Effects of cephalexin residues on the starter culture's microbial activity during the fresh cheese making process

Keywords: cephalexin, cheese, whey, carry-over, LC-MS/MS

1. SUMMARY

The aim of our study was to investigate the carry-over of cephalexin from cow's milk to cheese and whey, as well as, to study the potential impact of its presence on the microbial activity of the starter culture. Before cheese-making, the raw milk was artificially contaminated to different antibiotic levels. Cephalexin concentrations and the pH values were measured all along the process. It was found that cephalexin was transferred less into the cheese curd (1.8-4.3 % of the original amount) than into the whey (29.3-42.8 %). According to the results the concentration of cephalexin did not influence substantially the pH changes during curding nor the activity of the starter culture. However, pH of the fresh cheese showed significant ($p < 0.05$) differences compared to the control suggesting that antibiotic residues even below MRL level may influence the quality of product.

2. Introduction

Dairy products form an important part of the healthy everyday diet, being consumed in high and growing amounts worldwide. Cheese production process involves the formation of lactic acid from lactose as an essential step. Acidification of the cheesemilk is usually initiated by selected starter cultures of lactic acid bacteria. The rate and extent of acidification can significantly influence the quality of cheese, including its texture, by having effect on the microbial biota of the developing product. Whey is the main by-product of cheese making, with utilization options as an ingredient in human foodstuff and animal feed production as well as in agricultural applications **[1]**. Therefore, the presence of antibiotic residues in cheese whey could have negative implications for human or animal healthcare or environmental safety.

Ensuring livestock health is of utmost importance for combating hunger and assuring appropriate food for the population, and antibiotic treatments are still an important part of this effort. Therefore, the fate of veterinary drug residues in foodstuffs remains of increasing concern due to their possible negative implications for consumer health such as allergic reactions or disturbances in the intestinal flora **[2, 3, 4]**. In addition to the direct negative effects on human health, antibiotic residues may contribute to the development of antimicrobial resistance **[5, 6, 7]**.

In spite of the concerns outlined above, maximum residue levels (MRLs) of veterinary drug residues in dairy products, such as cheese or whey, is not regulated by the relevant Commission Regulation (EU) No. 37/2010 **[8]**. This lack of regulation is even more incomprehensible, when the actual data on antibiotic residues found in dairy products are taken into account **[9, 10, 11]**.

Cephalexin is a first-generation cephalosporin antibiotic belonging to the wider group of β-lactams with a broad spectrum of activity against both Gram positive and Gram negative bacteria **[12]**. In the European Union, it is set out in the Commission Regulation (EU) No. 37/2010 **[8]** with a maximum residue limit of 100 µg/kg for bovine milk. Since it can penetrate to soft tissues in significant ratio it has been widely applied in the treatment of dairy cow mastitis either alone or in combination products **[13]**.

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Our study was aimed to investigate the carry-over of cephalexin from cow's milk to cheese and the whey produced from it, as well as, to study the potential impact of its presence on the pH of the internal products and the fresh cheese made from raw and heat-treated cow's milk, respectively. Our further aim was to study the possible effects of cephalexin residues on the microbe count of cheesemilk and thus the initial operation of starter culture for cheese production.

3. Materials and methods

3.1. Milk spiking and cheese making

Raw cow milk was purchased at a local market at the beginning of every trial day. It was quick-tested for possible antibiotic residues by DelvoTest® (DSM Food Specialties B.V. Delft, The Netherlands). It was also tested specifically for residues of 11 veterinary antibiotics by an LC-MS/MS method developed previously **[14]**. From the raw milk no positive sample occurred during the trials.

Fresh cheese was prepared from 10 litres of control raw milk itself (without additives or heat treatment) in every trial, to serve as control samples for analysis. Furthermore, for each trial, 10 litres of milk were artificially contaminated to concentration levels of 50, 100 and 500 ng/mL cephalexin (by Fluka, purchased from Sigma-Aldrich, USA) respectively, resulting in theoretical contamination levels of 0.5·MRL, 1·MRL, 5·MRL values of this antibiotics (Low, Medium and High trials, respectively). After this, the milk was divided into two equal portions (two times 5 litres), from which one portion was subjected to heat treatment (72 °C, 15 sec) industrially applied in case of this cheese type, the other one was processed without it (see ***Figure 1***). Heat treatment was carried out in a temperature controlled cheese vat with continuous slow stirring in order to maintain balanced heat distribution. After heat treatment, fresh cheese was made from the milk with lyophilised starter culture (CHOOZIT™ RA 22 LYO 125 DCU by Danisco DuPont) containing *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subs. cremonis* and *Streptococcus salivarius subsp. thermophilus.* The starter culture was added in 0.313 DCU/5 L (260 mg/5 L) dosage. Every cheese making trial was repeated three times.

3.2 pH measurements

The pH of the milk was monitored during the cheese manufacture with Thermo Orion 2 Star benchtop laboratory pH meter. The pH values of the original milk and all milk batches serving as raw material were recorded. For milk portions used in spiked trials, the pH was recorded following the addition of cephalexin both before and after the heat treatment. The pH values of cheesemilk, whey and the fresh cheese were also recorded. The latter one was recorded immediately after the 30 minute pressing.

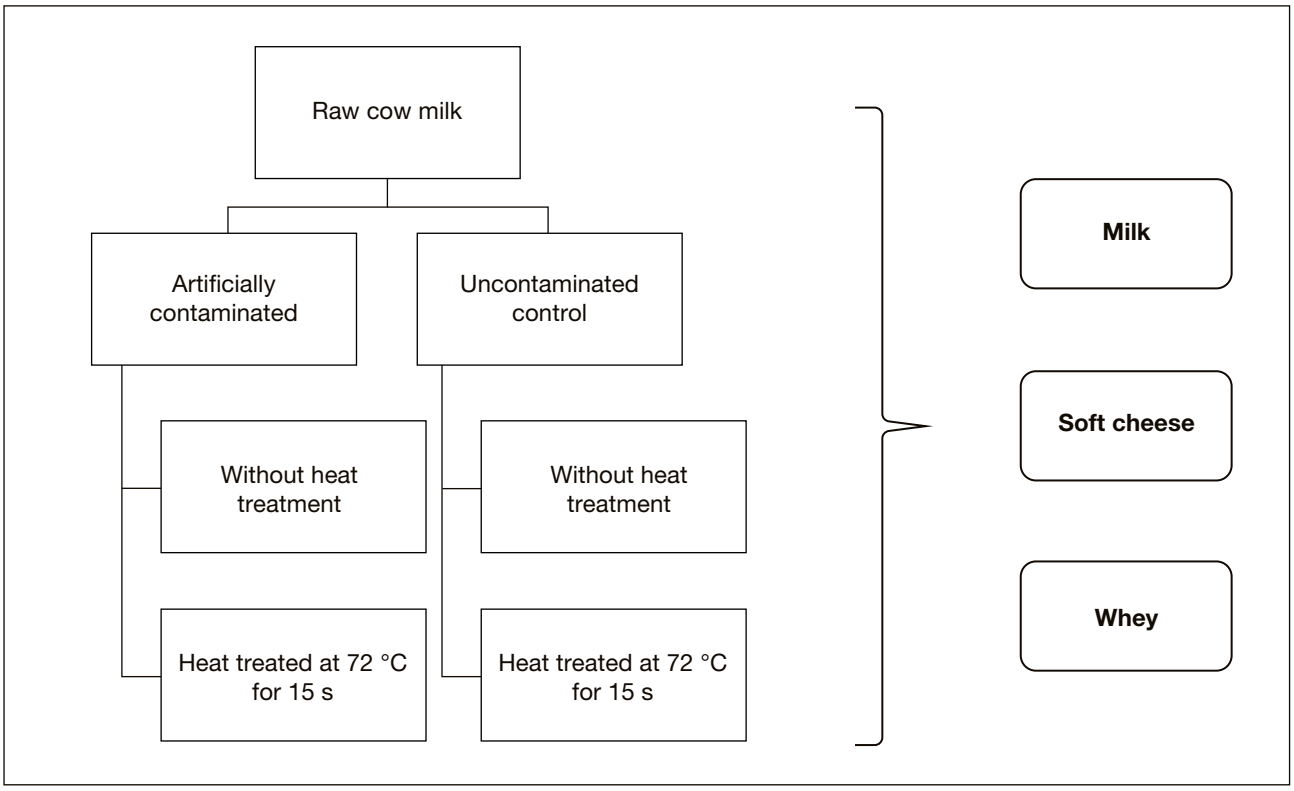


Figure 1. Design of the trials

3.3. Sample preparation and analysis

3.3.1. Milk and whey

The liquid samples (milk and whey) from each trial were kept in refrigerator (6 °C) before analysis and analysed together with the fresh cheese samples. From each individual milk and whey sample, nine sub-samples were taken. Internal standard (penicillin-V; by Fluka, purchased from Sigma-Aldrich, USA) was added to 1,000 μL of sub-samples to obtain 1,500 $ng \cdot mL^{-1}$ final concentration before sample processing. 80 μL 10 % (v/v%) acetic acid was added to 1,000 μL milk sample. It was vortexed for 30 s, and then 15 μL 1 M NaOH solution was added to it. The mixture was vortexed again for 15 s, and then it was centrifuged at 16,100 g and 15 °C for 10 min. The supernatant was filtered through 0.22 μm syringe membrane filter and then analysed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as described in section 2.4. All chemicals in the sections 2.3.1 and 2.3.2 excluding the analytical standards were from VWR International Ltd, Hungary.

3.3.2. Fresh cheese

Quantification of the cheese's antibiotic level was made via standard addition method. The calculation method for quantification is described in the section 2.4. Nine times 10 grams of fresh cheese were removed from randomly selected sites of the whole fresh cheese and measured into beakers. Three samples were treated in themselves, three of them had been spiked with medium levels of cephalexin (additional 80 μg/kg), and the last three had been spiked with high levels of the target compound (400 μg/kg). The spiking was carried out by a single-use syringe and needle, injecting small portions of the spiking solution (total volume 1.000 μl; cephalexin solved in water) into different parts of the fresh cheese pieces.

The samples were kept in refrigerator (6 °C) for a night and were processed on the next day. 20 ml extracting solvent consisting of 10 % acetonitrile, 89.5 % water and 0.5 % methanol with 5 % ammonia were added to every sample then manual homogenisation followed by stainless steel homogenising tools. Before the homogenization started 200 μL penicillin-V (50.000 ng/mL) solution was added to each sample as internal standard (ISTD). Homogenisation went until the size of fresh cheese pieces decreased below ~1 mm and the extracting solvent and fresh cheese pieces were mixed evenly.

Then the manually homogenised samples were replaced into Erlenmeyer flasks with screw caps. The content of beakers was rinsed with an additional 5 ml of extracting solvent into the Erlenmeyer flasks. The samples were shaken in a thermoset water bath shaker at 40 °C for 2 hours. Then the content of the Erlenmeyer flasks was transferred into centrifuge tubes and been centrifuged at 7,100 g and 15 °C for 10 min. After this 1,000 μL from the supernatant was subjected to the same procedure as described in the section 2.3.1 for milk and whey with except for internal standard addition.

3.4. Analytical chemical method

The cephalexin content of milk, whey and cheese extracts was analysed by a high performance liquid chromatograph coupled with a tandem quadrupole mass spectrometer (HPLC-MS/MS) system, according to a validated method described in details elsewhere **[14]**. A Shimadzu LCMS-8030 system with a Kinetex C18, 100 x 4.6 mm ID (2.6 μm particle size) column and a 4 x 2 mm C18 guard column (both from Phenomenex, Inc., USA) was used for the analysis. Lowest level of quantitation (LOQ) for cephalexin was 1.0 ng/mL in the case of milk and whey, and 2.5 μg/kg for fresh cheese.

The calibration curves of cephalexin measurements were carried out by the LabSolutions software of the Shimadzu 8030 LC-MS/MS apparatus using internal standard quantification method and $1/C^2$ weighing. The quantified data were then transferred and processed in MS Excel software. For the quantification of data for cheese, the standard addition method was used in order to minimise the mistakes that may originate from the heterogeneity of the sample. After the spiking and extraction described in the section 2.3 and analysis described above, the measured concentrations of cephalexin were plotted against the level of spiking made (i.e., zero, 80, 400 μg/kg), and the intercept of the straight gave the concentration level of the antibiotic in the given cheese.

3.5. Effect of cephalexin on the microbe count of the inoculated cheesemilk

The effect of cephalexin on cheese starter culture was investigated through measuring the microbe count (in colony forming units – CFU) of the inoculated cheesemilk with a MicroTester apparatus (Microtest Ltd, Hungary) operating on the principle that during growth in the test cell microorganisms decrease the redox potential of the medium as a consequence of their metabolic activity. Principles of operation and the methods of calculation are described in details elsewhere **[15]**. 10 mL sub-samples of inoculated cheesemilk were removed from the cheese vat just after the inoculation and homogenisation and sent to microbe count testing. Investigation was carried out both with the raw and heat treated milk trials. The total colony number of blank, raw milk was in the 10^3 CFU order, the same value for the heat treated blank milk was zero. The total colony number of the starter culture in the amount given to the milk (see section 2.1) was in the 10^8 CFU order.

3.6. Statistical analysis

Multi-way and one-way ANOVA methods were used to investigate the significance of differences ($p < 0.05$) between the measured parameters in the trials and those of the blank samples. Correlation of specific datasets and significance ($p < 0.05$) of correlation were also calculated. Statistical analysis of the results was performed by Microsoft Excel and R program (version 3.1.3.).

4. Results and discussion

4.1. Measured cephalexin concentrations

The cephalexin concentrations in milk, whey and cheese are presented in ***Table 1***. In every case the background cephalexin level of the untreated milk as well as the whey and cheese prepared from it (blank trial) were also checked. None of the blank trials showed cephalexin levels above the detection limit.

The differences between cephalexin concentrations in heat treated and untreated milk agree well with previous research in the field of heat stability of veterinary antibiotics **[14, 16]**. Based on these previous studies, cephalexin can be considered as a medium heat stable compound and this was supported also by our present results. At every concentration level the measured cephalexin concentrations of the heat treated milk differed significantly from the untreated portions' concentrations ($p = 2.92 \cdot 10^{-2}$, $5.22 \cdot 10^{-4}$ and $6.09 \cdot 10^{-7}$ for the Low, Medium and High concentration level trials, respectively). However, the extent of heat degradation is far from being adequate for completely removing cephalexin from the milk.

The cephalexin concentrations in whey and fresh cheese were in good accordance with the concentration of cephalexin in the milk itself with correlation factors of 0.9997 for whey and 0.9480 for cheese. Both correlations showed high significance ($p = 0.00001$ and 0.00399 for the correlations of milk-whey and milk-cheese concentrations, respectively). This supports that products originating from milk containing higher concentration of cephalexin will contain proportionally higher concentration of the antibiotics, too.

In every trial, whey contained significantly less cephalexin than the original milk ($p = 1.65 \cdot 10^{-7} – 2.64 \cdot 10^{-12}$), and cheese contained also significantly less antibiotics than the whey ($p = 1.49 \cdot 10^{-4} – 6.25 \cdot 10^{-12}$). This finding supports that cephalexin is retained less in the cheese curd and is transferred more into the whey. Giraldo et al. **[17]** found it differently in goat milk, concluding that cephalexin was the only β-lactam being retained in the cheese curd. Cabizza et al. **[18]** found that around 60 % of the original amount of oxytetracycline was found in the 1-day old cheese they studied. In our research, in the case of cephalexin, this ratio ranged between 1.8-4.3% by the original mass of cephalexin added to the milk, indicating a much lower retention of this compound in the cheese curd. On the other hand, whey contained 29.3-42.8 % of the original cephalexin amount. 52.9-67.7 % of the original cephalexin amount was lost during the cheesemaking process. As no significant microbial inhibitory effect was observed (see section 3.4) this loss may be attributed mainly to chemical decomposition occurring in the acidified environment. Investigation of the possible metabolites originating from this decomposition could be an important issue of future research.

The mass balance calculations of cephalexin process were carried out for the cheese making. The volumes of the original milk and the resulting whey , as well as the weight of the fresh cheese were recorded. From the measured cephalexin concentrations and the volume and weight data, amounts of cephalexin in every tested matrix were calculated in µg. Considering the cephalexin amount of the original milk as 100 %, carry-over ratios were also calculated (see ***Figure 2*** and ***Table 2***).

Table 1. Measured cephalexin levels

Trial	Heat treatment	$C_{N, ceph}$ [2]	Measured concentration in		
			milk	whey	soft cheese
		ng/mL	ng/mL	ng/mL	µg/kg
Blank control	untreated	0	< LOQ[1]	< LOQ[1]	< LOQ[1]
	72 °C		< LOQ[1]	< LOQ[1]	< LOQ[1]
Low	untreated	50	51.0±2.8	27.5±2.4	16.5±1.1
	72 °C		46.3±1.9	23.2±1.1	5.5±0.4
Medium	untreated	100	104.1±2.0	41.5±0.8	25.3±0.8
	72 °C		88.8±3.0	39.3±0.7	22.8±0.5
High	untreated	500	493.1±5.5	185.9±11.3	65.9±1.3
	72 °C		438.2±6.2	166.0±2.2	62.0±1.1

([1]LOQ: level of quantitation; [2]$C_{N, ceph}$: nominal cephalexin concentration in the milk)

Mass balance expressed as ratio of average total amounts of cephalexin in the given medium. The width of arrow heads is proportional to the average ratio of cephalexin.

