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Contents

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The first ten years of Acta Universitatis Sapientiae, Alimentaria

Dear reader,

The very first issue of *Acta Universitatis Sapientiae*, *Alimentaria* was published in the year 2008, followed by nine more volumes, which leads us to the present year celebrating the 10^{th} birthday of the journal. On this occasion, I have put together a brief summary that I would like to share with our readers.

In the course of these 10 years, 11 issues were brought to light on altogether 1,294 pages, amounting to a total of 93 articles. The average length of the publications is 14 pages. Regarding the authors' citizenship, the list is as follows: Romanian, Romanian and Hungarian (double citizenship), Hungarian, Serbian, Croatian, Macedonian, Ukrainian, Czech, Slovakian, and Iranian.

Members of the Editorial Advisory Board:

all of whom I would hereby like to thank for their work carried out in the past 10 years for providing valuable assistance in the publication of the journal with their experience, expertise, and creative ideas.

Further, I would like to express my gratitude to **proofreader** Annamária Balázs and **layout editor** Erzsébet Csizmadia. My special thanks go to Prof. Dr Zoltán Kása, Dr Angella Sorbán, Beáta Szabó and István Szász-Köpeczy from Cluj-Napoca as well as my former collaborators: my wife, Csapóné Dr Zsuzsanna Kiss and my former colleague, Judit Stanics, without whose assistance I could not have succeeded in having these 10 volumes published. I thank them kindly for their work.

Annexed to this brief summary, you will find the topics of the articles published throughout the ten volumes, in alphabetical order, as well as the contents of all issues and a list of the most frequently downloaded studies. Over the past ten years, we made every effort so that those interested in food science be provided with the latest research results in the field, which remains our most prominent endeavour in the future, too. Therefore, you are kindly invited to keep reading our journal.

Yours faithfully,

Prof. Dr János Csapó
Executive Editor

The topics of articles in alphabetical order:

V

vitamins, diet of the elderly, fat-soluble vitamins
vitamins, diet of the elderly, water-soluble vitamins

W

water soluble vitamins, microwave heating
watermelon, colour differences
wheat flour, farinograph test, quality determination
wheat grasses, wheat seeds, inorganic and organic selenium content
wheat grits, storage
wheat, Fusarium toxin contamination, milling technology
whole grain flour, basic chemical composition, different cereal grains
winter wheat flour, N and S contents
winter wheat flour, valorigraphic parameters
winter wheat grits, colour
winter wheat, aluminium toxicity
winter wheat, flour quality, kernel hardness
winter wheat, rheological properties, storage

Y

yoghurt production, various prebiotics, probiotics
yogurt, exopolysaccharides, oligosaccharides, connection status, configuration, phosphorous content, modification, structure

Contents of the ten volumes:

Alimentaria – Contents of Volume 1, 2008

Alimentaria – Contents of Volume 2, No 1, 2009

R. V. Salamon, Sz. Salamon, Zs. Csapó-Kiss, J. Csapó Composition of mare's colostrum and milk I. Fat content, fatty acid composition and vitamin contents. Acta Universitatis Sapientiae, Alimentaria, 2, 1 (2009) 119–131

J. Csapó, Sz. Salamon, K. Lóki, Zs. Csapó-Kiss Composition of mare's colostrum and milk II. Protein content, amino acid composition and contents of macro- and micro-elements. Acta Universitatis Sapientiae, Alimentaria, 2, 1 (2009) 133–148

Alimentaria – Contents of Volume 2, Number 2, 2009

Alimentaria – Contents of Volume 3, 2010

Alimentaria – Contents of Volume 4, 2011

Alimentaria – Contents of Volume 5, 2012

Alimentaria – Contents of Volume 6, 2013

Alimentaria – Contents of Volume 7, 2014

Alimentaria – Contents of Volume 8, 2015

Alimentaria – Contents of Volume 9, 2016

Alimentaria – Contents of Volume 10, 2017

Most downloaded articles

Production of selenium-enriched milk and dairy products by *Csapó, J./ Holló, G./ Holló, I./ Salamon, R. V./ Salamon, Sz./ Toró, Sz./ Kiss, Zs. Csapóné*

Aluminium toxicity in winter wheat by *Szabó, A./ Gyimes, E./ Véha, A.*

Lycopene and flesh colour differences in grafted and non-grafted watermelon by *Fekete, D./ Stéger-Máté, M./ Bőhm, V./ Balázs, G./ Kappel, N.*

Effect of oil-seed pressing residue on bread colour and texture by *Tarek-Tilistyák, J./ Tarek, M./ Juhász-Román, M./ Jekő, J.*

Colour characteristics of winter wheat grits of different grain size by *Horváth, Zs. H./ Véha, A.*

Experiences of using the fruit waste of the modern Hungarian pálinka fermentation technology for the foraging of extensively kept grey cattle by *Barabás, A./ Szigeti, J.*

Role of the farinograph test in the wheat flour quality determination by *Diósi, G./ Móré, M./ Sipos, P.*

Examination of the selenium content of wheat grasses produced in different soil types in Csík Basin by *Tamás, M./ Csapó, J.*

Somatic cell count of milk from different goat breeds by *Csanádi, J./ Fenyvessy, J./ Bohata, S.*

Investigation of colour agent content of paprika powders with added oleoresin by *Szabó, L./ Horváth, Zs. H.*

High hydrostatic pressure: Can we trust published data? by *Németh, Cs./ Castillo, L. A./ Horváth, F./ Zeke, I./ Friedrich, L./ Balla, Cs./ Németh, Z./ Póti, P.*

Milling technological experiments to reduce Fusarium toxin contamination in wheat by *Véha, A./ Szabó, P./ Bartók, T./ Gyimes, E.*

Investigation of rheological properties of winter wheat varieties during storage by *Móré, M./ Diósi, G./ Sipos, P./ Győri, Z.*

The role of vitamins in the diet of the elderly I. Fat-soluble vitamins by *Csapó, J./ Albert, Cs./ Prokisch, J.*

Production of highly nutritious functional food with the supplementation of wheat flour with lysine by *Albert, Cs./ Gombos, S./ Salamon, R. V./ Csiki, Z./ Prokisch, J./ Csapó, J.*

The role of vitamins in the diet of the elderly II. Water-soluble vitamins by *Csapó, J./ Albert, Cs./ Prokisch, J.*

The essentiality of selenium for humans, animals, and plants, and the role of selenium in plant metabolism and physiology by *Garousi, F.*

Toxicity of selenium, application of selenium in fertilizers, selenium treatment of seeds, and selenium in edible parts of plants by *Garousi, F.*

Production of a high-nutritional-value functional food, the Update1 bread, with the supplementation of the wheat flour with high-protein-content raw food materials by *Csapó, J./ Schobert, N.*

Simple utilization of lactic acid whey in dairy processing by *Csanádi, J./ Szász, G./ H-Bara, O.*

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Evaluation of the microbiological quality of some dairy products

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Abstract. Owing to their nutrient composition, dairy products ensure a favourable environment for different microorganisms. In our study, we investigated the microbiological quality of 22 different commercially available dairy products obtained from local stores and the open-air public market. Among the studied samples four were salty type soft cheese, two were fresh cheese, one was soft cheese (Mascarpone), one was feta-like cheese (Telemea), five were varieties of processed cheese, one was mozzarella, one was a semi-hard cheese, one was smoked cheese, five were cottage cheese, and one was a dairy spread. Samples were evaluated for the presence of *Pseudomonas* sp., total coliforms, *Escherichia coli*, *Salmonella* sp., S*taphylococcus aureus*, *Bacillus cereus* and *Clostridium perfringens*, yeast, and microscopic fungi.

Contamination level of the evaluated dairy products varied widely. Among the dairy samples one salty soft cheese, a processed cheese and one cottage cheese were the most contaminated, while low microbiological load was detected in the other samples.

Keywords and phrases: dairy products, microbiological load, pathogenic bacteria, spoilage

Based on our results, it can be concluded that the microbiological quality of the most studied samples is satisfactory, but there are samples with marginal characteristics too. In turn, three products can be declared potentially hazardous.

1 Introduction

Before consumption, food products are exposed to microbial spoilage during harvest, manufacture, storage, and distribution. The spoilage of foods threatens human health and leads to enormous economic loss. Worldwide, about 15–25% of foodstuffs deteriorate (*Deák & Farkas*, 2013).

Due to their nutritional value, especially the high protein and lipid content, dairy products are a suitable growth environment for a range of microorganisms. The type of dairy product mainly determines the type of the spoilage microorganism. The microbiological load of a product correlates with the steps of manufacturing. Cream cheese and processed cheese are associated with fungi, spore-forming bacteria. Psychrotrophs, coliforms, fungi, lactic acid bacteria, and their enzymatic degradation are responsible for the spoilage of soft, fresh cheese types. The typical types of spoilage microorganisms of cottage cheese are the psychrotrophs, coliforms, yeasts, and moulds (*Ledenbach & Marshall*, 2009).

Generally, in the microbial deterioration of dairy products, two main microorganism groups play a central role: psychrotrophs that grow at 5–7 °C and thermodurants that survive pasteurization. In dairy products, diverse bacterial species can be detected belonging to different genera: both Gram-positive (*Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus*) and Gram-negative species (*Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, *Flavobacterium*, *Burkholderia*, *Sphingomonas*, *Stenotrophomonas*). The predominant species altering milk and dairy products are considered those belonging to the genera *Pseudomonas* sp. The alteration is the result of the activity of a wide range of enzymes, such as protease or lipase, or the result of the production of organoleptic spoilage (*Raposo et al.*, 2017). *Pseudomonas* strains with pigment production are also involved in several cases of spoilage of dairy products. In fresh low-acid cheese, *P. fluorescens*, *P. brassicacearum*, and *P. putida* pigment compounds (indigoidine, pyoverdine) discolour the product (*Andreani & Fasolato*, 2017).

In addition to spoilage bacteria, dairy products may be carriers of pathogenic bacteria such as *Bacillus cereus*, *Brucella* sp., *Campylobacter jejuni*, *E. coli*

O157:H7, *Coxiella burnetii*, *Listeria monocytogenes*, *Mycobacterium paratuberculosis*, *Salmonella* sp., *Yersinia enterocolitica*, or *Staphylococcus aureus*. Some of this bacterial species have been associated with milkborne outbreaks (*Lu & Wang*, 2017; *Suilaiman & Hsieh*, 2017).

Among dairy products, cheese or cheese-related products have been mostly contaminated by different microbes. It was reported that cheese is very susceptible to *Salmonella* sp. (*Suilaiman & Hsieh*, 2017). According to *Suilaiman & Hsieh* (2017), between 1998 and 2014, several dairy outbreaks in the United States resulted from the consumption of raw milk or cheese contaminated with Shiga-like toxins producing *E. coli*. The presence of *E. coli* and total coliform bacteria refers to poor hygiene conditions.

Regarding the safety of dairy products, another foodborne pathogen is the enterotoxin-producing *Staphylococcus aureus*. The pathogenicity of these bacteria is mainly linked to toxin-mediated virulence, invasive capacity, and antibiotic resistance (*Carfora et al.*, 2015).

Species of *Bacillus cereus*, able to produce different toxins (cereulide, cytotoxin K, haemolysin BL, and non-hemolytic enterotoxin) are responsible for food poisoning. These bacteria are thermoduric spore formers, therefore challenging the dairy industry. Dairy products are on the *B. cereus*-contaminated food list (*Tirloni et al.*, 2017; *Grutsch et al.*, 2018).

Some species of yeast, such as *Geotrichum candidum*, *Pichia* sp., and *Candida* sp., contribute to a variety of defects in cheese. The production of their metabolites, such as sulphides and other compounds, results in off-flavours and gas production as well (*Lu & Wang*, 2017).

There are few studies on the prevalence of spoilage and foodborne bacteria in locally available dairy packed products or dairy products sold by weight. The aim of the present study was the evaluation of the microbiological quality and safety of different dairy products originated from local stores or the open-air public market.