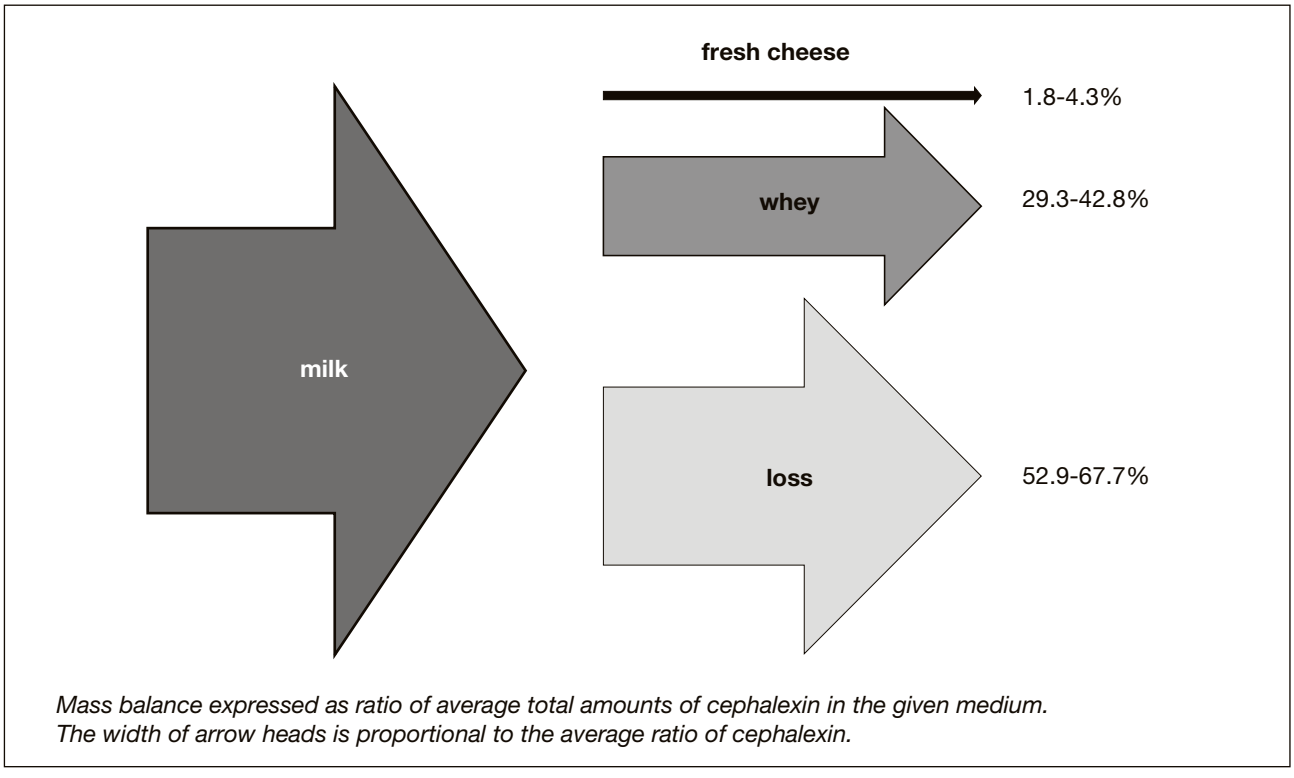
Since the legal background for maximum residue levels of veterinary antibiotics in processed foodstuff is incomplete, it is hard to compare the cephalexin levels measured to any official requirements. Acceptable daily intake (ADI) values as set by of Australian **[19]** and European **[12]** competent organisations mention 0.01 mg/kgbw/day (bw: body weight). However, the documents emphasise that since the limited toxicology data are not sufficient to establish a toxicological ADI, therefore microbiological ADI is given. The cephalexin levels measured in our trials can contribute to the acceptable daily intake amount up to 0.3 % in the case of an adult of 60 kg average body weight and 29.8±0.9 g fresh cheese consumption per day **[20]**; or up to 0.9 % in the case of a child of 20 kg average body weight and 25.5±1.1 g fresh cheese consumption per day **[20]**.

4.2. pH measurements

The optimal pH range for the fresh cheese we prepared is between 5.7-5.8. The pH of fresh cheese prepared in our trials was 5.24±0.03, 5.46±0.02 and 5.78±0.05 for the Low, Medium and High concentration level trials, respectively, in the case

Table 2. Carry-over ratios of cephalexin

Trial	Heat treatment	Volume of original milk	Volume of whey	Weight of cheese	Cephalexin concentration in the milk	Cephalexin concentration in the whey	Cephalexin concentration in the cheese	∑ cephalexin in the milk	∑ cephalexin in the whey	∑ cephalexin in the cheese	∑ cephalexin in the process milk	∑ cephalexin in the process whey	∑ cephalexin in the process cheese	∑ cephalexin in the process loss
		mL	mL	g	ng/mL	ng/mL	mg/kg	µg			%			
Low	untreated	5000 ± 2	3967 ± 53	670 ± 6	51.0 ±2.8	27.5 ±2.4	16.5 ±1.1	255.1 ± 5.5	109.1 ± 3.2	11.1 ± 0.6	100.0	42.8 ± 1.7	4.3 ± 0.4	52.9 ± 2.1
Medium	untreated	5000 ± 2	3679 ± 71	700 ± 31	104.1 ±2.0	41.5 ±0.8	25.3 ±0.8	520.3 ± 4.0	152.5 ± 2.8	17.7 ± 0.9	100.0	29.3 ± 1.1	3.4 ± 0.2	67.3 ± 3.2
High	untreated	5000 ± 2	4022 ± 53	718 ± 20	493.1 ±5.5	185.9 ±11.3	69.0 ±1.3	2465.4 ± 11.0	747.7 ± 6.1	49.6 ± 1.1	100.0	30.3 ± 1.4	2.0 ± 0.1	67.7 ± 2.8
Low	72 °C	4791 ± 6	3644 ± 27	738 ± 16	46.3 ±1.9	23.2 ±1.1	5.6 ±0.4	221.6 ± 8.3	84.6 ± 1.9	4.1 ± 0.1	100.0	38.2 ± 1.0	1.8 ± 0.1	60.0 ± 2.5
Medium	72 °C	4796 ± 9	3627 ± 24	690 ± 13	88.8 ±3.0	39.3 ±0.7	22.8 ±0.5	425.9 ± 15.2	142.4 ± 3.0	15.7 ± 1.7	100.0	33.4 ± 1.9	3.7 ± 0.1	62.9 ± 1.9
High	72 °C	4808 ± 5	3953 ± 18	624 ± 34	438.2 ±6.2	166.0 ±2.2	62.0 ±1.1	2107.0 ± 22.4	656.3 ± 4.3	38.7 ± 2.1	100.0	31.1 ± 1.4	1.8 ± 0.2	67.0 ± 2.1



Mass balance expressed as ratio of average total amounts of cephalexin in the given medium. The width of arrow heads is proportional to the average ratio of cephalexin.

Figure 2. General mass balance pattern of cephalexin in the cheese making process

of raw milk; 5.81±0.12, 5.71±0.05 and 5.91±0.02 for the Low, Medium and High concentration level trials, respectively, in the case of heat treated milk. The pH of the original milk was 5.99±0.13 and 6.15±0.06 for the raw and heat treated milk, respectively. As it can be seen, the optimal pH range was reached in some trials, but in specific trials the pH of product fell outside of this range (see the details in ***Table 3***). In each trial carried out with cephalexin in the milk, the pH of fresh cheese differed significantly from the blank trial's value irrespectively to the concentration of antibiotic ($p=7.76 \cdot 10^{-5}$ and $2.42 \cdot 10^{-4}$ for the raw and heat treated milk trials, respectively). It may suggest that cephalexin residues even well below the MRL level can change the pH of resulting cheese into unfavourable directions. Too low pH of cheese may result in texture problems during the ripening process.

Contrary to the expectations, presence of the antimicrobial agent did not lead to considerable delay or reduction in pH-changes during the cheese making process that would indicate lower level of activity of the starter culture. Our results showed that the concentration levels of cephalexin tested in the trials did not influence substantially the way as pH changed during the cheese making process. On the one hand, it is generally accepted that antibiotic residues in the milk may cause significant technological problems in the dairy industry **[21]**. Our results concerning the pH of fresh cheese seem to support this opinion. In the case of more susceptible yoghurt starter strains **[22]** it was proved among laboratory conditions that the presence of cephalexin in the ewe's milk causes imbalances in pH and in the L(+)/D(–) ratio of lactic acid isomers, resulting in yogurts less assimilable for consumers **[23]**. On the other hand, it was proved that lactic acid bacteria in cheese may be more resistant to certain individual antibiotics **[24]**, and it is also discussed **[12]** that cephalexin causes disturbances in pH development during cheese making only at higher concentrations (range of mg/kg). Our results are in accordance with this finding.

4.3. Effect of cephalexin on the microbe count of the inoculated cheesemilk

Testing the possible effects of cephalexin residues in milk on the cheese starter culture according to the examinations described in the section 2.6 (see ***Figure 3***.) did not result in significant reduction in the starter culture's microbial activity. The microbe count of cheesemilk (N; expressed in CFU – colony forming unit per mL) was not affected by the level of cephalexin in the medium.

Changes of pH values during cheese making are in strong relation with the activity of the starter culture's microbes, therefore this finding about the unchanged microbe count is in accordance with the fact that the pH changes were very similar in the case of control trials and those made with cephalexin containing milk. Moreover, this result is in accordance with previous findings **[12]** and supports also earlier research's conclusions that veterinary antibiotics alone may be less effective in inhibiting dairy industrial starter cultures than added in mixture **[24]**. As it is described in the section 3.2, it is a widely accepted supposition that antibiotic residues are harmful for the dairy product technologies without considering deeper the chemical group of the drug or the microbe strains [**25, 26]**. It is an undisputable fact that certain antibiotic residues may have severe impact on the final quality of dairy products or may even render the whole production process to fail **[23, 24]**. However, it shall be noted that the chemical-toxicological characteristics of the given antibiotics may strongly influence this picture as well as the microbiological sensitivity of the affected microbe strains **[22]**.

Table 3. Changes of pH values during and after the cheese making process

Trial	Heat treatment	$C_{N, ceph}$ [1]	pH of					
			milk	cheesemilk at			whey	cheese
				start of the process	half of the process	before cutting		
		ng/mL						
Blank control	untreated	0	6.81±0.02	6.79±0.02	6.48±0.05	6.37±0.03	6.38±0.08	6.30±0.03
	72 °C		6.74±0.09	6.80±0.04	6.55±0.03	6.48±0.03	6.52±0.06	6.62±0.08
Low	untreated	50	6.84±0.11	6.67±0.02	6.58±0.04	6.45±0.05	6.53±0.05	5.24±0.03
	72 °C		6.79±0.04	6.84±0.11	6.59±0.10	6.55±0.02	6.50±0.06	5.81±0.12
Medium	untreated	100	6.56±0.04	6.69±0.02	6.35±0.07	6.34±0.06	6.34±0.03	5.46±0.02
	72 °C		6.66±0.08	6.66±0.05	6.58±0.09	6.48±0.03	6.48±0.07	5.71±0.05
High	untreated	500	6.62±0.01	6.79±0.14	6.52±0.06	6.50±0.11	6.21±0.09	5.78±0.05
	72 °C		6.77±0.02	6.49±0.07	6.20±0.08	6.21±0.08	6.52±0.08	5.91±0.02

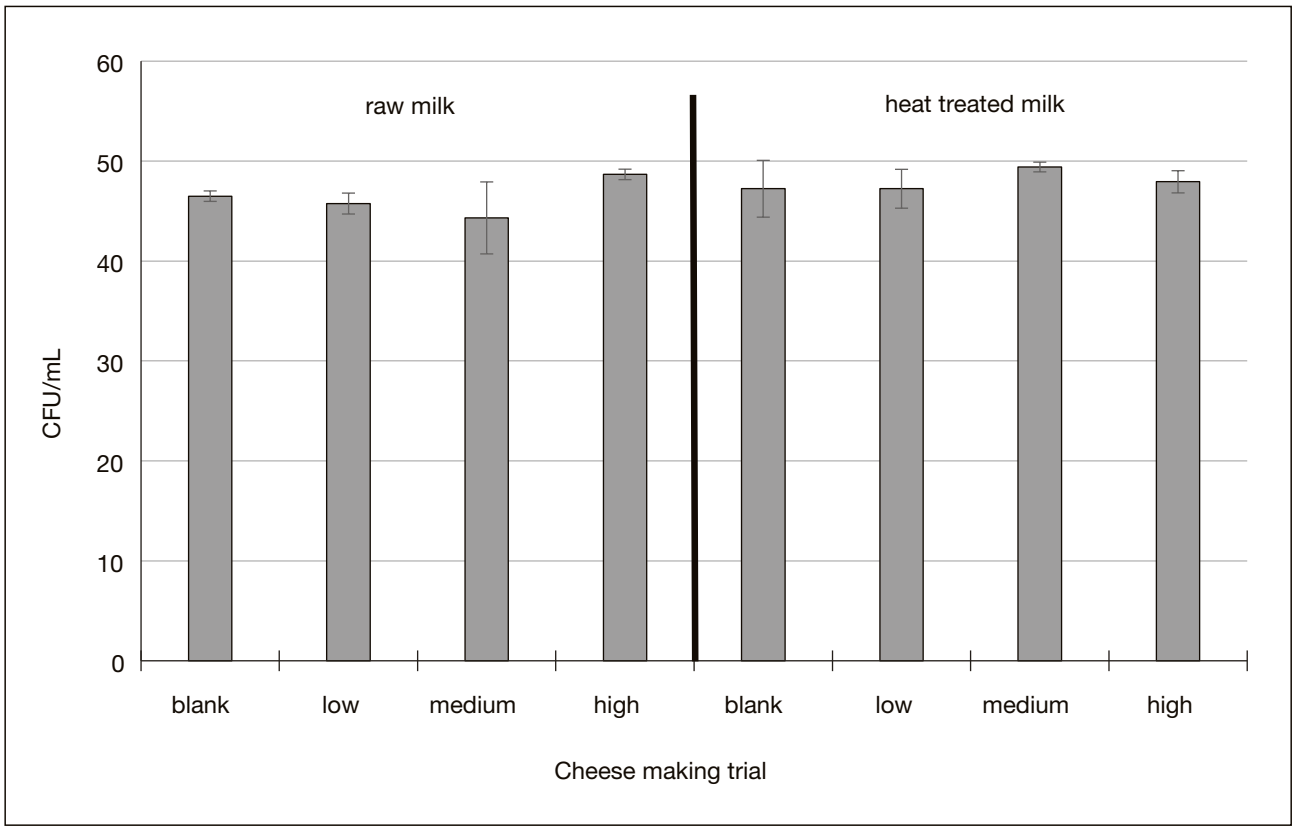
([1] $C_{N, ceph}$: nominal cephalexin concentration in the milk)

5. Conclusions

From the results outlined above it may be concluded that the effect of certain veterinary antibiotic residues in the milk cannot be described by general observations, and statements aiming at all antibiotics may be misleading. The cross-effects between the given antibiotic ingredient – or veterinary treatment – and dairy industrial culture, or process shall be investigated in themselves. Further research on the fate of veterinary antibiotics during dairy manufacturing processes is needed to clarify all the important interconnections that may occur. On the other hand, it is important to emphasise that dairy products made from milk containing antibiotic residues will also contain these residues although in some cases in lower concentrations. This fact is independent of the effect these residues may have on the texture and quality of the product. By-products and dairy industrial wastes may also be contaminated with antibiotics in this way, thus posing further threats to human health and the environment. These consequences draw our attention to the importance of keeping the withdrawal periods and examining the milk for antibiotic residues before letting it into the food chain.

6. Funding

The research was supported by the European Union and co-financed by the European Social Fund (grant agreement no. EFOP-3.6.2- 16-2017-00012, project title: „*Development of a product chain model for functional, healthy and safe foods from farm to fork based on a thematic research network*.")



Number of colony forming units per 1 mL (CFU/mL) as a function of cephalexin concentration in the milk just following the inoculation.

Figure 3. Connection between cephalexin level and cheese starter culture microbe count

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Awareness of lactose-free products and pro-, pre- and synbiotics among consumers

Picture is for illustration only

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Awareness of lactose-free products and pro-, pre- and synbiotics among consumers

Keywords: lactose intolerance, consumer awarness, lactose-free products, probiotics, prebiotics, synbiotics

1. SUMMARY

In our article, the extent of consumer awareness related to lactose intolerance and the consumption of lactose-free products is examined from the perspective of the development of milk-based lactose-free products. The survey was conducted with in-person questionnaire interviews, in which we inquired, among other things, how well consumers are aware of the symptoms of lactose intolerance, its treatment options, as well as how much they know about products that support the intestinal flora (pro-, pre- and synbiotics), which can help digestion. This provides useful information about the potential range of consumers and can also contribute to the successful planning of product marketing.

2. Introduction

2.1. Lactose intolerance and lactose-free products

Lactose, a disaccharide, is broken down in the human body by the enzyme lactase into the monosaccharides glucose and galactose during digestion, and these can be absorbed from the intestinal tract and then utilized at various locations in the body **[1]**. In lactose-free products, lactose is broken down into the two above-mentioned monosaccharides by some method (e.g., enzymatic hydrolysis) **[2]**, so in practice, such products can be utilized more easily even by people who are not lactose intolerant with one fewer degradation process and less energy consumption.

In the body of a lactose intolerant person, the enzyme lactase to be produced in the small intestine is congenitally absent or dysfunctional (primary lactose intolerance) **[3, 4]**, or its functioning is inadequate because of intestinal problems or antibiotic treatment (secondary lactose intolerance) **[5]**. Lactose, which enters the body, thus proceeds from the duodenum to the jejunum without being broken down, where it causes diarrhea or borborygmus through its osmotic effect, and then its degradation by the microflora produces gases and acids in the distal ileum and the colon, causing bloating, intestinal cramps and abdominal pain. This type of digestion may also cause constipation, nausea or vomiting. In addition, extraintestinal symptoms such as headache, memory impairment, fatigue, muscle and joint pain, allergy, arrhythmia and enuresis may occur **[6]**.

2.2. Pro-, pre and synbiotics

Probiotics are living food ingredients, strains of bacteria of human origin, that have a beneficial effect on the human body and are able to colonize the intestinal mucosa. Most of the experience have been accumulated from the use of Lactobacillus and Bifidobacterium found in yogurt and other fermented dairy products **[7]**. Prebiotics are natural nutrients, which are typically the exclusive nutrients of probiotics, they promote their proliferation and predominance **[8]**. These are oligosaccharides (e.g., fructooligosaccharides, lactulose) that inhibit the colonization by pathogens, but help the colonization by and growth of probiotic bacteria **[9]**. Synbiotics are a combination of pro- and prebiotics, such as dairy products for the preparation of which both types of substances are used **[8]**.

The complex, dynamically changing community of microorganisms living in the human intestinal system exerts its biological effect by forming a close unit with the entire body. Its formation begins at birth with the

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passage through the birth canal, then the quantitative and qualitative composition of the initial microflora changes under the regulation of the B lymphocytes of the immune system. Among other things, the intestinal flora inhibits the growth of pathogens, enhances intestinal motility, stimulates the immune system, ensures the permeability of the mucosa. Human data suggest that the probiotics used are effective in cases of antibiotic-induced diarrhea, travelers' diarrhea and pouchitis, they help to restore the microbiological balance of the intestinal tract **[9]**.

Each person's intestinal flora is quite individual, so it is difficult to determine a single, generalizable intestinal flora composition, and to define a specific healthy one. However, it is characteristic that the optimal intestinal flora shows a high degree of stable species diversity. The opinion on their significance is influenced by research findings, which make them seem increasingly important, for example because of their role in the development of the central nervous system and the regulation of behavior (anxiety, depression, social bonding, eating disorders, risk-taking behaviors) **[10].**

2.3 Consumer awareness and decision making

Consumer product choice is fundamentally determined by their subjective perceptions and preferences, so adequate market segmentation is key to the proper marketing of functional foods, for which it is essential to study the habits and expectations of the target consumer groups **[11]**. One of the biggest difficulties in communication is conveying reliable, understandable and credible information to consumers. In addition to research, development and innovation, raising consumer awareness and their continuous education play a key role in the market success of functional foods **[12, 13, 14]**.

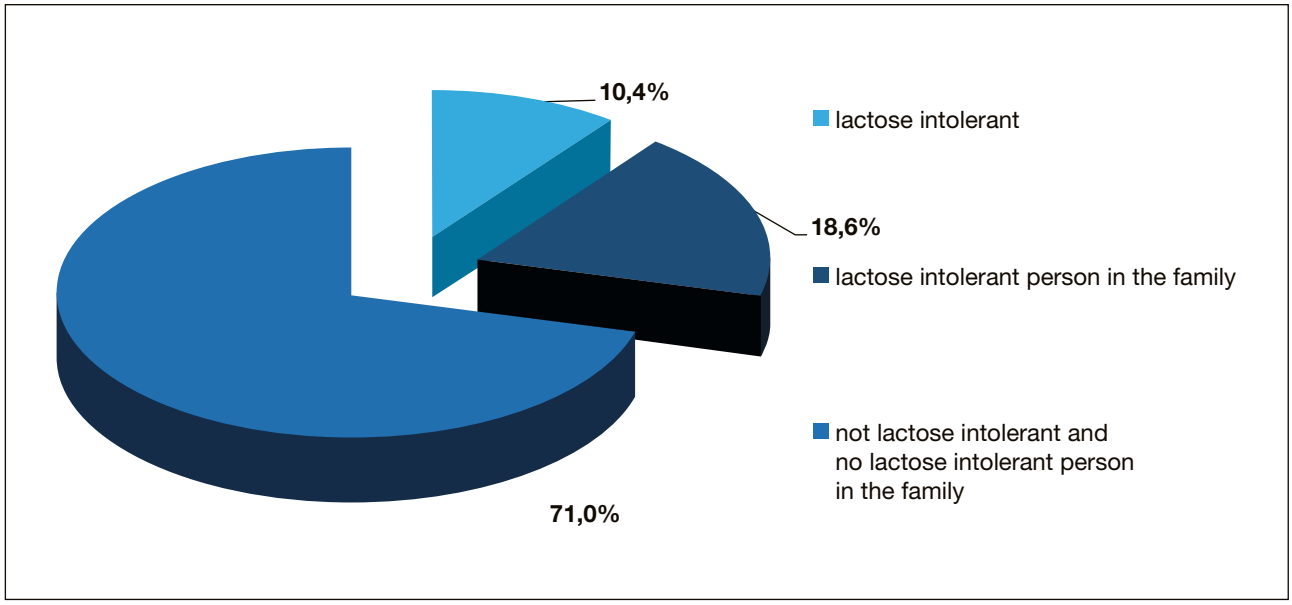

Figure 1. Involvement of the whole sample in lactose intolerance (n=952)

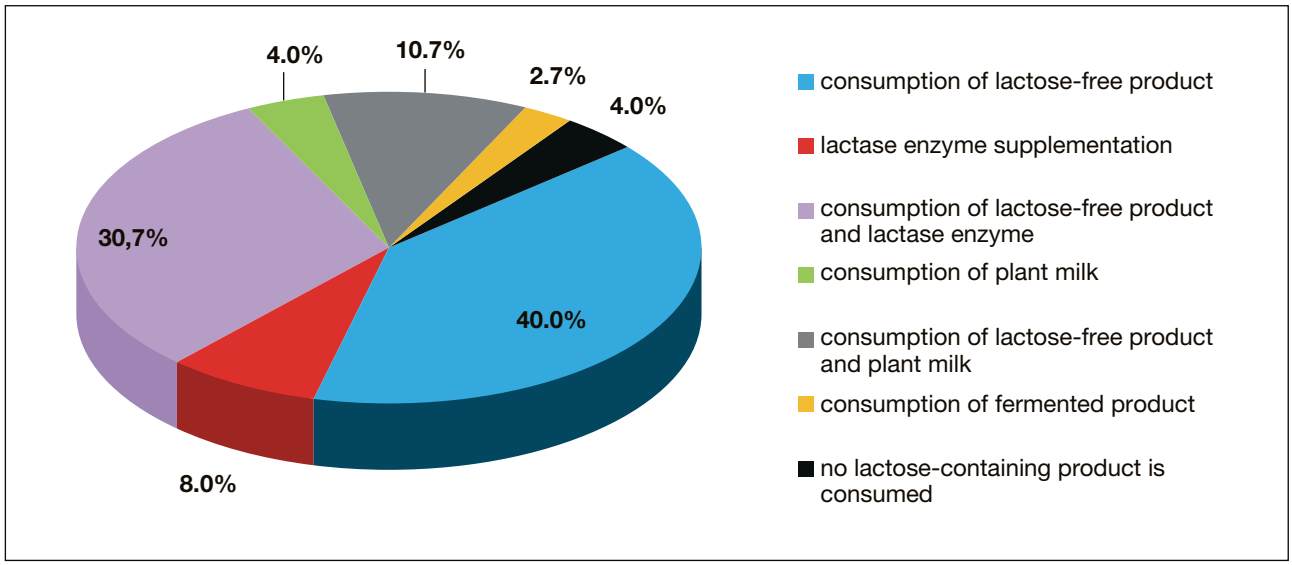

Figure 2. Distribution of responses of lactose intolerant consumers regarding symptom-free consumption of dairy products (n=75)

The aim of our study was to assess the level of consumer awareness of lactose intolerance and the consumption of products that support the intestinal flora and aid digestion (pro-, pre-, synbiotics, lactose-free products), to promote the development of milk-based lactose-free products **[15].**

3. Materials and methods

From a methodology point of view, the research consisted of in-person questionnaire interviews with a large number of people (1002). Based on the 2016 CSO microcensus, data collection was representative in terms of age, gender and place of residence by planning-strategic regions (NUTS-2). Sampling locations included major cities and smaller settlements from all over the country. Sampling took place in July and August of 2018. The opinions recorded in the questionnaires were registered and analyzed using a spreadsheet program (Microsoft Office Excel). A qualifying variable was inserted next to text answers, in accordance with the answer being correct, incorrect or close. This variable was compared to the corresponding multiple-choice question.

4. Results and discussion

4.1. Consumer awareness of lactose intolerance

In our current study, consumers' knowledge of lactose intolerance was assessed, based on their answer to the question „What do you think the symptoms of lactose intolerance are?". Consumers were examined dividing them into groups on the basis of their answer to the question „Is there a lactose intolerant person in your family?", since being involved in lactose intolerance may affect knowledge of its symptoms (**Figure 1**).

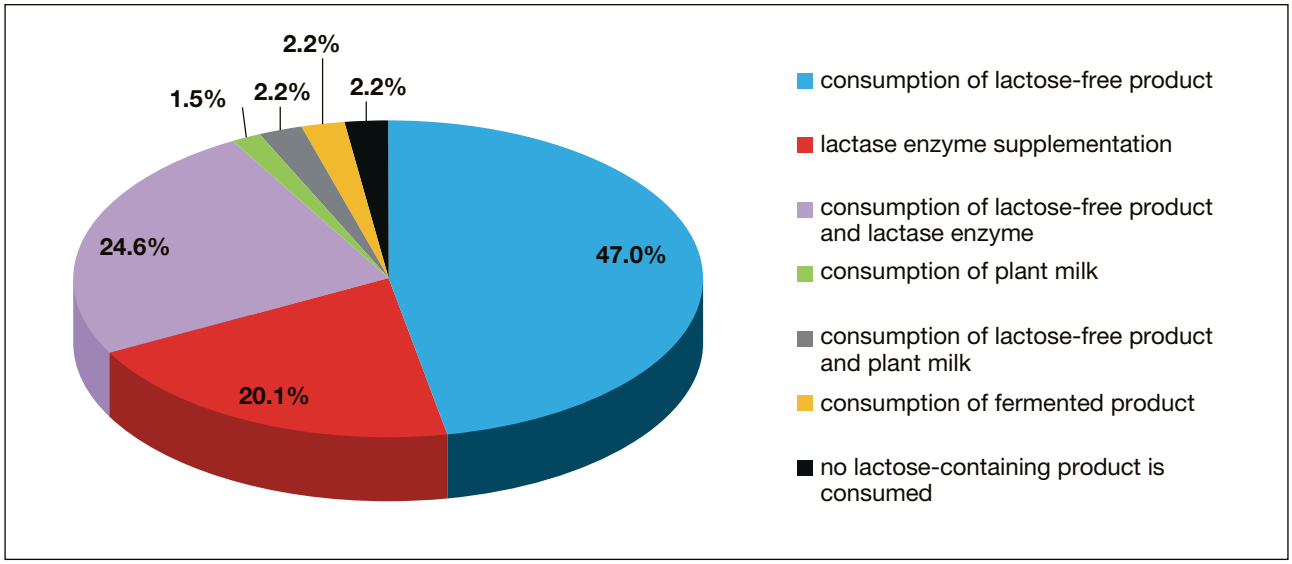

Figure 3. Distribution of responses of consumers with lactose intolerant family members regarding symptom-free consumption of dairy products (n=134)

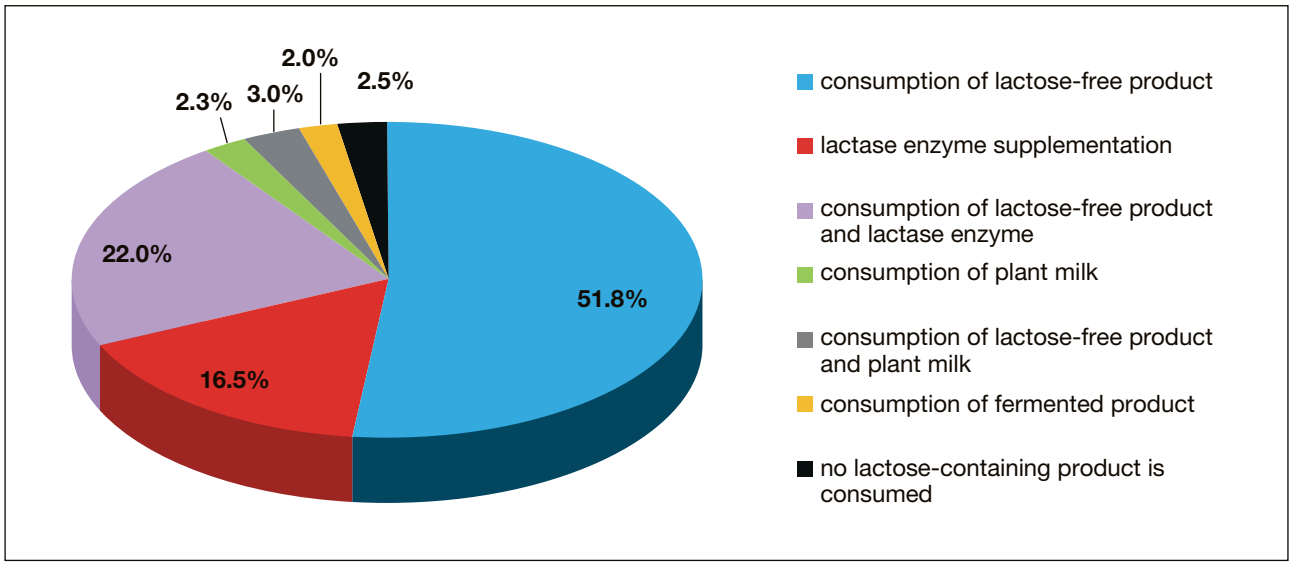

Figure 4. Distribution of responses of consumers not involved in lactose intolerance regarding symptom-free consumption of dairy products (n=400)

The responses to the question "What are the symptoms of lactose intolerance?" of the groups based on the involvement in lactose intolerance were evaluated quantitatively and, based on this, the responses within each group were divided into three further groups: correct (e.g., „indigestion", „bloating", „diarrhea"), close (e.g., „depending on the level of intolerance, immunosuppressive") and incorrect (e.g., „not processed by the body") answers. The result thus obtained (**Table 1**) shows that the consumers interviewed by us are aware of the symptoms of lactose intolerance, regardless of their involvement in it. The symptoms of lactose intolerant people, indigestion, diarrhea, bloating, cramps and the appearance of skin rashes after the consumption of milk or dairy products have been correctly described by them.

We were also curious about what customers think about the possibilities of consuming dairy products related to lactose intolerance, so their answers to the question „In your opinion, how can a lactose intolerant person consume dairy products without exhibiting symptoms?" were surveyed. Once again, consumers were compared in the above-mentioned three groups according to their involvement in lactose intolerance, and response categories were formed according to the textual responses (**Figures 2-4**). Regardless of the involvement in lactose intolerance, for the way lactose intolerant persons can consume dairy products, most people listed lactose-free products, followed by this and enzyme supplementation, and the third most frequent response was enzyme supplementation alone.

Dietitians primarily recommend the consumption of lactose-free milk and dairy products for lactose intolerant people, as well as the supplementation of the enzyme lactase and, to diversify the diet, plant drinks enriched with vitamins and minerals. Depending on the degree of individual tolerance, traditional sour milk products, semi-hard, hard and long-maturation cheeses can also be consumed **[16]**.