2 Materials and methods

During our work, 22 different dairy products (salty type soft cheese, fresh cheese, soft cheese, feta-like cheese, processed cheese, mozzarella, semi-hard cheese, smoked cheese, cottage cheese, dairy spreads) have been studied microbiologically with cultivation methods. The selection of the dairy product samples was made at random. Detection of the bacteria important for spoilage or for health and hygienic reasons, such as *Pseudomonas* sp., total coliforms,

Escherichia coli, *Salmonella* sp., *Staphylococcus aureus*, *Bacillus cereus*, or *Clostridium perfringens*, has been carried out with bacteriological cultivation methods on different selective culture media. One gram of each sample was aseptically transferred into a 9 ml physiological solution. From this, serial dilutions to 10^{-1}–10^{-2} were prepared, and a volume of 0.1 ml was spread on the selective agar mediums. The inoculated culture media have been incubated for 48 h at 37 °C and 44 °C for total coliforms and *Clostridium perfringens* respectively. The following selective culture media were used during the determination of the bacteria: Pseudomonas Selective Agar (Biolife), ChromoBio^{R} Coliform (Biolab), TBX Chromo-Agar (Carl Roth), Brilliance^{TM} Salmonella Agar Base (Oxoid), Mannitol Salt Agar (Oxoid), ChromoBio^{R} Cereus Base (Biolab), and Clostridial Differential Broth (Fluka Analytical).

In the case of detecting microscopic filamentous fungi, the studied samples have been inoculated on Czapek-Dox Agar (Oxoid) nutritive medium, and the determination has been done based on colony and cell morphology properties, with the help of microscopic preparations.

Statistical Analysis

Principal component analysis (PCA) has been performed using PAST Software for graphical description and to categorize the studied samples based on microbiological quality. The first three principal components from the microbiological counts of the dairy products have been obtained with PCA.

3 Results and discussion

During this study, we have evaluated the microbial contamination of 22 different commercially available dairy products obtained from local stores and the open-air public market. Among the studied samples, four were salty type soft cheese, two were fresh cheese, one was soft cheese (Mascarpone), one was feta-like cheese (Telemea), five were processed cheese varieties, one was mozzarella, one was a semi-hard cheese, one was smoked cheese, five were cottage cheese, and one was a dairy spread.

Samples have been analysed for the presence of *Pseudomonas* sp., total coliforms, *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, yeast, and microscopic fungi. The results of the bacteriological evaluation are presented in *Table 1.* Contamination level by bacteria of the evaluated dairy products varied widely.

Table 1: Bacteriological results of dairy products

Studied samples	Sample code	*Pseudo-monas* sp.	Coliforms	*Escherichia coli*	*Salmonella sp.*	*Staphylococcus aureus*	*Bacillus cereus*	*Clostridium perfringens*
				CFU/g				
Salty type soft cheese 1	SSFC1	$3{\cdot}10^3$	$1{\cdot}10^4$	$5{\cdot}10^3$	$2{\cdot}10^3$	<10	$3{\cdot}10^2$	-
Salty type soft cheese 2	SSFC2	4·10	n	$>3{\cdot}10^2$	<10	<10	$>3{\cdot}10^2$	-
Fresh cheese 1	FC1	$9{\cdot}10^2$	$4{\cdot}10^3$	$7.5{\cdot}10^3$	<10	<10	<10	-
Fresh cheese 2	FC2	$2{\cdot}10^2$	n	$>3{\cdot}10^2$	1·10	$2{\cdot}10^2$	$>3{\cdot}10^2$	-
Soft cheese Mascarpone	M	1·10	$2{\cdot}10^3$	1·10	$2{\cdot}10^2$	$3{\cdot}10^3$	<10	-
Feta-like cheese –Telemea	F	<10	$1{\cdot}10^3$	<10	<10	<10	<10	-
Processed cheese 1	PC1	1·10	810^2	<10	<10	<10	9·10	-
Processed cheese 2	PC2	<10	4·10	7·10	<10	<10	<10	+
Processed cheese 3	PC3	<10	$8{\cdot}10^3$	<10	<10	$1{\cdot}10^4$	4·10	+
Processed cheese 4	PC4	<10	<10	<10	$6{\cdot}10^2$	<10	<10	-
Processed cheese 5	PC5	<10	n	<10	<10	$>3{\cdot}10^2$	$>3{\cdot}10^2$	-
Dairy spread	DS	<10	$4{\cdot}10^2$	<10	3·10	3·10	<10	-
Mozzarella	MO	<10	$5{\cdot}10^3$	<10	<10	<10	<10	-
Smoked cheese	SC	<10	7·10	<10	<10	5·10	<10	-
Semi-hard chees	ShC	<10	n	<10	<10	10	$>3{\cdot}10^2$	-
Cottage cheese 1	CC1	$8{\cdot}10^2$	$7{\cdot}10^2$	$3{\cdot}10^3$	7·10	<10	<10	+
Cottage cheese 2	CC2	<10	<10	$1{\cdot}10^2$	<10	<10	6·10	-
Cottage cheese 3	CC3	<10	4·10	$1{\cdot}10^4$	$1{\cdot}10^3$	<10	$2{\cdot}10^2$	-
Cottage cheese 4	CC4	<10	$1.{\cdot}10^3$	<10	$2{\cdot}10^3$	$1{\cdot}10^3$	$3{\cdot}10^2$	-
Cottage cheese 5	CC5	<10	n	<10	<10	1	$9{\cdot}10^3$	-
Salty type soft cheese 3	SSFC3	<10	$8{\cdot}10^2$	<10	<10	<10	<10	-
Salty type soft cheese 4	SSFC4	4·10	n	<10	<10	$2{\cdot}10^2$	$>3{\cdot}10^2$	-

n: not evaluated

The *Pseudomonas* sp. on selective agar have been detected in eight samples. The number of this bacterial species varied between 10 and $3{\cdot}10^3$ CFU/g. The most contaminated product proved to be a salty type soft cheese with $3{\cdot}10^3$ CFU/g. In the case of both types of fresh cheese obtained from the open-air market, this bacterial isolate has also been detected. Among the five varieties of the studied processed cheese samples, only one (PC1) turned out positive for *Pseudomonas* sp. (10 CFU/g). Among the five evaluated samples, only one (CC1) has been detected $8{\cdot}10^2$ CFU/g. The physico-chemical properties (including pH, salt content) of cottage cheese support the growth of this group of bacteria. Also, they determine the shelf life of the product (*Ledenbach* & *Marshall*, 2009). In the case of a salty type soft cheese (SSFC2) obtained from the store, 4·10 CFU/g *Pseudomonas* sp. has been detected.

Pseudomonas sp. are the most dominant psychrotrophic microorganisms isolated from milk. Low temperatures like 3–7 °C and the ability to use large lipid and protein molecules favour their growth. It has been shown that these bacteria can reduce the diacetyl content of some dairy products, resulting in a green or yogurt-like flavour. The appearance of *P. fluorescens* strains is due to post-process contamination. The refrigeration and protein-rich content of the product is beneficial for this microorganism (*Ledenbach* & *Marshall*, 2009; *Andreani* & *Fasolato*, 2017; *Brasca et al.*, 2018).

It has been shown that different species of *Pseudomonas* genus caused the enzymatic spoilage of dairy products; *P. fluorescens*, *P. fragi*, and *P. putida* caused an alteration in the texture of soft and fresh types of cheese (*Andreani* & *Fasolato*, 2017).

The cell number for the initiation of spoilage by psychrotrophs is about 10^6 CFU/ml (*Brasca et al.*, 2018). In our studied samples, the incidence of *Pseudomonas* sp. did not reach this cell count. For the inhibition control of this group, the suggested solution is the proper combination of time and temperature (*Brasca et al.*, 2018).

Besides pseudomonads, coliform bacteria represent a quality indicator in the dairy industry. Among the evaluated samples, only two were free of coliforms. The highest total coliform load has been determined in the case of the salty type soft cheese from the open-air market, containing 10^4 CFU/g. In six dairy products, the coliform level has been 10^3, varying between $1{\cdot}10^3$ and $8{\cdot}10^3$ CFU/g. In the case of four products, the number of coliforms has been around $4{\cdot}10^2$–$8{\cdot}10^2$ CFU/g. In the case of a variety of processed cheese and a cottage cheese, the detected total coliform number has been 4·10 CFU/g (CC3), while in the case of smoked cheese it has reached 7·10 CFU/g (SC). The coliforms cause blowing defect in different types of brined cheese (*Pintado*

et al., 2015). Among the studied samples, the salty dairy products reached high counts of this group.

The presence of a coliform group in a dairy product reflects the unhygienic conditions of the production process. Coliforms could be eliminated by compliance with personal hygiene and sanitation processes. This group of bacteria includes bacterial species of *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia*, *Hafnia*, *Citrobacter* genera (*El-Ziney*, 2018; *Moatsou* & *Barbaros*, 2015), and many others. The negative impact of this group consists in their enzymatic activity and the production of metabolic by-products influencing the quality of the dairy product. The reduction of coliforms in cheese can be achieved by the reduction of temperature and drop in pH (*Martin et a*l., 2016). *Trmčić et al.* (2016) revealed that the presence of coliforms in a wide variety of cheese made from different kinds of milk is correlated with water activity. They also concluded that the type of the milk influenced the prevalence of this group of bacteria.

Escherichia coli represents a hygienic indicator organism in cheese production, reflecting faecal contamination. In reference to specific virulence factors and phenotypic characteristics, these bacteria have different groups such as enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and so on. Globally, *E. coli* O157:H7 serotype is responsible for foodborne disease outbreaks (*Law et al.*, 2017). 13 dairy samples have turned out free of *Escherichia coli*. The presence of *E. coli* has been found in nine studied samples. In the case of six samples, CFU/g of this bacterium has been higher than 300. Mascarpone contains 10 CFU/g, and in one of the processed cheese types 7·10 CFU/g (PC2) contamination has been found. Different serotypes of *E. coli* with virulence genes have been detected in dairy products by *Douëllou et al.* (2016).

Dairy products can be contaminated with *Salmonella* sp. Species of these bacteria are the major foodborne pathogens. Eight dairy products have been contaminated with *Salmonella* sp. The detected CFU/g of this bacteria varied between 10 and 10^3. The highest total *Salmonella* sp. load has been found in the case of one cottage cheese (CC4). Among the cottage cheese types, two were free of this bacteria. In the case of one salty type soft cheese (SSFC1), the *Salmonella* sp. colony counts have reached 10^3 CFU/g, in dairy spread, (DS) it was 3·10 CFU/g, while in one cottage cheese (CC1) the analysis indicated $7 \cdot 10$ CFU/g.

Species of the genus of *Staphylococcus* are proteolytic enzyme producers in milk and milk-derivate products yet also bacteria of potential danger concerning public health. *Staphylococcus aureus* is a zoonotic pathogen, causing differ-

ent infections (*Biswas & Mandal*, 2017). Based on coagulase production, they are divided in coagulase-positive and coagulase-negative strains. Coagulase-positive strains harbour virulence factors such as a heat-resistant nuclease, catalase, coagulase, haemolysins, protein A, lipase, leukocidin, staphylokinase, toxic shock toxin, or exfoliative toxins A and B. Some of these factors have also been detected in coagulase-negative strains (*Chajęcka-Wierzchowska & Zadernowska*, 2017).

The presence of *Staphylococcus aureus* has been found in seven dairy products. In this case, the highest bacterial load has been detected in two varieties of processed cheese (PC3, PC5), where the CFU/g resulted higher than 300. This bacterial isolate has also been present in Mascarpone (M $3 \cdot 10^3$) and a fresh cheese (FC2) obtained from the open-air market ($2 \cdot 10^2$ CFU/g). The value of 10^4 CFU/g is associated with the result of foodborne illnesses (*Biswas & Mandal*, 2017). In the case of our samples, this critical cell number has been detected in two varieties of processed cheese (PC3, PC5).

According to *Al-Khafaji* and *Flayyih* (2015), in three hundred milk and cheese samples, *Staphylococcus aureus* was the predominant species. This bacterium can originate from humans or biofilms, possibly from insufficient acidification during cheese production (*Kümmel et al.*, 2016). With the control of temperature, time, and pH, the growth and toxin production of this bacterium can be prevented.