4.2. Consumer awareness of pro-, pre- and synbiotics

In the questionnaire, the questions were compiled in a way that allowed us to identify the superficial and real knowledge of the respondents. For the first case, 3 multiple-choice questions were used, in which consumer awareness could be provided by

Table 1. Responses regarding the symptoms of lactose intolerance according to the involvement in lactose intolerance (n=687)

Answer regarding the symptoms of lactose intolerance:	Is there a lactose intolerant person in your family?		
	respondent	family member	no
correct	**100.0%**	**98.0%**	**98.0%**
close	0.0%	2.0%	1.1%
incorrect	0.0%	0.0%	0.9%

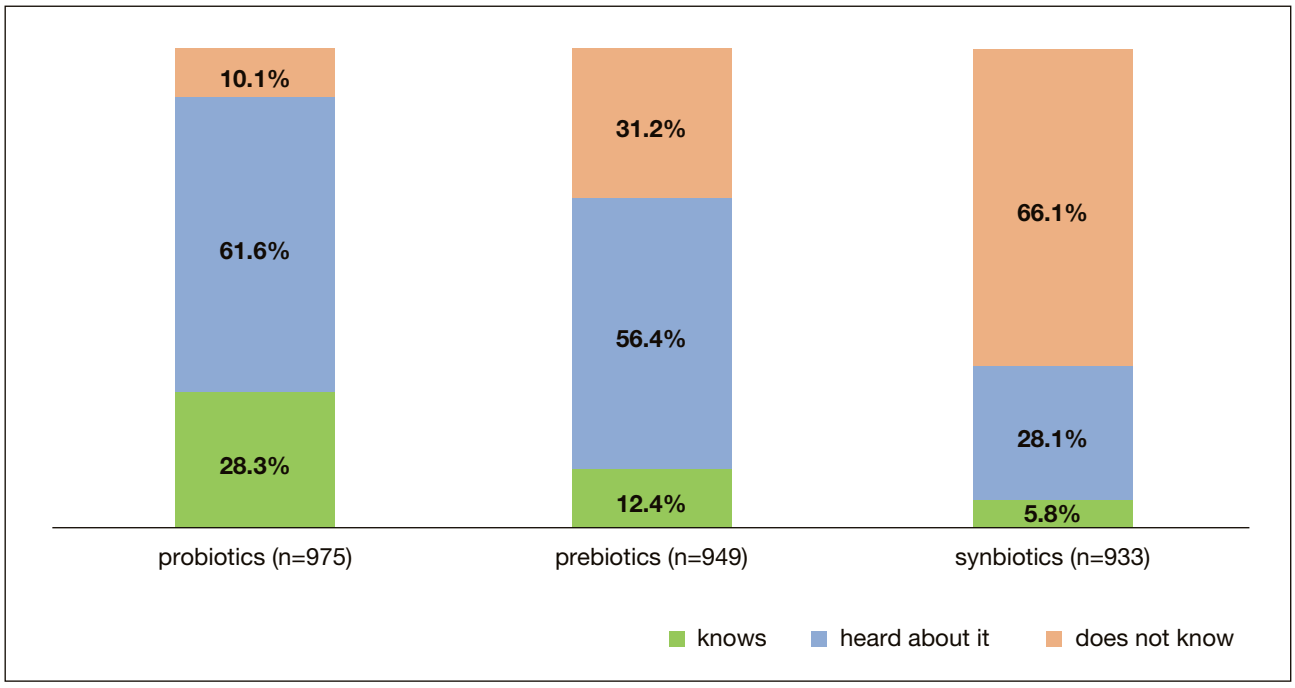

Figure 5. Consumer knowledge on the role of pro-, pre- and synbiotics in nutrition, based on a yes/no question

self-declaration (e.g., „Do you know what role pro/pre/synbiotics play in nutrition?"). Real knowledge was assessed by open ended questions (e.g., „If you know the role pro/prebiotics play in nutrition, please summarize your knowledge in a few words."). In the case of yes/no questions, responses of the whole sample were evaluated (**Figure 5**), and the responses (knows, heard about it) were compared to the text answers, which were evaluated quantitatively (correct, close, incorrect) (**Figure 6**) and the correctness of the knowledge was determined.

Of the entire sample, 28.3% said in the multiple-choice question that they know what a probiotic is, of these, 84.1% answered the following open ended question, and 85.3% of them were indeed aware of the role of probiotics in nutrition. For the entire sample (n=1,002), 19.8% knew correctly what probiotics were for. A previous study in Hungary found that 57.8% of respondents knew what the term „probiotic" meant **[17]**, and in another Hungarian study, the proportion of those who knew about the beneficial effects of digestion promoting foods (live flora, probiotic yogurts), based on self-declaration, was 76% **[18]**. Of those who were unaware of the role of probiotics in nutrition but had already heard of them, 16.1% answered the open ended question. 7.9% of the total sample (n=1,002) were uncertain but knew correctly the role of probiotics.

Of those who believed they knew the role of prebiotics (12.4%), 73.7% answered the open ended question, and 43.7% of these had the correct knowledge. For the whole sample (n=1,002), their proportion was 3.8%. Of those who did not know exactly the role of prebiotics in nutrition, but already heard of them, 7.7% answered the open ended question. They were uncertain in their knowledge, but still 29.3% of them correctly described the role of prebiotics. For the whole sample (n=1,002), their proportion was 1.2%.

In the case of multiple-choice questions, there is a clear decline in consumers' knowledge of pro-, pre- and synbiotics. Respondents are most aware of the role of probiotics, 28.3% of the total sample says they know them, while only 12.4% know prebiotics and 5.8% know synbiotics. For probiotics, 19.8%, and for prebiotics, 3.8% of the total sample have the correct knowledge. Those who have only heard of these but do not know them thoroughly represent 61.6% and 56.4% for pro- and prebiotics, respectively, while 28.1% for synbiotics. Accordingly, the proportion of those unfamiliar with the above concepts increased inversely (10.1%, 31.2% and 66.6 %).

The lack of knowledge regarding the effects of foods containing pro-, pre- and synbiotics on the intestinal flora is evidenced by the responses of the consumers interviewed regarding these products. Awareness of probiotics is the greatest, presumably due to their presence in advertisements for a longer period of time. Information about pre- and synbiotics were almost completely missing from consumers' knowledge.

In a consumer study of the role of functional foods (such as pro-, pre- and synbiotics, as well as lactose-free products) in disease prevention, consumers have identified functional foods as a way to treat gastrointestinal problems after a lifestyle change **[19]**, thus, these types of products are considered to be useful despite the fact that they are not fully aware of their exact mechanisms of action **[20]**.

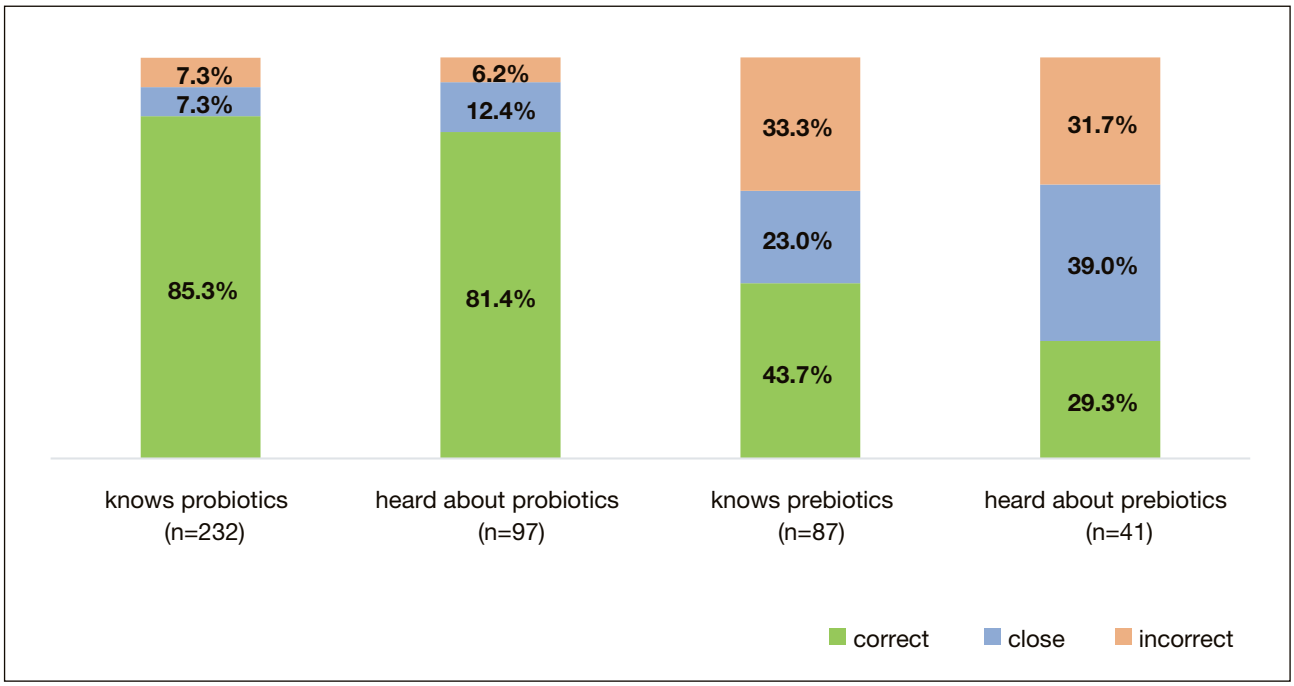

Figure 6. Consumer knowledge on the role of pro- and prebiotics in nutrition, based on an open ended question

According to the survey of Jasák **[21]** in Hungary, consumer knowledge of the benefits of the products has the strongest impact on the consumption of functional foods and they trust this category, however, the study of Szűcs et al. **[22]** showed that Hungarian educational programs on health care and conscious disease prevention are incomplete and, accordingly, children's knowledge of foods shows a mixed picture **[23, 24]**. According to domestic surveys of food consumer behavior, communication on food safety should be multifaceted and should be targeted at different consumer groups **[12, 25, 26, 27]**). The surveys of Kiss A. et al. **[28, 29]** have shown the extent to which incorrect product information influences the perception and consumption of dietary supplements of adolescents engaged in sports activities in their spare time, while the sources of this information are often the professionals who deal with them. An assessment of the factors influencing the purchase of a dairy product imitation resulted in the fact that the willingness to purchase is significantly influenced by the perceived value for money of the product, the culinary ability, knowledge, awareness of the respondent, and the general preference of the original product, as well as the often misleading packaging **[30]**.

In the case of Hungarian products, it may be particularly important for consumers to be properly informed about dairy products that support the intestinal flora, as a domestic survey showed that consumers prefer milk and dairy products of domestic origin **[31]**, thus, in certain health conditions, their beneficial effects may provide an incentive to choose them.

The consumer group studied by us is basically well informed about lactose intolerance, regardless of personal involvement, so consumers are well aware of this condition and its symptoms. More than 60% of the respondents consider lactose-free products and the supplementation of lactase enzyme to be the appropriate way for lactose intolerant people to consume dairy products. Consumers are therefore aware of the existence of milk-based lactose-free products and link it to lactose intolerance. Lactose-free milk and dairy products, on the other hand, can be useful not only for organisms unable to break down lactose, but also for people that possess the lactase enzyme, since they facilitate digestion by removing a breakdown step, which can be especially beneficial for organisms with other intestinal problems. Identification and exploitation of such value-added factors can be a key issue for the domestic dairy industry **[32]**. Since such a property of a product is not highlighted in marketing communication, the goal of lactose-free products is only associated with lactose intolerance by consumers, which may affect product consumption. When planning marketing, it could be worthwhile to draw consumers' attention to this feature of the product.

5. Conclusions

Due to the importance of the role of the intestinal flora in the human body and the effect of the diet on its composition, it would be important to increase consumer knowledge regarding foods that support the balance of the microflora. The effects of products that support the intestinal flora and aid digestion are directly related to the functioning of the intestinal system and, thus, to food digestion and the maintenance of human health, their knowledge can greatly influence product choice, so it would be important to fill this gap, for example, with educational materials, advertisements and other information opportunities.

6. Acknowledgment

"The project was supported by the European Union, co-financed by the European Social Fund (ESF, grant agreement no.: EFOP-3.6.2-16-2017-00012, Development of a functional, healthy and safe food product path model based on the farm-to-fork principle in a thematic research network)."

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Examination of the redox status of calves during the milk feeding period in a Hungarian large-scale dairy farm

Picture is for illustration only

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Examination of the redox status of calves during the milk feeding period in a Hungarian large-scale dairy farm

Keywords: Reactive Oxygen Species (ROS), free radicals, antioxidants (AO), plasma AO capacity (PAT), oxidative stress (OS), oxidative stress index (OSI)

1. SUMMARY

During the milk feeding period (from birth to weaning, generally for 60-70 days), calves receive feed milk or milk replacer rich in both protein and energy. Young animals show intense physical development and growth. Intensive oxidative metabolic processes, inadequate antioxidant defense system, oxidative stress can develop, which adversely affects the health and productivity of calves due to its cell-damaging effects. This justifies continuous monitoring of the redox status of the animals during the calf rearing period for early detection of oxidative stress. This may provide a basis for targeted antioxidant treatments to reduce calf disease-related losses.

2. Introduction

High Reactive Oxygen Species (ROS) are constantly formed in aerobic organisms, especially in the intracellular mitochondria. These radicals are required in controlled quantities for proper functioning of the body, for example in phagocytosis, apoptosis, but also in the maturation process of oocytes **[3, 12, 23, 29]**. However, if they are present in excessive amounts, they can damage the most important structures of living cells, such as lipids, proteins, or nucleic acids **[20, 21, 29]**. The body's antioxidant (AO) protection systems ensure control on acceptable safety concentration of ROS. Primary AO protection is provided by various enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx), while secondary AOs are usually microelements (such as Copper, Iron, Zinc, Selenium), vitamins and provitamins (e.g. Vitamin E, Vitamin C, carotenoids) and other substances with AO function (e.g. albumin, flavonoids, uric acid, bilirubin, etc.) necessary for the proper operation of primary AOs **[11, 23, 29]**. When the amount of ROS in the body exceeds the ROS-elimination capacity of AO systems, so-called oxidative stress (OS) develops **[27]**.

In the calves, with the onset of own respiratory at birth, OS may develop **[14, 22, 25]**. In this case, the AO up-taken from colostrum plays an important role in the protection against OS **[1, 15]**. After birth, the amount of AO usually decreases and only increases over time, with the complete functionality of young animals' own AO defense system **[8, 30]**.

Three factors can cause OS to develop. At first, when the body suffers from a lack of energy and tries to compensate by mobilizing the body reserves. In association with lipolysis, the amount of free fatty acids in the blood increases, which can serve as a substrate for lipid peroxidation. At the same time, energy production in mitochondria may also be intensified, which may lead to an increase in the amount of ROS in the blood. Together, these two factors predispose to OS development. Third predisposing factor is depleted AO system with reduced functionality **[2, 16, 29]**. Hence, OS can develop when the body is exposed to metabolic stress for example in early lactation **[6, 24]**. Furthermore, in weaning period, the feeding of animals changes significantly, and this change also can cause metabolic stress, when the animal is characterized by a negative energy balance **[7, 30]**.

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Intense growth in young animals, due to the body's high protein demand and in parallel intensive energy production in cells, OS may develop with high probability. This is indicated by the fact that advanced protein oxidation products (AOPP) are often shown young animals at higher concentrations while the rate of AOPP/albumin (ALB) is decreasing **[8]**.

During the milking period (from birth to weaning, usually from 60 to 70 days), calves receive feed milk or milk replacers rich in both, proteins and energy. Young animals show an intensive physical development and growth. Due to the intensive oxidative metabolic processes, an improper AO defense system, OS may develop, which, due to its cell-damaging effect, adversely affects the health and productivity of calves. This justify the continuous monitoring of the redox status of animals during the calf rearing period for the early detection of OS. This can provide a basis for targeted AO treatments to reduce losses associated with calf diseases **[2, 9, 10, 13, 18, 19, 28]**.

3. Material and method

The aim of the study was to determine through monitoring of redox status, that are the ROS and AOs in equilibrium during the milk feeding period, or else OS may be considered under the ordinary feeding and housing conditions. The link between some biochemical (albumine (ALB), total protein (TProt), blood urea nintrogen (BUN), glucose (GLU), beta-hydroxybutirate (BHB), aspartate-transaminase (AST)) and OS parameters were examined too.

The tests were performed on a large-scale dairy cattle farm in Hungary. The study included 26 clinically healthy, female Holstein-Friesian calves delivered through a normal calving. The animals were individually housed in a straw bedded 147 x 109 x 117 cm Calf-Tel® Compact plastic calf houses (Calf-Tel, Germantown, Wisconsin, U.S.), which included a 109 x 320 cm fenced unroofed pen. Calves receive ad libitum milk replacer formula with 22% raw protein (Rosalac Red,Schils B. V., GM Sittard, Netherlands) following the intake of colostrum .

Blood samples were collected from the calves 4 times at a similar time after morning feeding session from the jugular vein, in week 1, 2, 3 and 6. The samples were refrigerated and transported to the lab, where the Free Radical Analytical Sytem 4 Evolvo (FRFAS4, H&D s.r.l., Parma, Italy) was used to measure the amount of reactive oxygen metabolites (dROM) and plasma AO capacity (PAT), from which the oxidative stress index (OSI) was calculated using the formula dROM/PAT x 100. From the samples, we measured some parameters of protein metabolism (albumine, (ALB), total protein (TProt) and urea (BUN)), some parameters of energy metabolism (glucose (GLU), beta-hydroxybutyrate (BHB)) and the parameter of liver health (Aspartate Amino Transferase (AST) enzyme activity). We determine the statistical distribution of redox status indicators (dROM, PAT and OSI) and tested the relationship with the metabolic parameters described above. The data was stored in Microsoft® Excel (Microsoft Corporation, Redmond, USA). For analysis version 3.1. R statistical program was used (R Core Team, 2018).