The spore-forming bacterium *Bacillus cereus* is one of the most dominant spoilage microorganisms in the dairy industry. Alteration caused by these bacteria appears due to the enzyme activities, such as proteinases, lipases, or phospholipases, also determining the off-flavours of products. In the case of cheese, a negative impact of *Bacillus* sp. consists in the reduction of nitrate to nitrite that limits its preservative effectiveness. A wide variety of dairy products, including cheese and processed cheese, was contaminated by this bacterium (*Lopez-Brea et al.*, 2017). Nine dairy products turned out to be free from *Bacillus cereus*. High counts of this bacteria has been found in four of the studied samples, where the CFU/g resulted higher than $3 \cdot 10^2$ CFU/g. Also, a low contamination level has been detected in two cases of processed cheese (PC1, PC3) (9·10 and 4·10 CFU/g). *B. cereus* is also responsible for the emergence of foodborne diseases on a level of 5 log to 8 log cells/spores/g food. It has been shown that rapid cooling, low pH, and water activity contribute to the inhibition of this bacterium (*EFSA*, 2005).

The spoilage microbiota constituent of milk and dairy products under anaerobic conditions is *Clostridium perfringens*, which is one of the most common foodborne pathogens with low generation time (*Lopez-Brea et al.*, 2018). The

presence of *Clostridium perfringens* has been detected in three dairy samples: in two varieties of processed cheese (PC2, PC3) and one cottage cheese (CC1). The species of *Clostridium* genus are responsible for the late swelling spoilage of different types of cheese. One spore per millilitre milk can already cause alteration. These microorganisms metabolize the residual sugar and citrate with the production of organic acids. Discolouration of soft ricotta cheese has been caused by *Bacillus cereus* and *Clostridium* sp. (*Andre et al.*, 2017; *Remize*, 2017; *Ledenbach & Marshall*, 2009).

The pH and the nutritional characteristics of fermented dairy products favour the growth of yeasts, resulting in fungal spoilage. In these products, yeasts metabolize diacetyl, causing off-flavour, yeast taste, and alteration in colour and texture. In these cases, yeast count can reach 10^5–10^6 CFU/g.

In the different types of cheese, yeasts produce CO_2 and alcohol, altering the taste. The ethanol reacts with short-chain fatty acids, resulting in fruity esters. Egg odour can be the result of the sulphides produced by some proteolytic yeast strains (*Ledenbach & Marshall*, 2009).

Alteration in colour can be caused by *Yarrowia lipolytica*, resulting from the formation of pyomelanin, a brown pigment. Some yeasts can also produce biogenic amines (*Garnier et al.*, 2017).

The results of the evaluation of yeasts and moulds are presented in *Table 2*. In the case of our studied samples, yeast has been present in 13 samples. Except for a cottage cheese, the CFU/g has resulted higher than 10^2. The highest yeast contamination has been detected in the case of the salty soft cheese (SSFC3) $2{\cdot}10^4$CFU/g. The CFU/g has also resulted 10^4 in Mozzarella (MO), a variety of processed cheese (PC1), fresh cheese (FC1), and Mascarpone (M). Consequently, it can be summarized that the yeast count is smaller than the number that leads to deterioration. According to *Ledenbach* and *Marshall* (2009), the common spoilage yeast of cheese includes *Candida* sp., *Kluyveromyces marxianus*, *Geotrichum candidum*, *Debaryomyces hansenii*, and *Pichia* sp. The common yeast *Geotrichum candidum* caused spoilage in a variety of cottage cheese.

For moulds, the surface of different types of cheese represents a favourable environment. Due to oxygen availability, the vacuum package inhibits the growth of these microbes, but some species can support low oxygen tension. Microscopic fungi commonly found and growing in vacuum-packaged varieties of cheese include *Penicillium* sp. and *Cladosporium* sp. *Penicillium* is the mould genus most frequently occurring on cheese (*Ledenbach & Marshall*, 2009). Microscopic fungi have been detected in seven studied dairy products.

Table 2: The yeast and microscopic fungi count of some dairy products

Studied samples		Yeasts	Microscopic fungi
		CFU/g	
Salty soft cheese 1	SSFC1	$5{\cdot}10^3$	6·10
Salty soft cheese 2	SSFC2	$3{\cdot}10^2$	10
Fresh cheese 1	FC1	$1{\cdot}10^4$	1·10
Fresh cheese 2	FC2	<10	0
Soft cheese Mascarpone	M	$1{\cdot}10^4$	0
Feta like cheese	F	$8{\cdot}10^3$	0
Processed cheese 1	PC1	$1{\cdot}10^4$	0
Processed cheese 2	PC2	<10	<10
Processed cheese 3	PC3	<10	$1{\cdot}10^3$
Processed cheese 4	PC4	<10	<10
Processed cheese 5	PC5	<10	<10
Dairy spread	DS	$1{\cdot}10^2$	1·10
Mozzarella	MO	$1{\cdot}10^4$	<10
Smoked cheese	SC	$4{\cdot}10^3$	1·10
Semi-hard cheese	ShC	<10	<10
Cottage cheese 1	CC1	$5{\cdot}10^3$	8·10
Cottage cheese 2	CC2	$1{\cdot}10^3$	<10
Cottage cheese 3	CC3	<10	$2{\cdot}10^3$
Cottage cheese 4	CC4	<10	$2{\cdot}10^2$
Cottage cheese 5	CC5	6·10	<10
Salty soft cheese 3	SSFC3	$2{\cdot}10^4$	<10
Salty soft cheese 4	SSFC4	<10	<10

The highest microscopic fungi load has been found in one cottage cheese (CC3) ($2{\cdot}10^3$ CFU/g) and a variety of processed cheese (PC3) ($1{\cdot}10^3$CFU/g).

A negative impact on moulds' deterioration is in the case of products containing sorbate. Some of the microscopic fungi transform it into trans-1,3-pentadiene, causing an alteration in flavour. Some of them can produce mycotoxins.

The heat-resistant microscopic fungi, such as *Byssochlamys nivea*, can deteriorate cream cheese (*Ledenbach & Marshall*, 2009). This microorganism could be detected in our case in two heat-treated products (PC3, DS). For the prevention of the fungal spoilage, control over some factors is considered critical: air treatment, cleaning, and disinfection procedures, heat treatment, water activity reduction by brining, refrigeration, and modified atmosphere packaging (*Garnier et al.*, 2017). In our work based on colony and cell morphology properties, the following microscopic filamentous fungi have been identified: *Aspergillus niger* in the dairy spread, *Penicillium* sp., *Cladosporium* sp., and *Aspergillus* sp. in the cottage cheese, *Penicillium* sp. and *Mucor mucedo* in the salty soft cheese, and *Penicillium* sp. in the smoked cheese sample.

From the analysed samples, not all the products meet the compliance requirements by the European Regulation EC 2073/2005. It can be stated that one of the salty type soft cheese and one fresh cheese sample (both originating from the open-air market) are unsatisfactory. In these two products, the *E. coli* level is higher than the acceptable regulatory criteria. Mascarpone and one of the processed varieties of cheese samples have also been found unacceptable since the *S. aureus* count exceeds the standards. Regarding *Salmonella*, eight products have resulted unsatisfactory.

PCA and cluster analysis are useful methods for the classification of different types of cheese based on their quality (*Eroglu et al.*, 2015). The microbial contamination level of the studied dairy samples has been subjected to PCA. This statistical method reduces the dimensionality of the data, calculating the components that best describe the differences or similarities between samples (*Ercan et al.*, 2014). Three PCs have been established to be significant for the interpretation of microbial content. The eigenvalues and variance of PCs are shown in *Table 3*.

Table 3: Results of PCA analysis of the microbiological count

PC	**Eigenvalue**	**% variance**	**Cumulative variance** (%)
1	2.13042	26.63	26.63
2	1.82792	22.85	49.48
3	1.37301	17.16	66.64
4	1.04405	13.05	79.69
5	0.628574	7.86	87.55
6	0.529612	6.62	94.17
7	0.312203	3.90	98.07
8	0.154219	1.93	100

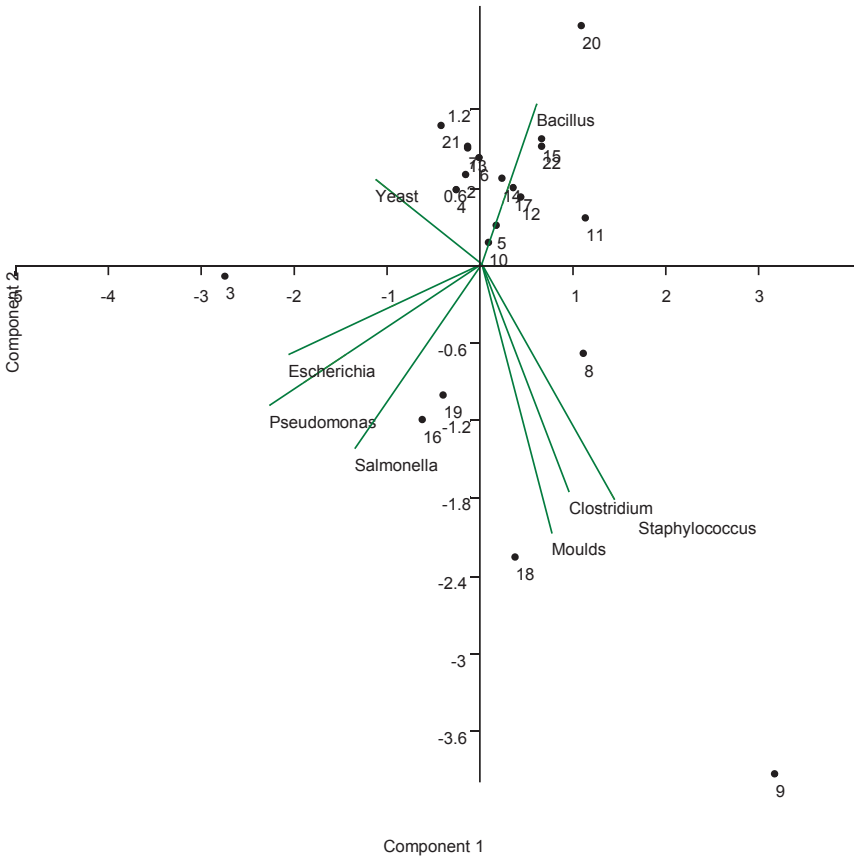
The principal components (PCs) are that eigenvalues resulted higher than 1. These PCs are adequate for the description of the variance. PC1 (26.63%), PC2 (22.85%), and PC3 (17.16%) explain 66.64% of the total variance. The tested variables can be grouped into three new variables (PCs).

According to *Table 4*, the most important variables for the first PC are *Pseudomonas* sp. and *Escherichia coli*, for the second PC, *Staphylococcus aureus* and moulds, and for the third PC *Bacillus cereus* and yeast.

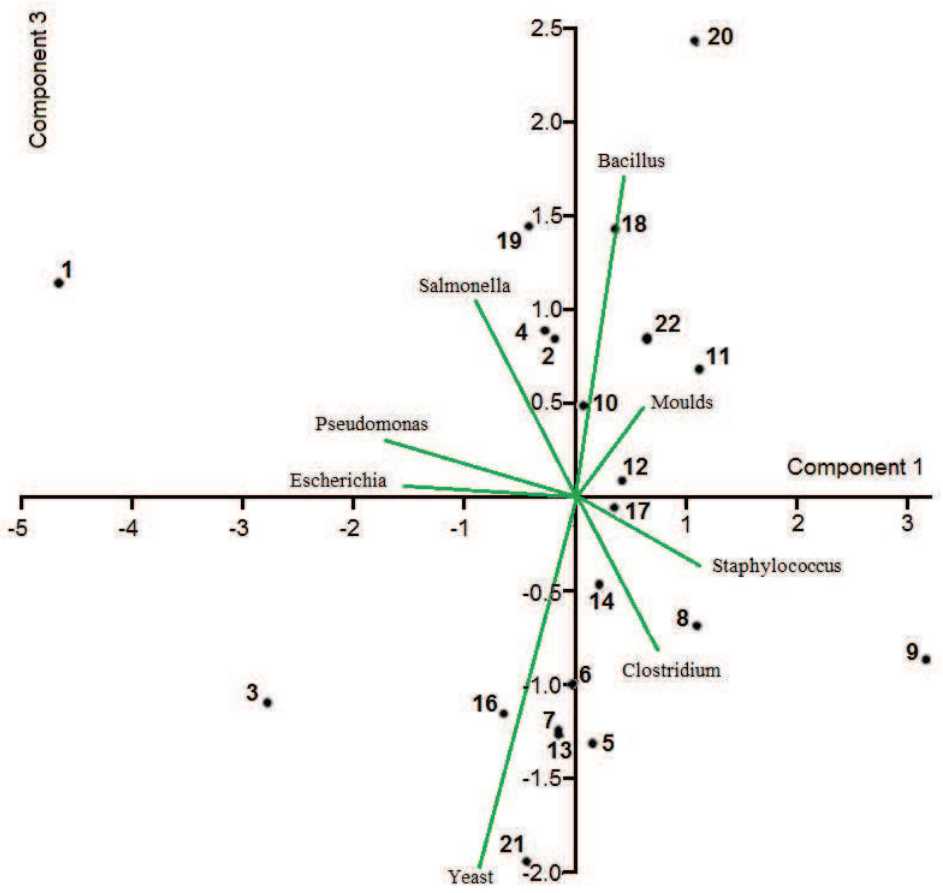
Score and loading plots are shown in *Fig. 1*. Samples coded with 1, 3, 16 (SSFC1, FC1, CC1), and 19 (CC4) are placed on the lower left quadrant (III) of the PC1-PC2 plot. These dairy products are sold by weight and the microbiological quality of these products is not satisfactory.

Table 4: Principal component coefficients (loadings)

	1st loading	2nd loading	3rd loading
Components	**PC1**	**PC2**	**PC 3**
Pseudomonas sp.	**-0.5609**	-0.2703	0.09455
Escherichia coli	**-0.5091**	-0.1721	0.01493
Salmonella	-0.3331	-0.35	0.3814
Staphylococcus aureus	0.3547	**-0.4502**	-0.1228
Bacillus cereus	0.1493	0.3077	**0.5661**
Clostridium	0.2322	-0.433	-0.2676
Yeast	-0.2802	0.164	**-0.6442**
Moulds	0.1863	**-0.5128**	0.1524

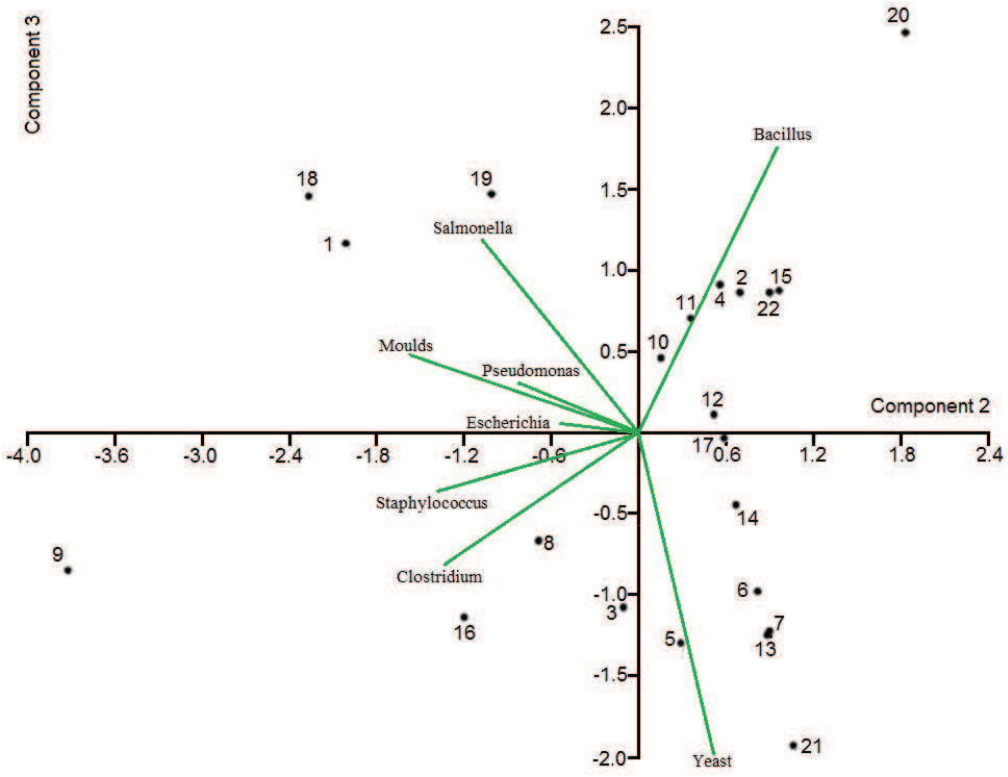


(a)



(b)

The presence of the *E. coli* and *Pseudomonas* in the first three products can originate from milk, from unhygienic conditions during manufacturing. Also, it can be influenced by household practices (*Agarwal et al.*, 2012). Samples coded with 4, 11, 10, 15 (FC2, PC5, PC4, ShC), and 22 (SSFC4) are placed on the upper right quadrant (I) of the PC3-PC2 plot. In four products, except for 10 (PC4), the *Bacillus cereus* count is high. The samples coded with 21, 13, 6, 14 (SSFC3, MO, F, SC), and 17 (CC2) are placed on the lower right quadrant (II) of the PC3-PC2. These products resulted in high yeast counts.



(c)

Figure 1: Score and loading plots of the PCs – (a) PC1-PC2, (b) PC1-PC3, (c) PC2-PC3 – obtained by the principal component analysis (PCA) of microbiological profiles of different dairy products

4 Conclusions

The nutrient content of dairy products favours the growth and development of pathogenic and spoilage microorganisms. Based on our results, the dairy products' microbiological quality varies. We may claim that the majority of the detected microorganisms is due to incorrect food storage, handling, or distribution practices. For maintaining the regulations, the control and prevention of undesirable microbes is also needed after manufacturing, during distribution and storage.

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Antimicrobial resistance of bacterial isolates from different dairy products and their emergence in the food chain

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Abstract. The antibiotic resistance of foodborne pathogens represents a healthcare concern globally. This phenomenon has an increasing impact on medicine and economy. A total of 26 spoilage and pathogenic bacterial isolates originating from different dairy products have been screened against eight different antibiotics. Based on the type of the selective agar medium used for their isolation, the isolates were: five staphylococci isolates, six *Vibrio* isolates, two *Pseudomonas* sp. isolates, three *Salmonella* isolates, five *E. coli* isolates, and five coliform isolates. The overall resistance to the tested antimicrobials of the bacterial isolates was 31.73%, the majority being susceptible. Based on the results, there are isolates with multiple antibiotic patterns that can be possible risk factors and may call for preventive measures.

Keywords and phrases: antibiotic resistance, bacterial isolates, Multiple Antibiotic Resistance

1 Introduction

Food may harbour antibiotic-resistant bacteria. The source of these microorganisms is the soil, the environment, or it can originate from humans or animals. The prevalence of these microbes can also result from cross-contamination. Meats are contaminated during slaughter or plants during irrigation, while processed food during manufacture (*Verraes et al.*, 2013).

The antibiotic resistance of foodborne pathogens entering the food chain has a growing impact on human health and the economy, too. The adequate use of antibiotics in agriculture, the spread of bacteria and their genetic determinants, and also the possible infection capacity result in the appearance of these bacteria in the food chain (*Founou et al.*, 2016).

The European Union has introduced different directives, requirements, and calls for monitoring the antibiotic resistance of bacterial species with public health concern (*EFSA*, 2018).

According to *EFSA*, 2018, antibiotic resistance is defined as "the ability of the bacteria to become increasingly resistant to an antimicrobial to which they have been previously susceptible". Natural selection or genetic mutation can lead to this phenomenon. The routine use of medicines increases the survival of bacteria in antibiotic environments acquiring antibiotic resistance genes (*Cole & Singh*, 2017).

Watkins and *Bonomo* (2016) reported different factors that contribute to the development of antibiotic resistance such as the number of bacteria in hospitals and their transfer, unsatisfactory disinfection, increase of high-risk patient populations, tourism, unsafe water, overdoses of antibiotics, inadequate diagnosis and the treatment with antibiotics, as well as lack of different authorized vaccines.

Bacteria with different biochemical mechanisms inactivate the antibiotic compound. These strategies are the alteration of antibiotic-bound protein, enzymatic inactivation of the antibiotic, prevention of the access into the cell, or elimination from the cell with (ATP)-powered drug efflux pump (*Cole & Singh*, 2017; *O'Bryan et al.*, 2018). *Friedman* (2015) reported foodborne antibiotic-resistant pathogenic bacteria such as *Campylobacter jejuni*, *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Vibrio cholerae*, or *Vibrio parahemolyticus*. *Friedman* (2015) also referred to the occurrence of antibiotic-resistant bacteria in dairy products, mainly from raw milk.

Phenotypically, the antibiotic resistance of a bacterial strain can be measured by the determination of inhibition zones or minimal inhibitory concen-

tration (MIC) for antibiotics (*Butaye et al.*, 2015). Even though antibiotic resistance is a health concern, there are few studies on antibiotic resistance in food-related bacteria in our region. Our study aimed to determine the antibiotic resistance of different spoilage and pathogenic bacterial isolates originating from different dairy products.

2 Materials and methods

The antibiotic resistance of the bacterial isolates originating from different dairy products has been determined using the disk diffusion method. The tested isolates were recovered from different dairy samples on selective mediums: Pseudomonas Selective Agar, ChromoBio^{R} Coliform, TBX Chromo-Agar, Brilliance^{TM} Salmonella Agar Base, Mannitol Salt Agar, ChromoBio^{R} Cereus Base, and Vibrio Selective Agar TCBS Agar. The presumptive isolates were tested from each selective medium.

Isolates have been inoculated into a physiological solution, and a suspension of 1 OD has been prepared. 0.1 ml of bacterial suspensions has been spread on Nutrient agar (Sigma-Aldrich) plates. From the eight different antimicrobial sensitivity discs (Oxoid Ltd. are used for antibiotic sensitivity testing), two and four discs (in two replicates) have been placed on Nutrient agar plate and have been incubated at 37 °C for 24 h. The applied antibiotics disks (belong to different classes of antibiotics) were: Ampicillin 10µg (Am), Amikacin 30µg (A), Tobramycin 10µg (T), Streptomycin 10µg (Str), Cefoperazone 75µg (Cef), Ofloxacin 5µg (O), Levofloxacin 1µg (L), and Ceftriaxone 30µg (C). After incubation, the diameters of the inhibition zones have been measured in millimetres. The results of the inhibition zones have been interpreted as sensitive, intermediate, or resistant, according to a Zone-Size Interpretation Chart (http://www.oxoid.com/UK/blue/techsupport/its.asp?itsp=faq&faq=tsfaq020&cat=antibiotic+sensitivity+testing&lang=EN&c=UK, HiMediaCatalogue 2017–18).

The Multiple Antibiotic Resistance (MAR) index has been calculated with the formula x/y, where x stands for the number of antibiotics to which the isolate exhibited resistance, and y is the number of total antibiotics for which the isolate was tested (*Adenaike et al.*, 2016).

3 Results and discussion

A total of 26 bacterial isolates originating from different dairy products have been tested for the resistance to eight antibiotics (*Table 1*).

Table 1: Antibiotic susceptibility pattern of the bacterial isolates originating from dairy products

Bacterial isolate		Ampicillin	Amikacin	Tobramycin	Streptomycin	Cefoperazone	Ofloxacin	Levofloxacin	Ceftriaxone
Staphylococcus isolates	S1	R	S	S	S	S	S	S	S
	S4	S	S	R	R	S	R	R	S
	S6	S	S	S	S	S	S	S	S
	S11	R	S	S	S	I	S	S	I
	MOMAN	S	S	S	S	S	S	S	S
Vibrio sp.	V2	S	R	R	R	R	R	R	R
	V8	S	R	R	R	I	S	R	I
	V13	S	R	I	R	S	S	I	S
	V17	S	R	R	R	S	I	R	S
	V18	S	R	R	R	I	S	I	I
	V15	S	R	R	R	R	R	R	I
Pseudomonas sp.	P5	R	S	S	R	S	R	R	S
	P14	R	I	R	S	S	S	S	S
Salmonella sp.	Sa7	R	S	S	S	S	R	R	S
	OSXLD	S	R	I	S	R	R	R	R
	Sa10	R	S	S	S	S	S	S	S
E. coli	E.C3	R	R	R	S	S	R	R	S
	EC12	R	R	R	R	R	R	R	R
	PTKOK	S	S	R	S	S	S	S	I
	EC16	S	R	R	R	R	R	R	R
	PTBX	S	S	S	R	S	S	S	S
Coliforms	MAKO	S	S	I	S	S	S	S	S
	TKO	S	S	R	R	S	S	S	I
	OSKO	S	S	R	R	S	S	S	S
	MOKO1	R	S	R	I	S	S	S	R
	MOKO2	S	S	S	S	S	S	S	S

R = Resistant, S = Susceptible, I = Intermediate

Bacterial isolates have been grouped into six groups based on the type of the selective agar medium used for their isolation. Five were staphylococci isolates (S1, S4, S6, S11, MOMAN), six *Vibrio* isolates (V2, V8, V13, V15, V17, V18), two *Pseudomonas* sp. isolates (P14, P5), three *Salmonella* isolates (Sa10, Sa7, OSXLD), five *E. coli* isolates (E.C3, EC12, EC16, PTKOK, PTBX), and five coliform isolates (MAKO, TKO, OSKO, MOKO1, MOKO2). The overall resistance to the tested antimicrobials of the bacterial isolates was 31.73% (66/208).