Table 1: means of dROM, PAT and OSI in each sampling

Parameter	**Sampling 1 3 - 8 days (n = 22)**	**Sampling 2 14 – 16 days (n = 26)**	**Sampling 3 21 – 25 days (n = 26)**	**Sampling 4 42 – 74 days (n = 26)**
dROM[U.Carr] mean (sd)	113 (21)	117 (17)	114 (26)	120 (42)
PAT [U.Cor] mean (sd)	2689 (316)	2439 (333)	2264 (165)	2822 (222)
OSI mean (sd)	4.24 (0.93)	4.85 (0.82)	5.03 (1.08)	4.29 (1.53)

Table 2: The difference in menas of dROM, PAT and OSI per sampling (ANOVA Tukey)

Samplings	**dROM p-value**	**PAT p-value**	**OSI p-value**
1st – 2nd	0.952	0.00871	0.2507
1st – 3rd	0.998	<0.001	0.0801
1st – 4th	0.782	0.31327	0.9982
2nd – 4th	0.973	<0.001	0.2945
2nd – 3rd	0.983	0.08872	0.9375
3rd – 4th	0.853	<0.001	0.0939

4. Results

The averages and standard deviation values of the OS monitoring parameters are presented in ***Table 1***. The mean dROM and OSI per sampling did not show a statistical difference ($p>0.05$). For PAT, there was a significant difference between the mean values per sampling ($p<0.05$), except for the first and fourth and second and third samples. The results are presented in ***Table 2.*** PAT values showed a downward trend during the first three samples and rose again only at the fourth sampling and exceeded the value of the first week.

The correlation test among observed redox- and metabolic parameters also was performed, the correlation matrix is shown in ***Table 3***.

5. Discussion

The mean of dROM values was the lowest at the first sampling and the highest at the fourth (113 sd 21 and 120 sd 42). Previously, it has been observed that the concentration of hydroperoxides was lower in the first 3 to 7 days of life than at birth, but rose again at 2nd to 3rd week of age [14]. In our study, the mean of dROM values (U.Carr 113 sd 21 and 114 sd 26) was similar in the first and third sampling, a slight increase was detected in the second (U.Car 117 sd 17) and at the fourth was the peak (U.Carr 120 sd 2), however the difference was not significant. Others also found dROM levels to be relatively stable during this period **[25]**. The dROM test can measure the amount of organic hydroperoxides **[4]**. Conjugate organic hydroperoxides are the primary products of peroxidation of polyunsaturated fatty acids **[17]**. Inconsistent results suggest that, maybe not the lipids are the primary substrates for peroxidation in this period, rather the proteins are that, and therefore, it is likely that the measurement of biomarkers of protein peroxidation products (e.g. AOPP) would be more appropriate for the detection of OS at this age.

The PAT averages showed a downward trend in the first three samples, then we recorded a significant increase in the fourth sampling, although the average values of the fourth sample were not statistically different from the first (2689 sd 316 and 2822 sd 222; $p = 0.311327$), so we can say that after the decrease in the second and third weeks, the amount of AOs returned from the sixth week to the first level following colostrum uptake. The difference between the second and third samples was trending, but not significant (2439 sd 333 and 2264 sd 165; $p = 0.08872$) when comparing the averages of the other samples (1-2, 2-4 and 3-4) the difference was significant ($p<0.05$) (See in ***Table 2***). This can be explained by the calves' own AO defense system at birth is underdeveloped and develops only in a few weeks, over time.

The OSI values varied similarly to PAT compared to each sampling. The lowest (4.24 sd 0.93) was the first, the highest (5.3 sd 1.08) at the third sampling, and the fourth was lower (4.29 sd 1.53). However, in any of cases was the difference significant. The value of the OSI is determined by the dROM and PAT. Although the average dROM increased at the fourth sampling, the decrease in PAT also reversed and showed a significant increase, i.e. the amount of AO was higher, followed by OSI decreases at the fourth sampling.

Table 3: Pearson correlations of observed parameters

	AST	BHB	BUN	OSI	PAT	Albumin	dROM	Glucose	Tprot
AST	1	0.5848	-0.1294	-0.2998	0.3141	0.3793	-0.1811	- 0.2784	0.0283
P value	-	*<0.0001*	*0.1996*	*0.0024*	*0.0015*	*<0.0001*	*0.0714*	*0.0050*	*0.7801*
BHB	0.5848	1	0.1366	-0.2090	0.3142	0.2509	-0.0565	-0.3474	0.0652
P value	*<0.0001*	-	*0.1755*	*0.0369*	*0.0015*	*0.0118*	*0.5768*	*0.0004*	*0.5191*
BUN	-0.1294	0.1366	1	0.0001	0.2112	-0.0363	0.1410	-0.0190	0.1972
P value	*0.1996*	*0.1755*	-	*0.9995*	*0.0349*	*0.7200*	*0.1617*	*0.8512*	*0.0492*
OSI	-0.2998	-0.2090	0.0001	1	-0.4285	0.3140	0.8678	0.0623	0.0899
P value	*0.0024*	*0.0369*	*0.9995*	-	*<0.0001*	*0.0015*	*<0.0001*	*0.5382*	*0.3735*
PAT	0.3141	0.3142	0.2112	-0.4285	1	0.2580	0.0594	-0.1432	0.3697
P value	*0.0015*	*0.0015*	*0.0349*	*<0.0001*	-	*0.0095*	*0.5569*	*0.1552*	*0.0002*
Albumin	0.3793	0.2509	-0.0363	0.3140	0.2580	1	0.4752	-0.3249	0.4041
P value	*<0.0001*	*0.0118*	*0.7200*	*0.0015*	*0.0095*	-	*<0.0001*	*0.0010*	*<0.0001*
dROM	-0.1811	-0.0565	0.1410	0.8678	0.0594	0.4752	1	-0.0210	0.3117
P value	*0.0714*	*0.5768*	*0.1617*	*<0.0001*	*0.5569*	*<0.0001*	-	*0.8360*	*0.0016*
Glucose	-0.2784	-0.3474	-0.0190	0.0623	-0.1432	-0.3249	-0.0210	1	-0.0374
P value	*0.0050*	*0.0004*	*0.8512*	*0.5382*	*0.1552*	*0.0010*	*0.8360*	-	*0.7117*
Tprotein	0.0283	0.0652	0.1972	0.0899	0.3697	0.4041	0.3117	-0.0374	1
P value	*0.7801*	*0.5191*	*0.0492*	*0.3735*	*0.0002*	*<0.0001*	*0.0016*	*0.7117*	-

Medium or higher (r >0.4) correlation was detected only in one case, between dROM and ALB. The other metabolic parameters shown no or just low association with redox parameters in our study. The medium positive correlation (r = 0.48, p<0.0001) ***(Table 3)*** between dROM-ALB were detected may be explained by the fact that ALB is one of the most significant AO factors in the blood **[26]**. Many of its physiological and pharmacological functions are known. By its structure, it has a significant binding capacity and an important function in the transport of metals, fatty acids, cholesterol, bile pigments, hormones and pharmaceutical active substances, and in addition, it plays a key role in the regulation of osmotic conditions. AO's significant role in defense is demonstrated by the fact that more than 70% of free radical binding is related to serum ALB. Protein-bound Copper and Iron ions are less likely to participate in the Fenton reaction, forming hydroxy radicals in the presence of Cu^{2+} and Fe^{2+} H_2O_2, thus ALB contributes in the AO defense.

Based on our the results it seems, that PAT and OSI may be used for numerical expression of efficiency of AO defense systems in calves, but the quantity of lipid-hydroperoxides indicated by dROM test did not show any difference during the sampling period, as proteins are still likely to be the primary substrate for ROS. It is worth considering how biomarkers (e.g. AOPP) change in the pre-weaning period.

6. Acknowledgement

The Project is supported by the European Union and co-financed by the European Social Fund grant agreement no. EFOP-3.6.2-16-2017-00012, project title: Development of a product chain model for functional, healthy and safe foods from farm to fork based on a thematic research network.

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Investigation of the nutritional and health values of apple land varieties

Picture is for illustration only

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Investigation of the nutritional and health values of apple land varieties

Keywords: old apple varieties, *Malus* × *domestica*, fruit quality, functional food

1. SUMMARY

In our research, we sought to answer the question whether the health-protecting values attributed to land varieties in folk medicine can be substantiated by laboratory analyses. In our experiments, nutritional (sugar and acid components) and health values (total antioxidant capacity and total polyphenol content) of eight apple land varieties were examined. Half of the land varieties investigated reached the fruit quality of the control 'Golden Delicious'. Due to their very favorable glucose-to-fructose ratio, the consumption of 'Bordás alma', 'Nyári édesalma', 'Jóalma' and 'Kapitány alma' is more favorable in the diet of diabetic patients. The antioxidant capacity of 'Pirosló bőralma' and 'Jóalma', as well as the polyphenol content of 'Piros pogácsa alma', 'Kapitány alma' and 'Vasalma' are outstanding, two to three times higher than those of the control varieties. Land varieties with a favorable sugar composition and high antioxidant and polyphenol content can be used as functional foods.

2. Introduction

Tens of thousands of apple varieties are known worldwide, but the variety use of industrial fruit production is predominantly limited to 5 to 10 varieties and their mutants, and variety use varies in part from country to country. Our old fruit varieties and land varieties cannot hold their own in industrial scale production, but they can play a significant role in local markets and fresh consumption.

According to Paragraph 1 of VM decree 27/2012 (III. 24.) on the state recognition of fruit land varieties and the conditions for the production and marketing of their propagating materials a land variety is „a fruit variety naturally adapted to regional, environmental and local ecological conditions and endangered by genetic erosion" **[1]**. The local variety is a variety grown in certain areas for subsistence or sale at nearby markets. By the 1800s, a special, unique variety structure evolved in Hungary. Landscape frameworks and characteristics had a strong determining effect on the development of land varieties. The exact way they originated is obscured by time, and today they are known as characteristic varieties of certain regions **[2, 3]**.

Old or land varieties that can still be found in some places in old orchards are of significant genetic and cultural value, which are of key importance for the conservation of biodiversity. However, the condition of the old scattered orchards has greatly deteriorated since the old owners stopped cultivating them. The condition of unpruned, unmowed orchards is getting worse year by year **[4]**. *In situ* conservation of old varieties is difficult to achieve due to environmental and anthropogenic factors. *Ex situ* conservation is possible in newly established land orchards, educational gardens or plant gene banks. There are many state and private fruit gene banks in Hungary **[5]**.

In his book "*Gyümölcsöző sokféleség"* (Fruitful Diversity) **[6]**, Surányi evaluates the advantages and disadvantages of old varieties and local varieties in terms of their scientific, economic and socio-cultural impacts. Overall, he finds that the use of historical and land varieties increases biodiversity, contributes to the success of landscape and nature conservation, provides healthier food and a better livelihood for the population of underdeveloped areas.

In terms of morphological properties, a relatively large number of textbooks are available **[5, 6, 7, 8, 9, 10,**

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11, 12, 13], although these pomological works were mainly created in the 19th century. Given our common historical past, the description of certain Hungarian varieties can be found in pomological works written in some neighboring countries **[14, 15]**.

There are only a few articles and studies on old Hungarian varieties, and they mainly report the results of morphological, phenological or resistance studies carried out in gene bank collections or scattered orchards **[2, 16, 17, 18, 19, 20, 21, 22, 23]**.

Apples are a popular fruit that can be consumed all year round, an integral part of a healthy diet. Apples have a water content of 90% and a carbohydrate content of 9 to 14%, hence their energy content is low, 31 kcal (i.e., 130 kJ), so those on a weight loss program can consume them in unlimited amounts. Their carbohydrate composition is very favorable, most of it is fructose (60%) **[24, 25]**. Due to the favorable glucose-to-fructose ratio, regular consumption of apples stabilizes blood sugar levels, they can be consumed by diabetic patients as well in controlled amounts. They also have excellent dietary and health effects due to their high fiber and pectin contents (0.8% and 1.3%). Of organic acids, malic acid has the highest proportion, and citric acid, succinic acid, phosphoric acid and chlorogenic acid also occur. In terms of minerals, potassium, calcium, magnesium, phosphorus and trace elements are present in significant amounts **[25]**. Due to the high antioxidant and polyphenol content of the apple fruit, it plays a significant role in the protection against damage caused by oxidative stress, in the elimination of free radicals and in increasing the effectiveness of the antioxidant protection system **[26]**.

Many of our land varieties have unique nutritional values and special areas of use, which has allowed these varieties to survive even today. Due to their nutritional and health protection values (e.g., outstanding polyphenol content), they can also be used as functional foods **[27, 28, 29, 30, 31, 32, 33]**.

Our study included apple land varieties, mainly from Western Hungary, which may be outstanding as functional foods due to their role in folk medicine. The water-soluble dry matter content, sugar and acid components, total polyphenol content and antioxidant capacity of the fruits were investigated.

3. Materials and methods

3.1. Varieties examined

The fruit samples (except for the control 'Golden Delicious') came from the Pórszombat gene bank of Gyula Kovács, where on sampling rootstocks are traditionally cultivated. The varieties examined are not found on the National List of Varieties, most of the varieties can be considered as typical Göcsej land varieties.

The fruit quality of eight apple varieties ('Jóalma', 'Rétesalma', 'Bordás alma', 'Pirosló bőralma', 'Nyári édesalma', 'Piros pogácsa alma', 'Vasalma', 'Kapitány alma') (***Figure 1***) was compared to the fruit of the control 'Golden Delicious' and 'Gala' varieties. Two samples of 'Golden Delicious' were examined: one from the Pórszombat gene bank, from traditional cultivation, and a commercially available one, presumably from industrial production.

Figure 1. Apple varieties included in the experiment (from left to right: 'Jóalma', 'Rétesalma', 'Bordás alma', 'Pirosló bőralma', 'Nyári édesalma', 'Piros pogácsa alma', 'Vasalma', 'Kapitány alma', 'Golden Delicious' (Pórszombat))

Our study included land varieties from Western Hungary with which certain folk medicinal effects are associated. For example, 'Jóalma' were taken to mothers-to-be at birth, because it was thought that if she consumed them, the child would not be colicky. 'Piros pogácsa alma' can be consumed by diabetic patients as well. The characterization and usability of the fruits can be found on the website of the "*Tündérkert"* (Fairy Garden) operated by Gyula Kovács **[34]**.

3.2. Examined fruit nutritional and health values

The measurements were performed at the Faculty of Horticulture and Rural Development of John von Neumann University and the Department of Food Science of the Faculty of Agricultural and Food Sciences of Széchenyi István University. The nutritional (water-soluble dry matter content (refraction), sugar and acid components) and health values (polyphenols, antioxidants) of the fruits of the varieties were investigated.

Measurement of water-soluble dry matter content

The fruits were harvested at consumption maturity (***Table 1***), and rapid laboratory tests (refraction) were carried out within 1 to 2 days after sample collection. 10 to 20 pieces of fruit per variety were available for testing. The water-soluble dry matter content (refraction) was determined from a homogeneous, filtered fruit juice with a hand-held refractometer, and the results were expressed in Brix%, i.e., g/100 g. With the sampling drill supplied with the device, samples were taken from several points from each fruit, so we were able to perform 4 to 6 measurements per fruit. The data obtained during the study were averaged by variety.

3.2.1. Determination of organic acid and sugar components

Fruits were frozen after harvesting. During sample preparation, the flesh of the fruit samples was minced and blended immediately before the measurement. For the measurements, 1 g of the samples was weighed into an Erlenmeyer flask, 50 ml of distilled water was added, then it was stirred on a magnetic stirrer for 60 minutes and, finally, centrifuged (30 minutes, 5,500 g). 1.5 ml was transferred into an Eppendorf tube and, after centrifugation (24,500 g, 20 minutes), 1 ml of the supernatant was filtered into a 1.8 ml vial. The samples thus prepared were injected into the HPLC instrument.

Organic acids were determined by ion exclusion liquid chromatography. The following standards were used: malic acid (95% pure), succinic acid (99% pure), citric acid (98% pure). To prepare standard stock solutions, standards were dissolved in 0.1% H_2SO_4 solution to the desired concentration (10 mg/ mL). The JASCO HPLC instrument consisted of the following units: pump (PU-980), autosampler (AS-950-10), degasser (VWR Model 2004), detector (UV-975), column thermostat (Jones Chromatography Model 7955), column (Bio-Rad Aminex HPX-87H). Measurement conditions were as follows: column temperature: 35 °C; flow rate: 0.6 ml/min; detection wavelength: 210 nm; eluent: 0.1% H_2SO_4 (isocratic).

Separation of the sugars was performed on an ion exclusion column by reverse phase liquid chromatography. The following standards were used: fructose (99% pure), glucose (99.5% pure), sucrose (99.5% pure). To prepare standard stock solutions, standards were dissolved in 100% H_2SO_4 solution to the desired concentration (10 mg/mL).

Table 1. Sample collection dates (Pórszombat, 2019)

Variety	**Harvest date**
Jóalma *(Good apples)*	July 16, 2019
Rétesalma *(Strudel apples)*	September 16, 2019
Bordás alma *(Ribbed apples)*	September 16, 2019
Pirosló bőralma *(Reddish skin apples)*	September 16, 2019
Nyári édesalma *(Summer sweet apples)*	September 16, 2019
Piros pogácsa alma *(Red cake apples)*	November 04, 2019
Vasalma *(Iron apples)*	November 04, 2019
Kapitány alma *(Captain apples)*	November 04, 2019
Golden Delicious *(Golden Delicious)*	November 04, 2019
Golden Delicious (kontroll) *(Golden Delicious (control))*	November 08, 2019*
*date of purchase, commercially available sample	

**date of purchase, commercially available sample*

The JASCO HPLC system used consisted of the following units: pump (PU-980); autosampler (AS-2055); degasser (DG-1580-53); detector (Refractive index detector, Merck RI 71); column (Supelcogel H, Sigma-Aldrich). Measurement conditions were as follows: column temperature: room temperature; flow rate: 0.5 ml/min; eluent: 100% H_2O (isocratic).