From the 26 bacterial isolates, 34.62% have shown resistance to Ampicillin, whereas most of the tested isolates exhibited susceptibility (*Table 2*).

Table 2: Percentage of antibiotic resistance, intermediate and susceptible isolates

Antibiotics	Resistant isolates (%)	Intermediate isolates (%)	Sensitive isolates (%)
Ampicillin	34.62	0	65.38
Amikacin	38.46	3.85	57.69
Tobramycin	53.85	11.54	34.62
Streptomycin	50.00	3.85	46.15
Cefoperazone	19.23	11.54	69.23
Ofloxacin	34.62	3.85	61.54
Levofloxacin	42.30	7.69	50
Ceftriaxone	19.23	23.08	57.69

The following isolates have been resistant to Ampicillin: two of the staphylococci isolates (S1, S11), two of the *Salmonella* isolates (Sa7, Sa10), two of the *E. coli* isolates (EC3, EC12), one of the coliforms (MOKO1), and the *Pseudomonas* isolates (P5, P14). All the tested *Vibrio* isolates resulted as sensitive to this antimicrobial. None of the isolates has shown intermediate susceptibility.

The majority of the studied bacterial isolates showed susceptibility to Amikacin (57.69%). None of the *Staphylococcus*, coliform, and *Pseudomonas* isolates exhibited resistance to this antimicrobial compound. One *Pseudomonas* isolate (P14) represents an exception as it has been detected to show an intermediate resistance. The other isolates have been sensitive to this antibiotic. *Vibrio* isolates showed resistance to Amikacin. Only one *Salmonella* (OS-

XLD) and three *E. coli* isolates (E.C3, EC12, EC16) have been resistant to this antibiotic.

14 out of 26 bacterial isolates exhibited resistance to Tobramycin. This phenomenon has been detected in the case of a *Staphylococcus* isolate (S4), five *Vibrio* isolates, one *Pseudomonas* isolate (P14), four *E. coli* isolates (E.C3, EC12, PTKOK, EC16), and three coliforms (TKO, OSKO, MOKO1). None of the *Salmonella* isolates has been resistant to this studied antimicrobial. Intermediate resistance has been detected in the case of three isolates.

Resistance to Streptomycin has been exhibited by half of the studied isolates. One *Staphylococcus* (S4) and one *Pseudomonas* isolate (P5), three *E. coli* (EC12, EC16, PTBX), and two coliforms (TKO, OSKO) were resistant to this antimicrobial agent. All the *Vibrio* isolates exhibited resistance. Intermediate susceptibility has been detected in the case of one bacterial isolate (MOKO1) obtained from selective agar medium for coliforms.

The highest level of susceptibility has been detected in the case of Cefoperazone. The majority of the tested isolates, 69.23%, exhibited sensitivity to this antibiotic. None of the *Staphylococcus* sp., *Pseudomonas*, and coliform isolates showed resistance against this agent. In the case of one *Staphylococcus* (S11) and two *Vibrio* isolates (V8, V18), intermediate resistance was detected, while the other isolates were susceptible to this antibiotic.

34.62% of the studied isolates obtained on selective agar media from different dairy products showed resistance to Ofloxacin. None of the coliforms showed resistance against it; they all have been susceptible. One isolate from the staphylococci group (S4), two *Vibrio* isolates (V2, V15), one *Pseudomonas* isolate (P5), two *Salmonella* isolates (Sa7, OSXLD), and three *E. coli* isolates (E.C3, EC12, EC16) showed resistance. One *Vibrio* isolate (V17) showed intermediate susceptibility to this antibiotics.

Around half of the studied isolates exhibited susceptibility to Levofloxacin. Resistance to this antibiotic compound was detected at a level of 42.30%. From the staphylococci, one isolate (S4), four of *Vibrio* isolates (V2, V8, V17, V15), one *Pseudomonas* isolate (P5), two *Salmonella* isolates (Sa7, OSXLD), and three from *E. coli* isolates (E.C3, EC12, EC16) showed resistance. None of the coliforms were resistant to Levofloxacin.

19.23% of the tested bacterial isolates showed resistance to Ceftriaxone. These isolates were: one *Vibrio* isolate (V2), one *Salmonella* isolate (OSXLD), two *E. coli* isolates (EC12, EC16), and one coliform isolate (MOKO1). None of the *Staphylococcus* and *Pseudomonas* isolates showed resistance. The lowest intermediate susceptibility has been detected in this case. Six bacterial isolates showed intermediate susceptibility.

Antibiotics with different mechanisms inhibit bacterial growth. These compounds prevent the synthesis of proteins involved in metabolism, DNA or RNA synthesis or have a negative impact on membrane permeability. Some bacteria with diverse strategies can avoid these effects. The dissemination of antimicrobial resistance in *Salmonella* species (and also in others) is due to horizontal gene transfer or the clonal spread of isolates. A high level of antibiotic resistance to fluoroquinolones (like Oxofloxacin, Levofloxacin) was detected in these bacterial species (*Ricke & Calo*, 2015). Similarly, the two *Salmonella* isolates exhibited resistance to these types of antibiotics. Resistance to fluoroquinolones can be resulted from target gene mutation or active efflux mechanisms, while the resistance to beta-lactams is caused by the secretion of beta-lactamases. In some reports, multidrug resistance in *Salmonella* species is determined on chromosome or plasmids (*Ricke & Calo*, 2015). It has been shown that serovars of this bacterium with multidrug resistance represent a public health concern. They appear not only in clinical samples, but they have been isolated from different foods (*Ricke & Calo*, 2015).

Vibrios appear in aquatic environments and can cause different infections. Species of this bacterium, such as *V. vulnificus*, have been isolated from different seafoods, for example, shrimp or oysters. *Vibrio casei* has been isolated and described from soft smear cheese (*Baker-Austin*, 2015; *Bleicher et al.*, 2010). It has been shown that tested *V. vulnificus* isolates exhibited different resistance to Amikacin, Ampicillin, and others; however, most of the isolates were susceptible (*Baker-Austin*, 2015). In contrast, in our results, most isolates exhibited resistance to the studied antibiotics. Our results are in accordance with researchers who detected multidrug resistance in these species (*Baker-Austin*, 2015; *Bleicher et al.*, 2010). Also, *V. parahaemolyticus* originating from cockles and seafood products carries varying antibiotic resistance (*Baker-Austin*, 2015). Antibiotic resistance of *V. cholerae* is encoded on plasmids. This strain with antibiotic resistance isolated from milk originated from the lack of good raw-milk-processing practices (*Sharma & Malik*, 2012).

The *Pseudomonas* group is involved in the spoilage of different dairy products. The species of this group usually exhibit resistance to β-lactam antibiotics (*Arslan et al.*, 2011). Our results are in accordance with this in the case of Ampicillin. In the case of Ceftriaxone, the isolates showed susceptibility (*Arslan et al.*, 2011). The *Pseudomonas* isolates showed intermediate susceptibility or strong susceptibility to Amikacin. That was like the results presented by *Arslan et al.* (2011) involving *Pseudomonas* isolates originating from homemade cheese. The mechanisms of antibiotic resistance of *P. aeruginosa* consist of outer membrane permeability, several efflux pumps, and

enzymes that inactivate the antibiotic. Acquired resistance is due to horizontal gene transfer (*Meletis & Bagkeri*, 2013). It has been shown that resistance to fluoroquinolones (Ofloxacin, Levofloxacin) is due to target-site alteration and potential efflux pump (*Meletis &Bagkeri*, 2013).

Food-related coliforms are hygienic and sanitary quality indicators. Most coliform isolates showed susceptibility to the tested antibiotics, what is in contrast with the results of *Zanella et al.* (2010), where raw milk harboured antibiotic-resistant coliforms.

Staphylococcus aureus is a commensal bacterium of the skin and nasal microbiota. It has been isolated from different types of food. It appears due to poor personal hygiene (*Castro et al.*, 2018). Several researchers from all over the world have reported the presence of multiresistant *S. aureus* in raw milk and dairy products (*Normanno et al.*, 2007; *Sasidharan et al.*, 2011; *Jamali et al.*, 2015; *Al-Ashmawy et al.*, 2016). Different mechanisms contribute to the resistance to β-lactams in *S. aureus* such as the production of penicillin-binding proteins. One of these proteins is coded by the *mecA* gene in Methicillin-Resistant Strains (MRS). This gene is spread on a mobile genetic element, designated as *SCCmec*. Diverse types of this genetic element are known in *S. aureus*. Some strains also produce β-lactamase. Other genes that are involved in methicillin and other β-lactam resistance in staphylococci have been described such as *mecB* and *mecC*. Nevertheless, penicillin susceptibility has also been observed in MRS (*Castro et al.*, 2018).

When a microorganism is resistant to more than two antibiotics, we can speak about Multiple Antibiotic Resistance (MAR). It can occur in cases when different antibiotic resistance determinants are spread on the same mobile genetic elements or due to overexpression of genes related to multidrug efflux pumps (*Blanco et al.*, 2016; *Nikaido*, 2009). *Figure 1* shows the Multiple Antibiotic Resistance (MAR) index of the studied bacterial isolates originating from dairy products. The calculation of Multiple Antibiotic Resistance index is a guide for the assessment of health risk (*Davis & Brown*, 2016). The *Staphylococcus* isolates exhibited two antibiotic resistance patterns (A, TStrOL) with MAR indices 0.5 and 0.125. The *Vibrio* isolates MAR index ranged between 0.25 and 0.875. In this case, five antibiotic resistance patterns have been detected (AmTStrCefOLC, AmTStrL, AmStr, AmTStr, AmTTStrCefOL). In the case of the two *Pseudomonas* isolates, the MAR index reached 0.25 and 0.5; the antibiotic resistance pattern of these isolates was AStrOL and AT. The MAR index of *Salmonella* isolates resulted in 0.125–0.5. These isolates exhibited three resistance patterns (AOL, AmCefOLC, A). Bacterial isolates obtained on selective agar medium for *E. coli* exhibited four antibiotic resis-

tance patterns (AAmTOL, AAmTStrCefOLC, T, AmTStrCefOLC) with the MAR index ranging between 0.125 and 1. The coliform isolates' MAR index ranged between 0 and 0.375. These isolates exhibited two antibiotic resistance patterns (TStr, ATC).