Malic acid, citric acid, succinic acid, fructose, glucose and sucrose concentrations of the apple samples were determined using the following formula:

$$C = \frac{(A-b)*V}{a*m} \qquad (1)$$

where:

- c: concentration of the given component
- A: peak area of the given component
- A_{ad}: peak area of the given additioned component
- b: intercept of the analytical measurement curve
- a: sensitivity of the measurement system (slope of the analytical measurement curve)
- V: dilution volume of the sample solution (100 mL)
- m: mass of the weighed fruit sample.

3.2.2. Determination of the total antioxidant capacity (FRAP) and the total polyphenol content

Fruits were frozen after harvesting. During sample preparation, the flesh of the fruit samples was minced and blended immediately before the measurement. 20 g of the minced sample was stirred on a magnetic stirrer for 1 hour with an extraction mixture containing methanol (70 ml), 37% hydrochloric acid (0.1 ml) and deionized water (29 ml), then it was centrifuged for 20 minutes at 10 °C and 5,500 g. The extract thus prepared was used to determine the antioxidant and polyphenol content.

Total antioxidant capacity was determined by the modified FRAP method of Benzie and Strain **[35]**. To 100 µl of the extract were added 3 ml of FRAP solution and 100 µl of high purity water. This was measured against an extract not containing fruit, at 593 nm, with a spectrophotometer.

For the determination of the total polyphenol content, to 100 µl of the extract 1.5 ml of high purity water was added, followed by the addition of the reagents. First, 2.5 ml of Folin reagent, then 2 ml of Na_2CO_3. Absorbance was measured after 90 minutes at 750 nm, against an extract not containing fruit, with a spectrophotometer.

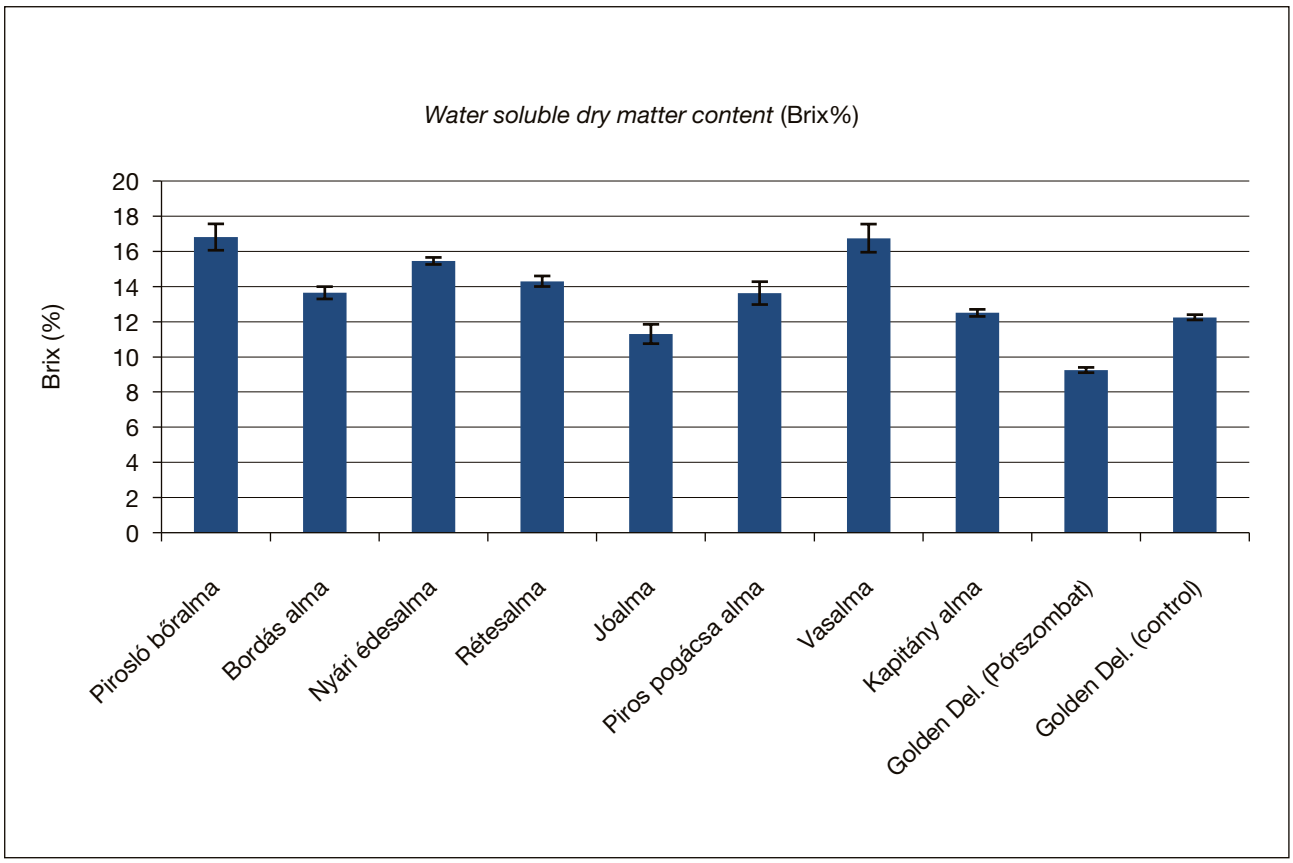

Figure 2. Water-soluble dry matter content of apple land varieties (refraction) (Pórszombat, 2019)

The same procedures were followed for the ascorbic acid solutions of 40, 60, 80, 100, 150, 200, 250 and 500 mg/l concentrations prepared for the calibration of the spectrophotometer for the determination of antioxidant capacity and the gallic acid solutions of the same concentrations prepared for the determination of polyphenol content. The total antioxidant and total polyphenol content of the apple samples were determined from the absorbance values measured for the apple sample solutions using the second order equation of the analytical curve fitted to the absorbance values measured for the calibration solutions and the corresponding concentration values using the nonlinear least squares method.

The total antioxidant and total polyphenol concentration of the apple fruits were determined using the following formula:

$$C = \frac{100*A}{a*m} \quad (2)$$

where:

- c: concentration of the given component
- A: absorbance of the given component
- V: dilution volume of the sample solution (100 mL)
- m: weight of strawberries measured
- a: slope of the analytical measurement curve.

4. Results and discussion

4.1. Water-soluble dry matter content

The water-soluble dry matter content (11-17 Brix%) of the land varieties examined reached or exceeded that of the 'Golden Delicious' control variety (***Figure 2***). The Brix% of the 'Golden Delicious' from the Pórszombat orchard was lower than that from the industrial orchard. In our previous work **[33]**, a water-soluble dry matter content of 12 Brix% was also measured for the 'Golden Delicious' variety. Varieties with a Brix% over 12 are suitable for the production of concentrate **[36]**. The land varieties examined, with the exception of 'Jóalma', are suitable for processing if their acid content is around 7 g/l. Previous Hungarian studies **[28, 29, 30, 31, 32]** also measured medium-high water-soluble dry matter contents (above 12 Brix%) in most old Hungarian apple varieties.

4.2. Sugar components

The measured sugar components (glucose, fructose, sucrose) account for 64 to 96% of the water-soluble dry matter content (Brix%). The sugar content of the varieties (glucose+fructose+sucrose) was 82-142 mg/g (average: 111 mg/g). The amounts of sugars were as follows (***Figure 3***): fructose: 50.4-72.6 mg/g (average: 63 mg/g), sucrose: 27.6-54.2 mg/g (average: 37,5 mg/g), glucose: 0.8-22.8 mg/g (average: 10.6 mg/g). When analyzing the sugar components, it is

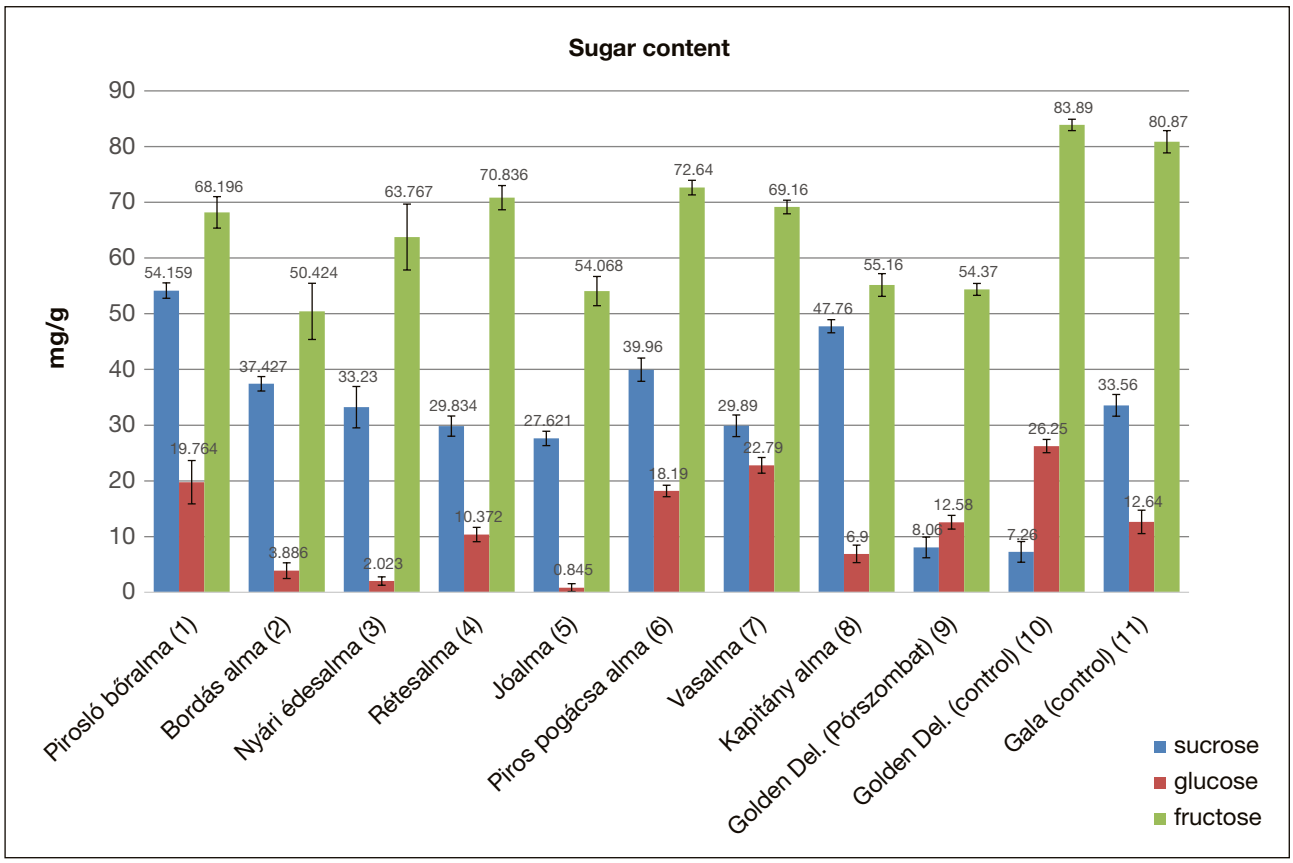

Figure 3. Sugar components of apple land varieties (Pórszombat, 2019)

clear that fructose (48-72%) is predominant in apple varieties, which is in agreement with the average value of 60% reported in textbooks (e.g., **[25]**). In land varieties, the proportion of sucrose was 27-41% and the proportion of glucose was 1-22%.

In addition to reducing the glucose intake in the diet of diabetic patients, the consumption of fructose and sucrose is more desirable. Fructose, as the sweetest sugar, imparts a sweet taste even at lower concentrations, but at the same time increases blood sugar levels more slowly due to its enzymatic conversion to glucose. Similarly, the disaccharide sucrose has a much smaller blood sugar raising effect. The glucose-to-fructose ratio of the fruits of the studied varieties was 0.02-0.33. Due to their very favorable glucose-to-fructose ratio (below 0.15), low glucose content (below 7 mg/g) and higher sucrose content, the consumption of 'Bordás alma', 'Nyári édesalma', 'Jóalma' and 'Kapitány alma' is more beneficial in the diet of diabetic patients. According to folk medicine, the 'Red cake apple' variety can also be consumed by diabetic patients. This was not clearly supported by our studies: its glucose-to-fructose ratio is medium (0.25).

4.3. Organic acid components

In the apple fruit, malic acid is predominant (80-95%), but it also contains citric acid, succinic acid, and smaller amounts of phosphoric acid and chlorogenic acid **[25]**. The total acid content (malic acid + citric acid + succinic acid) of the varieties examined was 10.2-18.7 mg/ml (average: 13.8 mg/ml). The fruits of 'Pirosló bőralma' (18.7 mg/ml) and 'Kapitány alma' (16.9 mg/ml) had exceedingly high acid contents. Despite its high acid content, 'Pirosló bőralma' has sweet and sour fruits because its sugar content is also high (142 mg/g).

The amounts of acids were as follows (***Figure 4***): malic acid: 6-12 mg/ml (average: 10.4 mg/ml), citric acid: 0-7.1 mg/ml (average: 2.7 mg/ml), succinic acid: 0.4-1.4 mg/ml (average: 0.7 mg/ml). Malic acid was dominant in the samples. The distribution of the organic acids in the varieties: malic acid (54-95%), citric acid: 0-44.3%, succinic acid: 2.1-10.3%.

4.4. Total antioxidant capacity (FRAP) and total polyphenol content

The total antioxidant capacity (FRAP) and total polyphenol content of the land varieties are shown in ***Figure 5***. The total antioxidant capacity of the varieties studied was 2.14-4.22 mg AS/g (average: 3.46 mg AS/g). The antioxidant capacities of 'Pirosló bőralma', 'Jóalma', 'Piros pogácsa alma', 'Vasalma' and 'Kapitány alma' were twice as high as that of the 'Golden Delicious' control variety. The total polyphenol content of the land varieties was 0.24-2.75 mg GAE/g (average: 1.19 mg GAE/g). The polyphenol content of 'Piros pogácsa alma' and 'Kapitány alma' was twice as high, the polyphenol content of 'Vasalma' was three times as high as that

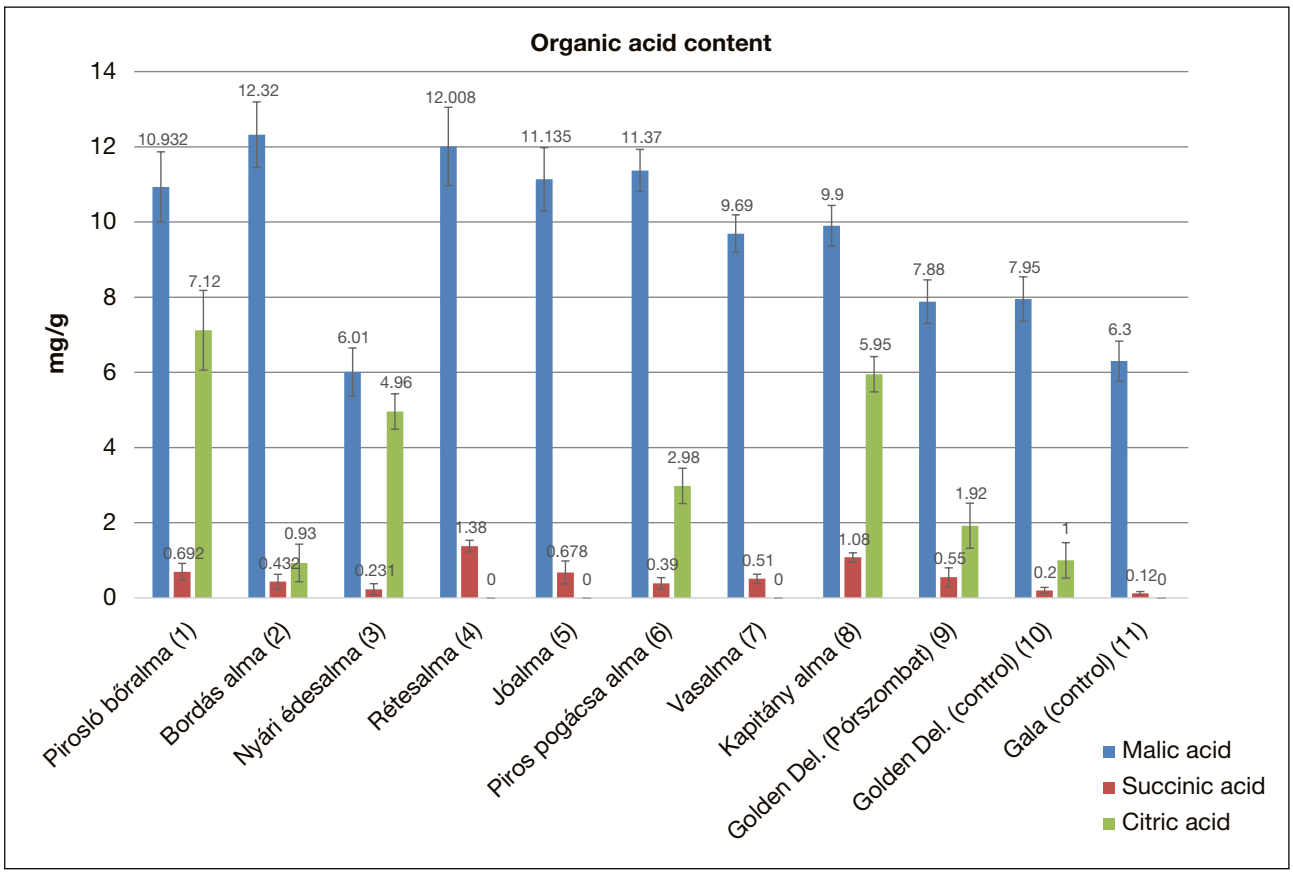

Figure 4. Acid components of apple land varieties (Pórszombat, 2019)

of the 'Golden Delicious' and 'Gala' control varieties. In contrast, the other land varieties have much lower polyphenol contents. Other researchers have found similarly high antioxidant and polyphenol contents in the case of many old Hungarian apple varieties **[28, 32, 33]**.

5. Conclusions

In our research, we sought to answer the question whether the health-protecting values attributed to land varieties in folk medicine can be substantiated by laboratory analyses. The water-soluble dry matter content (Brix%) of land varieties from scattered orchards was slightly higher than that of the control varieties from industrial fruit production. Due to their very favorable glucose-to-fructose ratios, the consumption of 'Bordás alma', 'Nyári édesalma', 'Jóalma' and 'Kapitány alma' is preferred in the diet of diabetic patients. According to folk medicine, the 'Red cake apple' variety can also be consumed by diabetic patients. In our study, its glucose-to-fructose ratio was moderate, which is still adequate, but it is advisable to give preference to the above-mentioned varieties. The average distribution of organic acids in the land varieties shows a similar result as in the control: malic acid 77%, citric acid: 18%, succinic acid: 5%. The antioxidant capacity of 'Pirosló bőralma' and 'Jóalma', as well as the polyphenol content of 'Piros pogácsa alma', 'Kapitány alma' and 'Vasalma' are outstanding, two to three times higher than those of the control varieties. 'Jóalma' were taken to mothers-to-be at birth, because it was thought that if she consumed them, the child would not be colicky. This can be explained by the favorable glucose-to-fructose ratio and perhaps the high content of malic acid and polyphenols. Land varieties with a favorable sugar composition and high antioxidant and polyphenol content can be used as functional foods.

6. Acknowledgment

We are grateful for the support of this research, which was implemented within the framework of the EFOP-3.6.2-16-2017-00012 project „Development of a functional, healthy and safe food product path model based on the farm-to-fork principle in a thematic research network". The project is implemented within the framework of the Széchenyi 2020 program, with the support of the Hungarian State and the European Union, and co-financed by the European Social Fund.

*In this paper, the authors report on the results of the examination of Hungarian apple varieties. Therefore, the Hungarian variety names are used in the text, but in **Table 1**. we also give their raw translation in English (A ed.).*

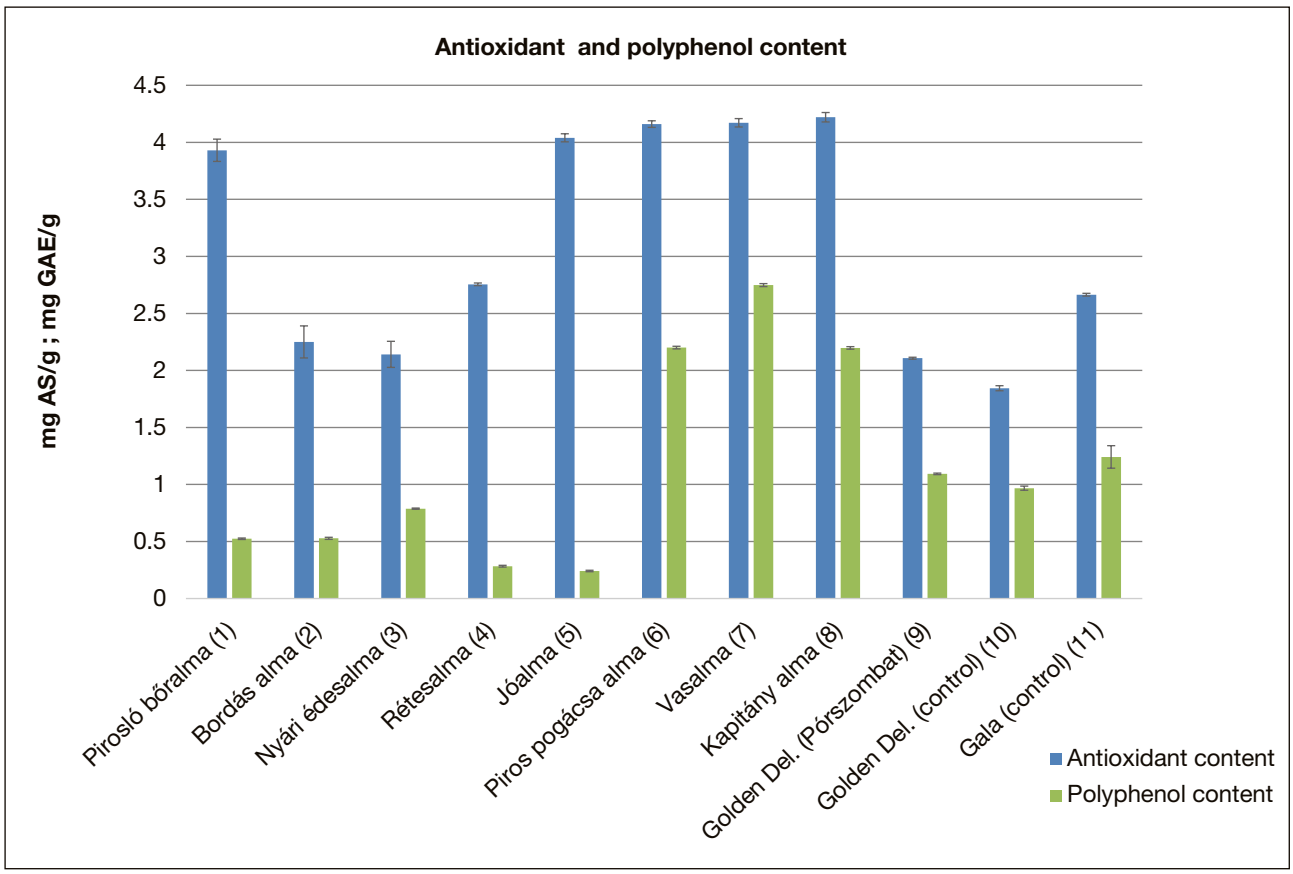

Figure 5. Total antioxidant capacity (FRAP) and total polyphenol content (Pórszombat, 2019)

7. Literature

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Preliminary results on the effects of different soil cover methods on the composition of nematode communities

Picture is for illustration only

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Preliminary results on the effects of different soil cover methods on the composition of nematode communities

Keywords: biodiversity, microfauna, healthy soil, production technology, sustainable agriculture

1. SUMMARY

In an experiment lasting for five months, changes in the soil nematode community composition were followed at an organic strawberry plantation. The aim of this study was to present the preliminary results of this investigation.
Strawberry (*Fragaria x ananassa* 'Asia') 'frigo' seedlings were planted at the end of March 2019, divided into nine plots (2.5 m × 2.2 m), after which they received three types of treatment in three replicates, in a random block arrangement: organic (hay) and inorganic mulch (black geotextile) and uncovered control.
Of the soil microfauna, nematode communities were studied. For their characterization, taxonomic and functional diversity indices (taxon richness, effective species number and functional dispersion index) were used, and the trophic composition of the communities and their changes were explored.
Soil nematode communities were affected by both the sampling period and the soil cover. The total nematode density decreased in all cases compared to the initial value (t0, pre-planting state). The effective species number was highest in the case of the geotextile cover. Both types of mulch increased the functional diversity of the communities over the five-month period, resulting in significant differences compared to the control plots. Furthermore, significant changes in the community structure were observed by trophic groups between both sampling times and treatments, which were mainly manifested in an increase in the proportion of plant parasitic and fungal-feeding nematodes and a decrease in the proportion of bacterial-feeding and omnivorous nematodes.
Although the results were not significant in all cases, they showed that soil cover helps to maintain the taxonomic and functional diversity of soil invertebrates (here, nematodes), which can contribute to the stability of the soil ecosystem and to providing an ecosystem with a positive impact on production.

2. Introduction

Maintaining soil health is essential for agricultural activities and for food safety. Of the many ecosystem services, soil-dwelling invertebrates are of primary importance for soil formation and degradation **[1, 2]**. Of these, the testing of nematodes as indicator organisms, also targeted by us, is recommended by the European Food Safety Authority (EFSA). Due to their species richness, lifestyle characteristics and diversity of feeding, there are various metrics and indices for the multifarious description of their

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communities, which allow the characterization of the processes and changes taking place in the soil food web **[3-5]**.

Soil cover is a common practice in certain agricultural production sectors to prevent moisture loss and crop contamination. In addition, the procedure may increase the organic matter content of the soil (see organic mulch), thus affecting soil biota, including soil-dwelling nematodes, and primary production as well **[6-8]**.

At the same time, the effects of different mulch types on nematode communities, especially their functional aspects, have been sparsely investigated. The aim of the present experiment was to explore the taxonomic and functional diversity of the nematode fauna in a strawberry plantation, the composition of the community, and its changes using organic (hay) and inorganic (geotextile) soil covers, compared to an untreated control in this respect.

3. Methods

3.1. Location and design of the experiment

The field experiment took place in Kecskemét, in the experimental garden of John von Neumann University (46° 55' 8.59" É, 19° 41' 8.3" K). The area falls within the temperate climatic zone, where the average annual rainfall is 530 mm and the average annual temperature is 11 °C **[9]**. It is important to note, however, that the year of the study was warmer (12.4 °C) and drier (500 mm) than the 30-year average. The soil of the area can be classified as calcareous sandy soil type ('Calcaric Arenosol') **[10]**, characterized by a slightly basic pH (8.02), and low nitrogen (8 mg/kg NO_3+NO_2) and organic matter content (0.77 g humus/100 g dry weight).

Before the study, fertilizer was applied twice (on March 18 and May 10; 350 kg/ha 'Phoenix' granulated poultry manure). 'Frigo' strawberry plants (*Fragaria x ananassa* 'Asia') with diameters between 9 and 13 mm were planted in each experimental plot on March 26 in 4-4 twin rows, with 30 × 25 cm row and plant spacing, with a 60 cm cultivation path. The crop was harvested by hand between June 3 and 19.

Soil cover was applied from the time of planting the seedlings. The area was divided into nine plots in a randomized block arrangement (2.5 m × 2.2 m), with three replicates per treatment. The treatments were as follows:

(1) organic mulch (hay), consisting of herbaceous plants, mainly grasses (Poaceae);

(2) inorganic mulch: a water-permeable black geotextile cover made of polypropylene; and

(3) uncovered control.

Geotextile was placed on the soil prior to planting, while hay was replaced roughly once a month so that a soil cover of at least 2 to 3 cm could be ensured at all times. During blooming and ripening, a hay cover was applied in the control plots to prevent contamination, and it was removed after the harvest. The area was not cultivated in the year prior to the experiment.

All cultivation activities (fertilization, irrigation, weeding) were carried out in accordance with the national and EU rules of organic farming. Irrigation was performed using a micro-nozzle system, always adjusted to the weekly rainfall. Weeding was carried out once a month by hand.

3.2. Nematode sampling and analysis

During the experiment, soil samples were taken twice for analyses of the nematode communities: before planting the strawberry seedlings (date t_0, March 20, 2019), and more than two months after the harvest (date t_1, August 28, 2019). Composite samples were mixed from ten random subsamples with diameters of 2 cm taken between the seedling rows from a depth of 0-15 cm, and these were stored in separate plastic bags at 4 °C until the nematode extraction.

Nematodes were extracted from 50 g subsamples by the standard Baermann funnel method over 48 hours **[11]**, they were counted under a stereomicroscope (×50 magnification), and the number of individuals per g was given for soil dry weight. The animals were killed by heat treatment and preserved in 4% formalin. To determine the community structure, at least 100 individuals per sample were identified at the genus or family level (except for samples with lower individual numbers where all individuals were identified) based on the identification key of Bongers **[12]**. The relative frequency of nematode taxa per 100 individuals was used to determine the population density of a given taxon.

Nematodes were classified into feeding/trophic groups (bacterial-feeders, fungal-feeders, herbivores and plant parasites, omnivores and predators), and into c-p ('colonizer-persister') 1-5 categories similar to the r-K lifestyle classification, making c-p 1 the extreme r strategist species and c-p 5 the extreme K strategist species **[3,13]**.

3.3. Statistical methods

For statistical analyses, version 3.6.2 of the R program (R Development Core Team, 2019) and its software packages 'FD', 'ggplot2', 'lsmeans','nlme' and 'vegan' were used **[14-18]**. During the statistical analyses, the assumptions of normality, homogeneity of variances, and independence of the samples were examined in each case. The significance level was determined at $p < 0.05$.

Table 1. Contributions (%) of nematode taxa to total soil nematodes averaged by trophic groups and their functional traits under different mulch treatments at two sampling times. Genera and family marked in bold were the most dominant nematode taxa during the survey.

Group and taxon	Body mass	c-p†	Control		Hay		Geotextile	
			t_0	t_1	t_0	t_1	t_0	t_1
	µg		%					
Bacterivores			60.55 aA‡	61.62 aA	62.21 aA	40.76 aB	72.20 aA	46.20 aB
Acrobeles sp.	0.6	2	32.76	26.14	38.12	16.82	46.24	8.42
Acrobeloides sp.	0.227	2	10.04	8.64	5.42	11.86	7.64	11.64
Bastiania sp.	0.161	3	0.00	0.28	0.00	0.00	0.00	0.40
Cephalobus sp.	0.257	2	4.30	12.98	5.27	5.34	5.46	13.26
Cervidellus sp.	0.152	2	6.80	4.33	3.31	1.90	6.11	4.70
Chiloplacus sp.	0.508	2	1.14	1.55	6.00	0.65	3.11	3.88
Eucephalobus sp.	0.243	2	0.00	0.38	0.00	0.63	0.56	1.61
Heterocephalobus sp.	0.356	2	0.29	4.60	1.76	0.00	0.33	0.40
Microlaimus sp.	0.146	3	0.00	0.28	0.00	0.00	0.00	0.00
Monhysteridae sp.	0.358	2	0.00	0.38	0.00	0.00	0.00	1.89
Panagrolaimidae sp.	0.655	1	1.17	0.38	0.00	1.66	0.89	0.00
Plectus sp.	0.902	2	0.00	0.69	0.87	0.00	0.31	0.00
Prismatolaimus sp.	0.413	3	2.34	0.99	0.29	1.90	1.27	0.00
Tylocephalus sp.	0.214	2	0.87	0.00	0.29	0.00	0.00	0.00
Rhabditidae sp.	5.037	1	0.84	0.00	0.88	0.00	0.28	0.00
Fungivores			6.91 aB	17.09 aA	5.03 aB	15.93 aA	5.13 aB	16.24 aA
Aphelenchoides sp.	0.145	2	1.42	0.56	0.90	5.45	0.00	0.00
Aphelenchus sp.	0.231	2	3.49	4.64	2.68	2.58	1.43	5.27
Ditylenchus sp.	0.494	2	1.42	10.93	1.17	3.07	0.28	5.30
Filenchus sp.	0.098	2	0.58	0.66	0.00	4.83	0.32	4.24
Tylencholaimellus sp.	0.564	4	0.00	0.30	0.28	0.00	3.10	1.43
Herbivores			11.3 aA	8.40 bA	8.06 aB	32.43 aA	6.93 aB	26.21 abA
Meloidogyne sp.	24.219	3	0.00	0.00	0.28	0.00	0.00	0.80
Pratylenchus sp.	0.126	3	2.52	3.85	0.61	3.40	1.64	10.71
Telotylenchidae sp.	0.46	3	8.78	4.25	7.17	29.03	4.98	14.70
Tylenchidae sp.	0.16	2	0.00	0.30	0.00	0.00	0.00	0.00
Xiphinema sp.	5.668	5	0.00	0.00	0.00	0.00	0.31	0.00
Mindenevők / Omnivores			19.03 aA	12.89 aA	23.56 aA	10.88 aB	15.74 aA	10.72 aA
Aporcelaimellus sp.	9.079	5	4.21	1.21	5.52	0.95	2.04	4.95
Carcharolaimus sp.	3.727	4	0.58	0.38	0.00	0.00	0.00	0.61
Ecumenicus sp.	0.705	4	5.49	0.97	7.22	3.33	6.92	1.26
Eudorylaimus sp.	3.09	4	3.44	8.47	4.39	6.28	2.59	2.24
Kochinema sp.	0.646	4	0.00	0.61	0.57	0.00	0.56	0.63
Microdorylaimus sp.	0.201	4	3.09	0.00	1.13	0.00	1.52	0.00
Paraxonchium sp.	5.7	5	2.22	1.25	4.73	0.32	2.11	1.03
Ragadozók / Predators			2.21 aA	0.00 aA	1.14 aA	0.00 aA	0.00 aA	0.63 aA
Discolaimus sp.	2.928	5	1.64	0.00	1.14	0.00	0.00	0.63
Mylonchulus sp.	1.745	4	0.57	0.00	0.00	0.00	0.00	0.00
Teljes taxon gazdagság / Total taxon richness			**24**	**27**	**24**	**18**	**24**	**23**

† c-p (colonizer-persister) 1-5 values: classification similar to r-K life history categories, 1=extreme r-strategists, 5=extreme K-strategists **[3]**.
‡ Lower case and upper case letters indicate differences among treatments within each sampling period and between sampling dates, respectively (Tukey-Kramer adjusted $p < 0.05$).