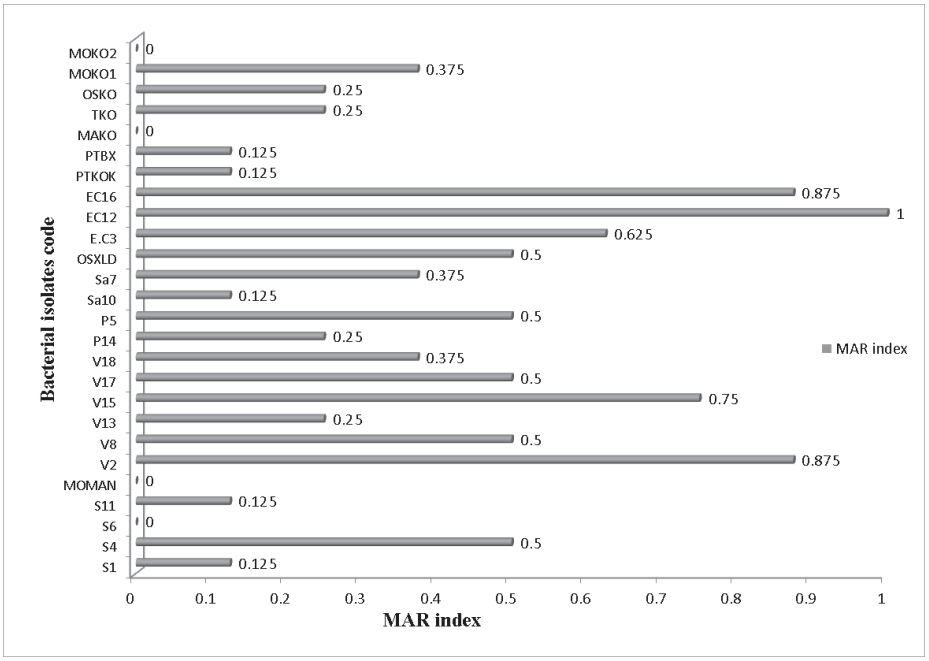

Figure 1: Multiple Antibiotic Resistance (MAR) index values of bacterial isolates

If the value of the MAR index is equal to or higher than 0.2, contamination with high risk is indicated. These values also reveal the frequent use of antibiotics in agriculture, livestock farming. Seven isolates possess values in the interval 0.2–0.4. If the value of the MAR index is equal to or higher than 0.4, faecal sources are indicated (*Adenaike et al.*, 2016). In dairy farming, antibiotics were used for prohylaxis or growth promotion (*Sharma et al.*, 2017). Ten of the studied isolates can be categorized into this group. Bacteria with a value below 0.2 can originate from an environment with less antibiotic usage. Five of the studied isolates belonged to this category. One of the *E. coli* isolates reached the value 1, which means a multidrug-resistant strain. This isolate is originated from an environment where antibiotics were often used.

4 Conclusions

According to the results of the present study, the tested bacterial isolates exhibited different resistance to the tested antibiotics. It can be summarized that the majority of the strains appeared to be susceptible to the tested agents. Also based on the Multiple Antibiotic Resistance index, there are bacterial isolates that are related to a conspicuous antibiotic use for human or animal medicine. This is an important food safety issue that calls for surveillance. Potentially, these strains can be carriers and transmission tools of multidrug resistance.

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Methods and procedures for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods

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Abstract. We have developed a new procedure for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods, through which soy trypsin inhibitor activity decreases to the tenth or twentieth part of the original value. We determined the optimal concentration of the applied chemicals (hydrogen-peroxide, ammonium-hydroxide) as well as the optimal temperature and duration of the treatment. The chemical procedure combined with heat treatment results in lower energy consumption as compared to the original heat treatment methods.

1 Introduction

Soy has a high protein and energy content, and it is a widely consumed food ingredient due to its low price range and especially its high lysine content. It

Keywords and phrases: trypsin inhibitor, inactivation by heat treatment, chemical methods, hydrogen-peroxide, ammonium-hydroxide

has recently become highly popular as, besides its excellent content values, it has been used efficiently for the treatment of various chronic diseases and different types of cancer (*Vagadia et al.*, 2017). Unfortunately however, soy also contains a great number of substances (protease inhibitors, phytic acid, isoflavones) that limit the use of soy owing to their adverse physiological effects. Inactivation of trypsin inhibitors, harmful enzymes, and bioactive components, increasing protein quality, improving texture, colour, and smell, enhancing functionality and digestibility are all decisive factors of the production of soy products (*Csapó* & *Csapóné*, 2006).

Trypsin and chymotrypsin inhibitors form inactive complexes with trypsin and chymotrypsin in the small intestine, thus inhibiting the hydrolysis and bioavailability of dietary proteins. Their presence stimulates an increased pancreatic activity, which may eventually cause pancreatic enlargement followed by pancreatitis. These inhibitors can be inactivated by heat treatment. Their thermal stability depends on molecular size and the number of disulphide bridges. One of the most stable trypsin inhibitors is the Bowman–Birk inhibitor found in soybean, with a molecular mass of 7,900 Da, consisting of 71 amino acids and containing 7 disulphide bridges. Dry-heated at 105 °C in aqueous solution, it can retain most of its activity even after ten minutes of boiling (*Csapó* & *Csapóné*, 2006). The Kunitz-type trypsin inhibitor, also isolated from soy, is markedly more thermolabile; it is irreversibly denatured at 90 °C, its molecular mass is 21,500 Da, it is made up of 181 amino acids, and it contains two disulphide bonds (*Kunitz*, 1947). Nevertheless, in acid environment, it is resistant to digestive enzymes, which is why it does not dissolve either in the stomach or later in the small intestine, following the enzymatic digestion (*Astwood et al.*, 1996).

The specificity of protease inhibitors is extremely varied – it can comprise different proteases and isodynamic enzymes as well. On the other hand, we have knowledge of inhibitors that neutralize two types of enzymes, e.g. trypsin and chymotrypsin – such is the Bowman–Birk inhibitor. Protease inhibitors are formed in floras first of all, but there are also a few animal and microbial variants. Among them, the best known is the ovomucoid and the ovoinhibitor found in egg white, which retain some of their activity during ordinary cooking but do not inhibit the human trypsin. Recognizing the adverse health effects of inhibitors made it necessary to limit the food inhibitor content. This has been made especially necessary by the widespread use of soy products, crude soybeans being one of the most relevant products containing antinutrients. In our country, only those ingredients can be used for direct human consumption whose trypsin inhibitor activity is under the value of 10 per mg (*Csapó* &

Csapóné, 2006).

Heat-sensitive antinutrient substances include trypsin and chymotrypsin inhibitors, which are proteins in terms of their composition and are primarily formed in legume seeds as well as in cereal grains and potatoes. Their molecular mass is 6–64 kDa. A single plant may contain several types of antinutrient substances that differ in molecular mass, isoelectric point, and thermal stability.

Serine protease inhibitors occur naturally in large numbers and diverse forms, and they inhibit protein catabolism by the inactivation of serine protease enzymes (*Silverman et al.*, 2001; *Rawlings et al.*, 2004). In the largest quantities, they can be found in cattle pancreas, egg, lima bean, and soy (*Zhou et al.*, 1989). Every inhibitor is a substrate analogue, which, linked to the enzyme, forms an inactive complex with the latter, inhibiting protein hydrolysis to amino acids.

Inhibitors may take on a great number of diverse, varying roles in nature. They inhibit the proteolytic activity of serine proteases once these have fulfilled their role in various parts of the organism, while in the seeds of different legumes and plants they inhibit the protein digestion of the insects consuming the seeds. So, they actually constitute an integral part of the plant's defence mechanism against the animal pests consuming them. In the case of consuming improperly heat-treated or crude soybean, the trypsin inhibitor found in high concentrations in soybean contributes to increased pancreatic activity, while, if consumed in large quantities over a long period of time, it may cause pancreatitis and reduce animals' feed intake, but the Bowman–Birk trypsin inhibitor can also play a role in preventing cellular proliferation and combating cancer (*Kennedy*, 1998).

The trypsin inhibitor of cattle pancreas is made up of 158 amino acids and contains three disulphide bridges. Both in cattle and in humans, it inhibits trypsin, chymotrypsin, and plasmin activity, but it does not inhibit elastase activity in pigs (*Huber et al.*, 1971). The glycoprotein of hen's ovomucoid egg white is made up of 186 amino acids (*Stadelman & Owen*, 1995), and it functions as a trypsin inhibitor. It contains three tandem domains, each of which having three disulphide bridges (*Salahuddin et al.*, 1985; *Cooke & Sampson*, 1997). The ovoinhibitor in the egg white has five binding sites, inhibiting cattle trypsin, chymotrypsin, and pig elastase activity (*Begum et al.*, 2003). Ovomucoid and ovoinhibitor make up 11% and 15% of the egg white respectively (*Gertler & Ben-Valid*, 1980; *Kinoshita et al.*, 2004).

Kunitz- and Bowman–Birk-type protease inhibitors (BBI) are present in the largest quantities in soybean. The BBI molecular mass is 87 kDa, which,

having independent binding sites, is highly bound to trypsin and chymotrypsin alike. The Kunitz inhibitor's molecular mass is 20.1 kDa, has a single binding site, is highly bound to trypsin, but it only slightly inhibits chymotrypsin. The Kunitz inhibitor was first prepared in pure form by Kunitz in 1945 – it is a single-chain protein made up of 181 amino acids, having two disulphide bridges (*Steiner*, 1965; *Koide & Ikenaka*, 1973; *Kim et al.*, 1985), and inhibiting mainly trypsin and slightly chymotrypsin and plasmin (*Naninga & Guest*, 1964; *De Vonis Bidlingmeyer et al.*, 1972). By a trypsin-like mechanism, it inhibits other enzymes as well, but it has no effect whatsoever on metallic, acidic, and thiol proteases.

Trypsin inhibitor and the enzyme form a stoichiometric complex, which has the ability to split the inhibitor between the arginine and isoleucine. Enzyme inhibition is reversible and pH-dependent – the dissociation of the enzyme-inhibitor complex releases the inhibitor in its initial form (*Ozawa & Laskowski*, 1966; *Finkenstadt & Laskowski*, 1967).

The trypsin-chymotrypsin Bauman–Birk inhibitor is a monomer protein with 71 amino acids and containing seven disulphide bridges (*Kay*, 1979). It has a separate binding site for trypsin and another one for chymotrypsin. It has a one to one binding with protein, that is, one molecule of inhibitor inhibits one molecule of enzyme. This inhibition is not competitive, the inhibitor being able to form tertiary structure with both enzymes (*Birk*, 1985). One mg of inhibitor is able to inhibit 0.5 mg of trypsin and 1 mg of chymotrypsin.

DiPietro and *Liener* (1989) carried out a heat treatment of the Kunitz inhibitor, the Bowman–Birk inhibitor, and crude soybean extract at 100 °C, and they established that the soybean extract rapidly lost its enzyme inhibitor activity, the Kunitz inhibitor added to the soybean extract could only become inactive with difficulty, while the Bowman–Birk inhibitor remained mostly active in the soybean extract even after a rather long period of heat treatment. Taking a look at the clear solution of the inhibitors, a rather rapid inactivation at a temperature ranging between 75 °C and 90 °C was observed with both inhibitors, which contradicts the results obtained in relation to the soybean extract. According to Kassell's research (1970), besides soy, all the other legumes contain trypsin in significant quantities, but the soy inhibitor was found to contain by far the largest quantities.

High temperatures and steam injection technique were also applied by many in an attempt to inactivate the trypsin inhibitor. Blanching was adopted as well, while, adopting preliminary heat treatment, they also achieved successes at temperatures below 100 °C (*Yuan & Chang*, 2010), and the high temperature–short time period (137 °C, 70 seconds) technique paid off, too

(*Rouhana et al.*, 1996). *Kwok et al.* (1993) treated soymilks of various pH (2.0, 6.5, 7.5) and established that trypsin inhibitor is less stable at a higher pH level as compared to a lower one. At higher temperatures (143 °C, 154 °C), pH level had much less effect on activity.

Van der Ven et al. (2005) studied trypsin inhibitor activity in minced meat, where 30% of the meat was replaced by soy preparations (concentrate, isolate, textured flour). Heat treatment carried out at 70, 80, 90, and 100 °C for 15 minutes reduced trypsin inhibitor content to such a degree that caused no more indigestion in consumers. Under similar conditions, the components of the developed meat-structure model provided better protection for the trypsin inhibitor than during heat treatment in aqueous solution.

Johnson et al. (1980) used direct steam injection to produce soymilk out of soybean extract. Carrying out their experiments within the temperature range of 99–154 °C, they found that at a pH value of 6.7 with every 11 °C temperature rise trypsin inhibitor activity was halved. At this pH level, at 154 °C, and in 40 seconds, the same amount of decrease in activity was obtained as at 99 °C in 60 minutes. At an increasing pH level (pH = 9.5), activity declined at an even faster pace.

There has been a recent spread of modern techniques that do not follow traditional procedures but almost always entail heat treatment. Such is, for instance, the ohmic heating treatment, during which electric current goes through foodstuffs and the generated heat produces its effect (*Wang et al.*, 2007). Dielectric heat treatment is widely adopted in the industry, during which all types of frequencies were used from radio waves (42 MHz) to microwaves (2450 MHz). Microwave treatment following soaking inhibited the trypsin inhibitor with high efficiency, while not causing any significant changes in the composition of the soybean (*Hernandez-Ifante et al.*, 1998). Applying a treatment of 2500 MHz for 30 minutes, *Zhong et al.* (2015) managed to inactivate a significant amount of the trypsin inhibitor. In a layer of 30 cm, the pre-soaked soybean was heated by microwave to 120 °C, which resulted in the inactivation of 93% of the trypsin inhibitor (*Petres et al.*, 1990).

Radio frequency treatment is also a dielectric heat treatment technology, which is a lot less time-consuming than traditional heat treatment, and, combined with high temperatures (80–120 °C), they were able to reduce trypsin activity in a short amount of time (90–120 s) to 7–12% of the original value (*Vearasilp et al.*, 2005). In the course of infrawave treatment, energy is transferred at a low temperature to the treated foodstuff, during which inhibitor activity declines but food components are not damaged. The treatment improves water adsorption, reduces cooking time and the concentration of antin-

utrients (*Kayitesi et al.*, 2013). *Abu-Tarboush* (1998) used gamma radiation to reduce the activity of defatted soy flour inhibitor, during which he was able to reduce the inhibitor content by 34% with a radiation intensity of 10 kGy, while there was a 4% increase regarding the digestibility of the nutrients.

Applying an ultrasonic treatment at 20 kHz for 20 minutes, *Huang et al.* (2008) achieved a 55% decrease of inhibitor activity, a decrease influenced by ultrasound amplitude and treatment duration. They established that inactivation in the inhibitor's construction was caused by the digestion of disulphide bridges and various structural changes. Extrusion is another widely used method in food industry, being able to significantly reduce inhibitor quantity to safe levels while protein quantity suffers no changes at all during the procedure (*Clarke & Wiseman*, 2007). Adopting instantaneous heat treatment at 150 °C, during extrusion, inhibitor activity decreased under 5% of the initial value.

Haddad & Allaf (2007) developed a procedure for reducing inhibitor activity, during which the soaked soybean was heated to appropriate temperature (170 °C), and, alternating pressure values of 8×10^5 and 50×10^2, they were able to neutralize 94% of the inhibitor in one minute and 99% of it in six minutes. *Osela et al.* (1997) used fluidized bed, which is suitable not only for the drying of the crop but also to inactivate the inhibitor. Adopting this procedure, they were able to carry out the entire inactivation process at 140 °C in 10 minutes.

A common trait of the chemical methods used to inactivate the trypsin inhibitor is that they somehow digest the disulphide bridges. The activity of soy protease inhibitor can be reduced with Escherichia Coli or the nicotinamide adenine dinucleotide phosphate (NADP)-thioredoxin system found in plants, which contains NADP, thioredoxin, NADP-thioredoxin reductase, reduced lipoic acid, and dithiothreitol. The system is targeting the Kunitz and the Bowman–Birk inhibitors, whose 45% and 32%, respectively, are dissolved during a 1–2-hour treatment at 30 °C (*Jiao et al.*, 1992).

Sessa et al. (1988) used sodium bisulphite to inactivate the trypsin inhibitor content of flattened and whole soybeans. Following a 2-hour heat treatment at 65 °C in a 0.1 molar solution, they managed to inhibit 94% of the soy's initial trypsin inhibitor content. The excess sodium bisulphite was removed by extraction.

In a model medium, *Sessa et al.* (1990) successfully applied the combination of L-ascorbic acid and copper sulphate to inactivate the Kunitz and the Bowman–Birk trypsin inhibitors. However, when they treated soy flour under similar conditions, most of the inactivating effect did not occur, which

they could attribute to the reactions occurring between the ascorbic acid and other components of the soy. In a model medium, the inactivating effect was accounted for by the pro-oxidant effect of the ascorbic acid and copper ions, which digested the trypsin inhibitor structure. In a model system as well as in a lyophilized, alkaline soymilk extract, *Sessa & Ghantous* (1987) used sodium metabisulphite and glutaraldehyde, separately and in combination, to inactivate the Kunitz trypsin inhibitor of the soy. The glutaraldehyde treatment reduced only 60–75% of the trypsin inhibitor activity, which was not significantly changed even by increasing the temperature from 25 °C to 75 °C. Under similar circumstances, the sodium metabisulphite treatment resulted in 96% loss of trypsin inhibitor activity. The two reagents were more efficient in the inactivation process when used together as compared to their separate use.

Huang et al. (2004) performed an experiment with tea polyphenols and established that these are able to inactivate the Kunitz inhibitor at 30 °C in 30 minutes. *Lei et al.* (1981) managed to accelerate trypsin inhibitor inactivation through heat treatment by adding cysteine. During a heat treatment at 80 °C for 10 minutes at a pH level of 9.0, they used 2.5 mM of cysteine to inhibit 90% of the trypsin inhibitor activity. This significant decrease attained at a relatively low temperature during the treatment can be explained by the digestion of disulphide bridges. A thiol–disulphide exchange takes place between the inhibitor protein and the free sulfhydryl groups of the cysteine, in which the inhibitor is inactivated.

Taking a look at the changes in the amino acid composition of complete soybean according to crude protein content, *Csapó & Henics* (1990) established that the increasing protein content is accompanied by a decrease of the protein's biological value, that is, there is no change in the concentration of the essential amino acids, whereas that of the non-essential amino acids increases. Following these observations, *Varga-Visi et al.* (2005, 2006, 2009a, 2009b, 2009c) investigated the effect of the various treatments on the antinutrient substances of the soy as well as the degree of racemization taking place during the time of the heat treatment. They concluded that during optimal heat treatment, when trypsin inhibitor content falls below 10% of its initial value, amino acid racemization is not significant and no relevant changes occur in the soy protein composition.

After an analysis of literature data, the structure and chemical properties of trypsin inhibitors can be summarized as follows. The molecular mass of the Kunitz-type trypsin inhibitor derived from soybean is 21,000 Da, and its isoelectric point is pH = 4.5; regarding the structural characteristics: it includes the two disulphide bridges and does not contain a free sulfhydryl group.

Its N-terminal amino acid is aspartic acid, while its C-terminal amino acid is leucine. Trypsin inhibitor derived from 1 mg of soybean inhibits 1.5 mg of trypsin stoichiometrically, reversibly, and in a pH-dependent reaction, forming a trypsin–inhibitor complex. During this reaction, it blocks the enzymatic activity of the trypsin, and, besides trypsin, it also inhibits chymotrypsin and other proteolytic enzymes.

The molecular mass of the Bowman–Birk trypsin inhibitor found in soy is 7,900 Da, it is made up of 71 amino acids, and it contains seven disulphide bridges. After dry-heating at 105 °C in an aqueous solution and boiling for ten minutes, it is still able to retain much of its activity. It is highly bound both to trypsin and chymotrypsin with independent binding sites. It has a one to one binding with protein, that is, one molecule of inhibitor inhibits one molecule of enzyme. This inhibition is not competitive, the inhibitor being able to form tertiary structure with both enzymes. One mg of inhibitor is able to inhibit 0.5 mg of trypsin and 1 mg of chymotrypsin.

The trypsin inhibitor derived from soybean is thermolabile, sensitive to high pH levels and protein precipitation reagents. However, it dissolves faster under stable acidic conditions, in an alkaline environment. Regarding the structure and characteristics of the trypsin inhibitor and in terms of inactivation, the most important features are as follows:

- it contains disulphide bridges,
- the active structure dissolves rapidly in alkaline environment, and
- it is sensitive to high pH levels.

The method adopted by *Lei et al.* (1981) appears to be the most promising one, the researchers being able to accelerate trypsin inhibitor inactivation through heat treatment by adding cysteine. This significant decrease attained at a relatively low temperature during the treatment can be explained by the digestion of disulphide bridges. A thiol–disulphide exchange takes place between the inhibitor protein and the free sulfhydryl groups of the cysteine, in which the inhibitor is inactivated.

The rest of the procedures introduce some substance into the system which was not there before and which needs to be removed subsequent to the treatment. Cystine/cysteine amino acids are natural components of soy, wherefore, even if a certain amount of "reagent" is retained, no further problems are caused; moreover, it increases the amino acid content of the soy, already low in sulphur. During our experiments, we were also trying to follow this path,

except that for the splitting of disulphide bridges we did not intend to apply reduction and thiol-disulphide exchange but oxidation.

Our experiments thus aimed at elaborating a method and procedure for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods. In practice, crude soybean can be used only to a very limited degree due to antinutrients, while the widely applied heat treatment is a costly solution. As most of the currently adopted procedures are highly energy-consuming and the quality of the end-product also leaves much room for improvement, we tried to obtain oxidation with hydrogen-peroxide combined with heat treatment to reduce soy trypsin inhibitor activity, expecting significant energy savings. Since literature data suggested higher inhibitor instability in an alkaline environment, we combined hydrogen-peroxide treatment with ammonium hydroxide, expecting a better efficiency.

Based on our pre-trials, it seems certain that chemical treatment (ammonium hydroxide, hydrogen-peroxide) is conducive to significant energy savings, and the biological value of the obtained heat-treated soy protein declines to a lesser degree during the procedure owing to less heat exposure. Pre-trials have been successfully performed, and we are about to report on their results in our article.

2 Methods and materials

Measurement of trypsin inhibitor and urease enzyme activity and of the degree of heat treatment

We have verified the efficiency of inactivation methods in two ways: determining urease enzyme activity and measuring trypsin inhibitor content. We can estimate trypsin inhibitor activity with the much more easily measurable urease enzyme activity as the two enzymes have very similar heat sensitivity, and, having analysed the measurement results of several hundred samples, we found a linear relationship between the degree of trypsin inhibitor and urease enzyme activity.

Determination of the trypsin inhibitor activity of minced and flattened soy products as well as of samples heat-treated with extruder was performed according to the EN ISO 14902 (2002) standard. The determination is based on that the inhibitors are extracted from the substance under examination, followed by the measurement of the degree to which in the solution containing the extract, incubated with trypsin, p-nitroaniline is released from the benzoyl-L-arginine-p-nitroanilide substratum (L-BAPA).

When estimating urease enzyme activity (*Csapó & Csapóné*, 2010a), we added 50 cm^3 of phosphate buffer (pH = 7.5) and 50 cm^3 of buffered urea solution (30 g urea/1,000cm^3 phosphate buffer) to two times 1,000 mg of soybean meal. After mixing, incubation was performed at 35 °C for 30 minutes, and then the pH of the solutions was measured right away. The bigger the difference in pH was between the solution containing the substratum and the one not containing it, the more ammonia was released as an effect of the urease enzyme. Determination of the degree of heat treatment based upon the measured difference in pH level is as follows: crude, not heat-treated: ΔpH = 1.7–2.5; partially heat-treated: ΔpH = 0.2–1.7; highly heat-treated (toasted): ΔpH = 0.0–0.2. In the examination of the degree of heat treatment (*Csapó & Csapóné*, 2010b), we applied the cresol red dye binding test.

Pre-trials

Since protein hydrolysis and the splitting of disulphide bridges are dependent on concentration, temperature, and time, we set up the following experiments. As the trypsin inhibitor is less stable in an alkaline environment and as digestion of disulphide bridges can also take place through oxidation, we started our experiments with these two chemicals, using the hydrogen-peroxide independently and the mixture of hydrogen-peroxide and ammonium-hydroxide. As a first step, we intended to have a look into whether hydrogen-peroxide digests disulphide bridges at all, whether inhibitor activity decreases upon treatment, and whether oxidation performed under alkaline conditions is any step forward compared to simple oxidation. When testing for the chemicals' effects and optimizing the concentration, we made use of 100 g of flattened soybean (dry matter content [hereinafter DM]: 92%) in each of our experiments (*Table 1*).

Optimization of treatment temperature

During the pre-trial phase, it turned out that it is not worth working with ammonium-hydroxide and hydrogen-peroxide concentrations of more than 1.5–3% and that the combination of the two chemicals gives the best result – in what followed, we carried out our experiments with 2.5% ammonium-hydroxide (decimal dilution of the concentrated solution) and 2.5% hydrogen-peroxide. In this experiment, the solution added to the 100 mg of soy contained the ammonium-hydroxide and hydrogen-peroxide as well (*Table 2*).

The treated soy obtained subsequent to pre-trials was immediately cooled down to room temperature, desiccated with the help of a ventilator, and the trypsin inhibitor activity of the samples was determined.