For each plot, the taxonomic and functional diversity of the nematode communities were estimated. The former was expressed by taxon richness and the effective species number or Hill index **[19]**, while the latter was expressed by a multivariate indicator based on multiple functional traits (body weight, c-p group, feeding strategy), the so-called functional dispersion (FDis) **[14]**. The values of the functional traits were obtained from the automatic calculation system of the NINJA (Nematode INdicator Joint Analysis) database **[20].**

Linear mixed models were used to test the effects of different mulch treatments (uncovered control, hay, geotextile), sampling times (t_0 and t_1) and their interactions on the nematode communities (diversity indices, total density, distribution of trophic groups). The lack of spatial and temporal independence between the individual samples resulting from the sampling design was taken into account by including the variables 'Plot' and 'Sampling time' as random factors. For multiple comparisons, the least square means method was used with Tukey's adjusted p values **[16]**.

To understand the effects of treatments and the sampling period on the taxonomic composition of the communities, nested permutational multivariate analysis of variance (PERMANOVA, Bray-Curtis index, number of permutations: 999) was performed, the results of which were plotted using non-metric multidimensional scaling (NMDS).

4. Results and discussion

4.1. Diversity and abundance

The study confirmed the presence of 34 nematode taxa (29 genera, 5 family-level categories), representing a total of 22 taxonomic families (***Table 1***). Taxon richness varied between 7 and 21 genera per plot. After the harvest, the highest taxon number (27) was found in the control, while the lowest (18) was shown by the plot treated with organic mulch (***Table 1***). After the five months, taxon richness was significantly reduced in the case of hay cover (***Table 2***).

According to the effective species number which, in addition to species richness, also takes into account the abundance of the different species, the soils covered with geotextile exhibited the most diverse nematode community (***Table 2***). This type of mulch had a positive effect on taxonomic α diversity by the end of August, showing a significant difference compared to the plots treated with hay.

Functional diversity (FDis) showed significantly higher values for both soil cover treatments compared to the initial state, as well as to the control (***Table 2***).

Regarding the total density of nematodes, as opposed to the soil cover treatments, the sampling period proved to be the determining factor, as the numbers of individuals decreased from 5.58-14.58 for the plots in the spring to 1.56-4.88 per g of dry soil by the end of the summer (***Table 2***).

The results supported our first hypothesis that the presence of cover is a dominant factor for the soil nematode community. In other words, the nematode taxon has been shown to be suitable for indicating changes in soil biota caused by agricultural practices **[21, 22]**. The diversity values of nematodes were in agreement with the results of a previous study, also involving strawberry plantations **[23]**.

4.2. Composition of the nematode communities

4.2.1. Trophic classification of nematodes and its taxonomic aspects

The most dominant taxa were the following: *Acrobeles* (29.9%), Telotylenchidae (9.79%), *Acrobeloides* (9.45%). Although the majority of nematodes were present in the case of each treatment and period (***Table 1***), the taxonomic composition of the communities was significantly affected by these factors (PERMANOVA: R^2=0.13, p=0.016 and R^2=0.28, p=0.004; ***Figure 1***). Representatives of the genera *Microdorylaimus*, *Mylonchulus*, *Tylocephalus*, *Xiphinema* and the family Rhabditidae were present only in spring, before the planting of the seedlings (t0), while *Bastiana*, *Microlaimus*, Monhysteridae

Table 2. The results of linear mixed models testing the effects of mulch treatments, sampling periods (t_0 and t_1), and their interaction on soil nematode communities (diversity indices and total density).

	Kontroll / Control		Széna / Hay		Agroszövet / Geotextile	
	t_0	t_1	t_0	t_1	t_0	t_1
Taxon richness	17.67 aA†	18.00 aA	17.67 aA	12.00 aB	16.67 aA	15.67 aA
Hill index	9.94 aA	9.62 abA	9.13 aA	7.68 bA	7.39 aB	11.94 aA
FDis‡	0.28 aA	0.23 bA	0.27 aB	0.35 aA	0.22 aB	0.35 aA
Total density (g^{-1} dry soil)	7.69 bA	3.25 aB	7.30 bA	2.37 aB	11.33 aA	1.71 aB

† Lower case and upper case letters indicate differences among treatments within each sampling period and between sampling dates, respectively (Tukey-Kramer adjusted $p < 0.05$).
‡ FDis: Functional dispersion

and Tylenchidae could only be detected in the post-harvest period (t1). While *Microlaimus*, *Mylonchulus* and Tylenchidae were present only in the control plots, the genus *Xiphinema* was present only in the case of the geotextile cover.

The trophic group of bacterial-feeding nematodes was the most diverse with 15 taxa, followed in number by omnivores (7), herbivores (5) and fungal-feeders (5). The predator group was represented by only 2 genera (***Table 1***).

Although bacterial-feeders dominated in almost all soil samples (25% - 80.4%), their proportion decreased significantly as a result of the treatments (***Table 1***). This negative trend was mainly due to a significant decline in the populations of the genus *Acrobeles* populous bacterial-feeding taxon (50.3%) (***Table 1***). An opposite change was observed for the genus *Acrobeloides*. The genus *Cephalobus* was present in higher proportions after the harvest period, with the exception of the hay cover. While *Heterocephalobus* appeared to prefer the control plots, the populations of the genus *Chiloplacus* were negatively affected by the hay cover (***Table 1***).

The proportion of fungal-feeding nematodes in the communities increased significantly over time (between 2.5% and 27.7%) (***Table 1***). The dominant genera were *Ditylenchus* and *Aphelenchus*, with 35.3% and 29.4% of the group, respectively. The former became more abundant in each summer end sample, regardless of the treatment. The percentage of the genus *Filenchus* increased within the communities for both mulch types (***Table 1***). Similar situations were observed for the genus *Aphelenchoides* in the plots with organic mulch and the genus *Aphelenchus* in the plots covered with geotextile.

Herbivores (or plant parasites) accounted for 1.9 to 40% of the nematode communities. Both cover types had a significant positive effect on the trophic group, which was more significant compared to the control primarily in the case of hay (***Table 1***). The most abundant taxon proved to be the family Telotylenchidae (72.9%). The genus *Pratylenchus* became more significant in post-harvest samples for each treatment, especially in the case of the geotextile cover. *Meloidogyne*, known as a strawberry pest, occurred only sporadically and only with low densities, irrespective of the treatment (***Table 1***).

Of the omnivorous nematodes, the genera *Eudorylaimus*, *Ecumenicus* and *Aporcelaimellus* were the most common (74.7%). They accounted for 1.8% to 29.2% of the total nematode community, which decreased significantly during the growing season, mainly in the case of organic mulch (***Table 1***). At the genus level, the presence of *Ecumenicus* decreased consistently for all treatments (***Table 1***). The presence of the genus *Paraxonchium* was adversely affected by the hay cover, while *Aporcelaimellus* responded differently to the different cover methods: positively to geotextile cover, negatively to hay mulch (***Table 1***). The lack of treatment had a favorable effect on the members of the genus *Eudorylaimus*.

Predatory nematodes were rare in our samples, they were mostly absent (their proportion was 0% to 5.7% for the whole community). They were represented by the following two genera: *Discolaimus* (84.6%) and *Mylonchus* (15.4%).

Various results have been reported in the literature regarding the application of similar treatments, ranging from an increase in nematode diversity **[24]**, through treatment resulting in no change **[25]**, to a decrease in the taxonomic diversity of nematodes

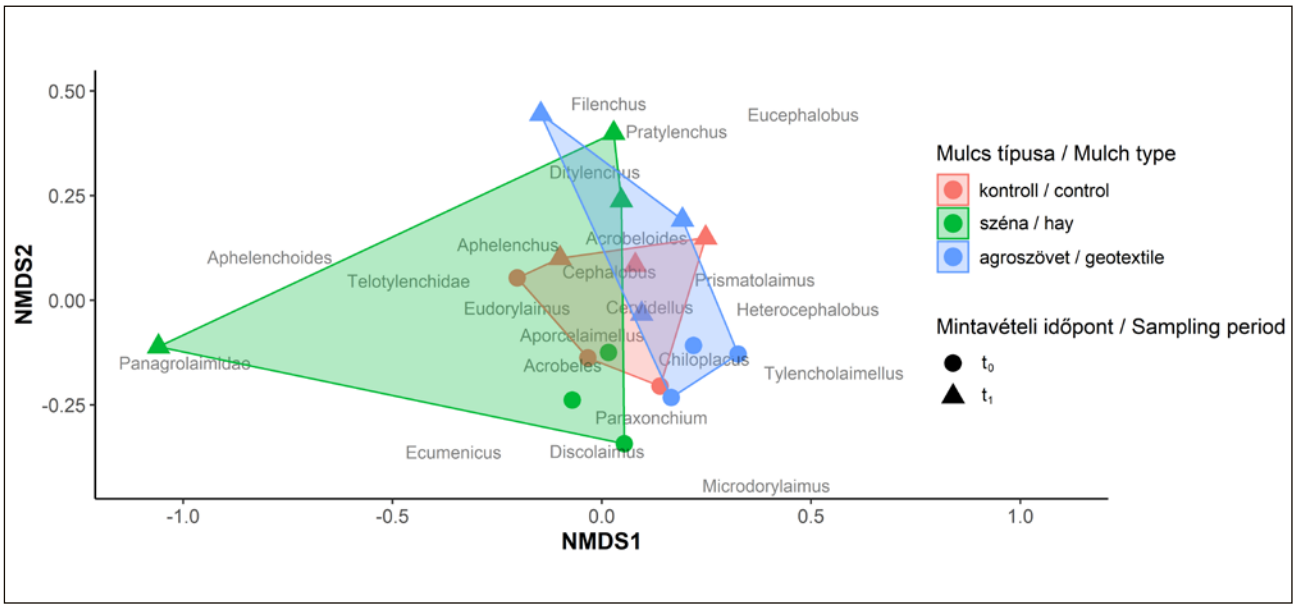

Figure 1. Non-metric multidimensional scaling (NMDS) ordination plots of nematode communities by mulch treatments (no: non-mulching, organic: mulching with grass hay, inorganic: mulching with black geotextile) at pre-plant (t0) and post-harvest (t1) time periods.

[e.g., **26]**. The effect of the organic mulch applied depends significantly on the quality and type of the organic matter that makes it up (C:N ratio, contaminants, nematotoxic components, etc.), the presence of antagonists, as well as the duration and frequency of the application **[24]**. In our case, the drought, the low number of rainy days and the low total precipitation during the critical period (45.8 mm during July and August) may have had a significant effect on the population dynamics of nematodes **[27].**

5. Conclusions

The results support that soil cover has a beneficial effect on soil fauna diversity. Contrary to our expectations, mulch could not offset the effects of the summer drought and thus increase the abundance of nematodes, but it did have a positive effect on the functional diversity of nematodes. This can lead to a more stable soil ecosystem, which increases the functional resilience and adaptive capacity of the soil biota, which can lay the groundwork for a more viable and sustainable agricultural production.

6. Acknowledgment

First of all, we would like to thank Dr. Péter Nagy (SZIE Department of Zoology and Animal Ecology, Gödöllő), who identified the nematodes. Ágnes Gortva-Vajda (ÁTE Institute for Biology, Budapest) participated in the fieldwork. The gardeners of John von Neumann University helped to carry out and maintain the experiments. The project was supported by the European Union, co-financed by the European Social Fund (ESF, grant agreement no.: EFOP-3.6.2-16-2017-00012, project title: Development of a functional, healthy and safe food product path model based on the farm-to-fork principle in a thematic research network).

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IMPRESSUM

Kiadó / Publsher: Wessling Nemzetközi Kutató és Oktató Központ Nonprofit Kft. / Wessling International Research and Educational Centre Nonprofit Ltd. / **HU ISSN 2676-8704**
Felelős kiadó / Director: Dr. ZANATHY László ügyvezető igazgató / CEO
Főszerkesztő / Editor in chief: Dr. SZIGETI Tamás János
Szerkesztő / Editor: KONECSNY Tímea, SZUNYOGH Gábor
Angol fordítás / English translation: Dr. HANTOSI Zsolt
Honlap adminisztrátor / web admin.: JUHÁSZ Péter

Nyomdai előkészítés / Layout dtp: Adworks Kft., E-mail: info@adworks.hu
Elérhetőségeink/Contact: H-1045 Budapest, Anonymus utca 6., Telefon/Phone: +36 1 872-3600, +36 1 872 3621; Fax: +36 1 435 01 00, Mobil phone: +36 30 39 69 109, E-mail: eviko@wirec.eu; Web: www.eviko.hu
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E-mail: eviko@wirec.eu, *Előfizetési díj egy évre/Subscription for one year: bruttó 4620 Ft. /15 €.*
A lap negyedévente jelenik meg. / This journal appears quarterly in a year.

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A szakfolyóiratot a következő figyelő szolgáltatások vették jegyzékbe és referálják / The Journal of Food Investigation is have been referred and listed by the next monitoring services: SCOPUS, SCIMAGO, MATARKA (Magyar folyóiratok tartalomjegyzéke/*Hungarian Periodicals Table of Contents*), Thomson Reuters, Elsevier's Abstracting and Indexing Database

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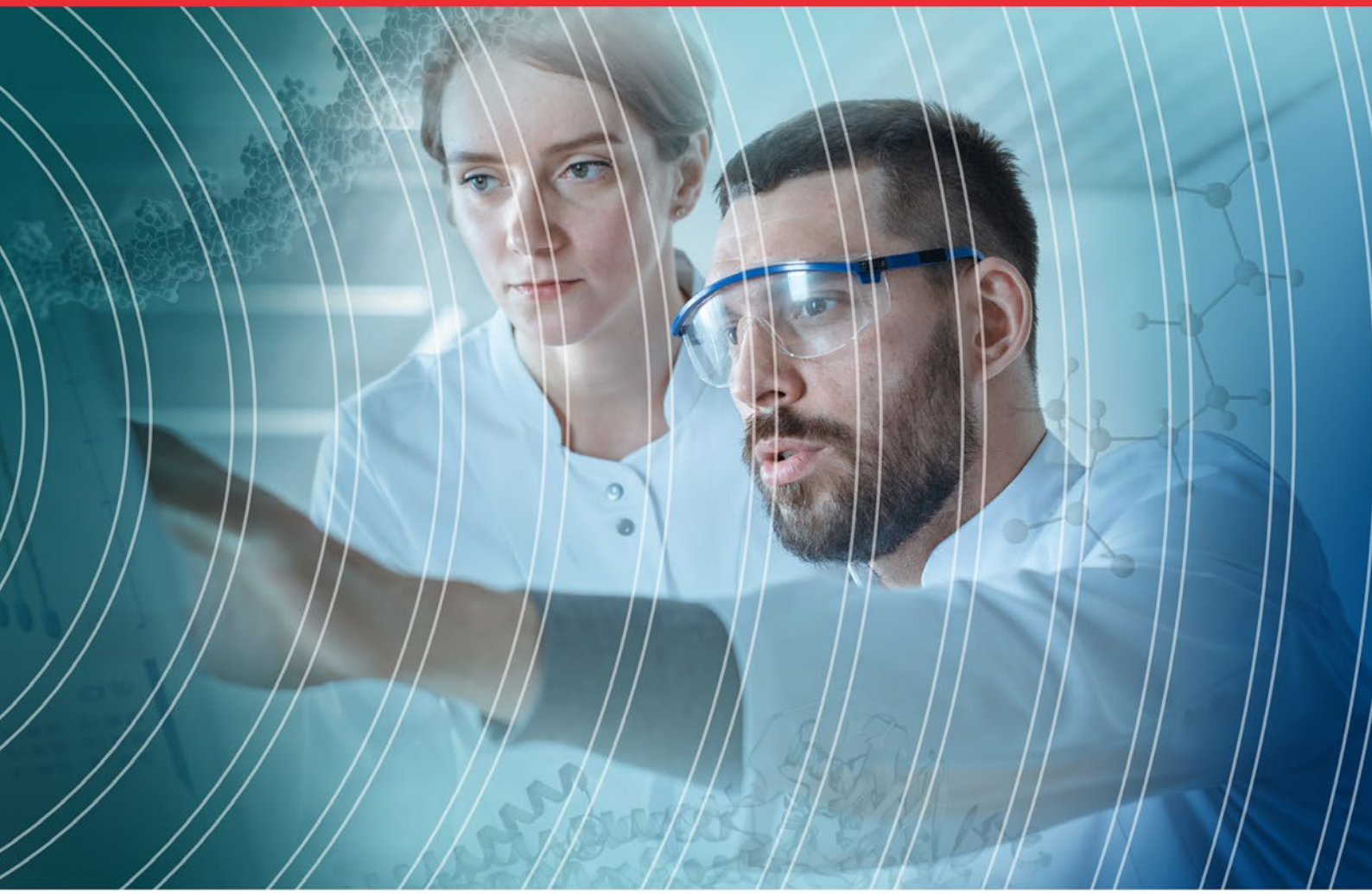

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