Table 1: Pre-trial set-up

Sample indication	Treatment	Duration (hour)	Temperature (°C)
1.1.	DM + 20 cm^3 25% ammonium-hydroxide	1	100
1.2.	DM + 20 cm^3 25% ammonium-hydroxide + 20 cm^3 25% hydrogen-peroxide	1	100
1.3.	DM + 20 cm^3 distilled water	1	100
1.4.	DM + 20 cm^3 12.5% ammonium-hydroxide	1	100
1.5.	DM + 20 cm^3 12.5% ammonium-hydroxide + 20 cm^3 12.5% hydrogen-peroxide	1	100
1.6.	DM + 20 cm^3 3.0% ammonium-hydroxide	1	100
1.7.	DM + 20 cm^3 3.0% ammonium-hydroxide + 20 cm^3 3.0% hydrogen-peroxide	1	100
1.8.	DM + 20 cm^3 1.5% ammonium-hydroxide	1	100
1.9.	DM + 20 cm^3 1.5% ammonium-hydroxide + 20 cm^3 1.5% hydrogen-peroxide	1	100
1.10.	DM + 20 cm^3 0.2% ammonium-hydroxide	1	100
1.11.	DM + 20 cm^3 0.2% ammonium-hydroxide + 20 cm^3 0.2% hydrogen-peroxide	1	100

Table 2: Heat optimization experiment – set-up

Sample indication	Treatment	Duration (hour)	Temperature (°C)
2.1.	DM + 20 cm^3 distilled water	1	20
2.2.	DM + 20 cm^3 reagent*	1	20
2.3.	DM + 20 cm^3 distilled water	1	40
2.4.	DM + 20 cm^3 reagent*	1	40
2.5.	DM + 20 cm^3 distilled water	1	60
2.6.	DM + 20 cm^3 reagent*	1	60
2.7.	DM + 20 cm^3 distilled water	1	80
2.8.	DM + 20 cm^3 reagent*	1	80
2.9.	DM + 20 cm^3 distilled water	1	100
2.10.	DM + 20 cm^3 reagent*	1	100

* Solution of 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide

Establishment of the optimal temperature was followed by determining the optimal duration of the treatment.

Optimization of treatment duration

As a starting-point of the experiments aimed at time optimization, we opted for 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide and a temperature of 100 °C. We set up the experiment as follows (*Table 3*).

Table 3: Experiment set-up for optimizing the duration of heat treatment

Sample indication	Treatment	Duration (hour)	Temperature (°C)
3.1.	DM + 20 cm^3 distilled water	20	1000
3.2.	DM + 20 cm^3 reagent*	20	100
3.3.	DM + 20 cm^3 distilled water	40	100
3.4.	DM + 20 cm^3 reagent*	40	100
3.5.	DM + 20 cm^3 distilled water	60	100
3.6.	DM + 20 cm^3 reagent*	60	100
3.7.	DM + 20 cm^3 distilled water	120	100
3.8.	DM + 20 cm^3 reagent*	120	100

* Solution of 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide

3 Results and discussion

Due to the structure of the trypsin inhibitor, we attempted to reduce its activity in three ways:

- splitting the disulphide bridges with oxidation,
- splitting the disulphide bridges with reduction,
- hydrolysis of the carrier protein under alkaline conditions, and
- the combination of the above methods.

Considering the examinations prior to our experiments, it has become obvious that reduction is complicated; it is difficult to get rid of the excess reagent and the by-products generated during reduction. Oxidation performed with hydrogen-peroxide does not generate by-products as water produced during oxidation evaporates in the drying process. Ammonium-hydroxide used in the alkaline treatment partly evaporates during the drying process and is partly adsorbed and thus increases the crude protein content on the surface of the

treated soybean; therefore, we went on to opt for the combination of these two chemicals.

Optimization of the chemical concentration

Regarding the optimization of the chemical concentration, the results obtained for urease enzyme activity and trypsin inhibitor quantity are included in *Table 4*. In each of the cases, heat treatment was carried out at 100 °C for 60 minutes.

Table 4: Results of experiments for the optimization of the chemical concentration

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
1.1. DM + 20 cm^3 25% hydrogen-peroxide	0	0
1.2. DM + 20 cm^3 25% ammonium-hydroxide + 20 cm^3 25% hydrogen-peroxide	0	0
1.3.* DM + 20 cm^3 distilled water	1.48	128
1.4. DM + 20 cm^3 12.5% hydrogen-peroxide	0	0
1.5. DM + 20 cm^3 12.5% ammonium-hydroxide + 20 cm^3 12.5% hydrogen-peroxide	0	0
1.6. DM + 20 cm^3 3.0% hydrogen-peroxide	0.22	14.7
1.7. DM + 20 cm^3 3.0% ammonium-hydroxide + 20 cm^3 3.0% hydrogen-peroxide	0.15	5.9
1.8. DM + 20 cm^3 1.5% hydrogen-peroxide	0.44	33.9
1.9. DM + 20 cm^3 1.5% ammonium-hydroxide + 20 cm^3 1.5% hydrogen-peroxide	0.17	12.8
1.10. DM + 20 cm^3 0.2% hydrogen-peroxide	0.51	40.2
1.11. DM + 20 cm^3 0.2% ammonium-hydroxide + 20 cm^3 0.2% hydrogen-peroxide	0.21	15.3

* Control

Both the 25% and the 12.5% concentration of hydrogen-peroxide and combined treatment respectively reduced both urease enzyme and trypsin inhibitor activity to zero as compared to the control, where pH difference was 1.48 and inhibitor activity 128 TIU/mg. When the quantity of hydrogen-peroxide was reduced to 3%, pH difference decreased to 0.22 and TIU to 14.7, which is 12% of the control. Using the same concentration with combined treatment, pH difference dropped to 0.15 and TIU to 5.9, 5% of the initial value. When the concentration of hydrogen-peroxide was reduced to 1.5%, both pH difference

and TIU value increased, which was significantly improved by the combined treatment, where pH difference decreased to 0.17 and TIU value to 12.8, which is exactly 10% of the original value. Chemical concentration being reduced to 0.12%, combined treatment resulted in a pH difference of 0.21 and a TIU value of 15.3.

The obtained results show that heat treatment performed at 100 °C for 60 minutes with a combination of 1.5% hydrogen-peroxide and ammonium-hydroxide reduces TIU value to safe levels, while during control treatment, using water instead of the reagents, much of the trypsin inhibitor activity was retained. Therefore, in our further experiments, also considering a safe inhibitor reduction, we used a 2.5–2.5% chemical concentration.

Temperature optimization

During temperature optimization, the results obtained for urease enzyme activity and trypsin inhibitor content are included in *Table 5*.

Table 5: Experiment results for temperature optimization

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
2.1. control	1.48	135.2
2.2. 20 °C	1.34	102.4
2.3. control	1.48	138.4
2.4. 40 °C	0.99	82.3
2.5. control	1.48	124.3
2.6. 60 °C	0.84	31.4
2.7. control	1.52	128.2
2.8. 80 °C	0.38	32.2
2.9. control	1.40	125.8
2.10. 100 °C	0.19	12.1

A one-hour chemical treatment at room temperature resulted in a minimal decrease of pH difference, while TIU value dropped by 24%. At 40 °C, TIU value dropped by 40%, at 60 °C and 80 °C, there was a 75% decrease, while heat treatment performed at 100 °C yielded a TIU value below 10% of the initial value. Thus, the experiment for temperature optimization demonstrated the following: decrease upon chemical treatment both at room temperature

and at 40 °C can be significant; 60 °C and 80 °C are not sufficient for a one-hour treatment; 100 °C is sufficient for the TIU value to be reduced to 10% and for urease enzyme activity to fall within the well-toasted range.

Time optimization

During time optimization, the results obtained for urease enzyme activity and trypsin inhibitor content are included in *Table 6.*

Table 6: Experiment results for time optimization

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
3.1. control	1.62	135.2
3.2. 20 minutes	0.67	44.1
3.3. control	1.43	129.3
3.4. 40 minutes	0.19	14.3
3.5. control	1.49	125.8
3.6. 60 minutes	0.17	12.2
3.7. control	1.17	82.2
3.8. 120 minutes	0.08	6.8

The table data show that in 20 minutes' time TIU value decreases from 135.2 to 44.1, which amount to an almost 70% decrease. In 40 minutes, this decrease is close to 90%, while it takes an hour for the TIU to drop below 10% of the initial value. After a 120-minute heat treatment, TIU value did not drop significantly and stayed constant at around 8% of the original value. We can also infer from the table data that by increasing the duration of heat treatment the TIU values of the control sample decreased from 135.2 to 82.2.

Overview of the procedure's optimal parameters

An optimal chemical concentration is 1.5–5.0% aqueous solution of the hydrogen-peroxide and ammonium-hydroxide, while the optimal solution quantity is 20% of the soy's dry matter content. The optimal temperature is 100°C, and the optimal time for heat treatment is 60–120 minutes. The reason why we specify ranges and not exact parameters is that values can change depending on the sample preparation, the initial moisture content, and the used equipment. Based on the above, one possibility/method for reducing the soybean's

trypsin inhibitor content and urease enzyme activity is as follows: Mix one tonne of flattened, air-dried soy with a 200 l solution containing 1.5–2.5% hydrogen-peroxide and 1.5–2.5% ammonium-hydroxide. Consequent upon gas evolution, an expansion occurs, wherefore the capacity of the apparatus has to be designed so that it can hold 2.0–2.5 times the soy's volume foreseen for the treatment. Subsequent to gas evolution, the initial volume returns to its original value in 10 minutes' time. After leaving it for 15–20 minutes, a heat treatment of 60–120 minutes at 100°C causes the soybean's urease enzyme activity and trypsin inhibitor content to fall below the tenth of their initial values.

We cannot evaluate our results in the light of the literature as we have not found a single reference at anyone using hydrogen-peroxide or ammonium-hydroxide to inactivate soy trypsin inhibitor content. Treatment performed using these two chemicals gives rise to several questions. What happens to the hydrogen-peroxide that is not used up in oxidation? During heat treatment, it most likely transforms into oxygen and water, wherefore no harmful substance is retained in the soybeans following such a heat treatment.

Does it lead to oxidation in the case of valuable soy components, sulphurous amino acids, vitamin E, and unsaturated fatty acids? As trypsin inhibitors occur in high concentrations in the soybean shell, the outer shell, and directly underneath it, it is not probable that the oxidative transformation would be a significant one. In the case of amino acids, these transformations – based on our examinations – are negligible; however, we did not assess the oxidation of fatty acids and vitamin E, which is why we have no data on these.

The question might be raised as to what happens to excess ammonium. In 60 minutes, it partially evaporates, and a small part of it may be bound to soy components via surface adsorption. If we regard soy as animal feed, especially the dairy feed of high-yielding cattle, then it causes no problem at all since microorganisms inhabiting the rumen can use both ammonia and non-protein nitrogen for protein synthesis. Nevertheless, finding answers to these questions is a task for the future.

Summary

Owing to its high trypsin inhibitor content, crude soybean meal can only be used with certain restrictions for human consumption and animal feeding. To reduce and optimize its trypsin inhibitor content, certain heat treatment procedures and more or less successful physical and chemical methods were developed. These procedures mostly entail high energy consumptions, while

the end-product's quality also leaves much to be desired. The abovementioned urged us to try out the chemical method using hydrogen-peroxide–ammonium-hydroxide combined with heat treatment to reduce soy trypsin inhibitor activity, expecting a significant amount of energy savings. We determined the optimal concentration of the chemicals applied, the optimal temperature and duration of treatment, in the course of which soy trypsin inhibitor activity decreased to tenth–twentieth part of the original value. The procedure results in lower energy consumption as compared to the original heat treatment methods.

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Methods and procedures for the processing of feather from poultry slaughterhouses and the application of feather meal as antioxidant

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Abstract. The research subject is the elaboration of a method and procedure for processing feather from poultry slaughterhouses and using it as antioxidant as well as for satisfying the sulphurous amino acid needs of ruminants. We investigated the level of digestion of the meal feather obtained with our technology, its antioxidant effect and role in the rumen fermentation of the ruminants. Making use of the digested feather meal's antioxidant effect and amino acid composition, we make a suggestion for the preparation to be used as antioxidant and for the satisfaction of the sulphurous amino acid needs of ruminants. By adopting this procedure, the valueless feather can be transformed into a useful feed supplement (natural antioxidant, sulphur source) that can bring about significant economic growth. Pre-trials have been performed successfully, and in what follows we'll need to prove through field trials and pilot-scale experiments that feather meal can be produced and utilized economically as antioxidant in monogastric animals and as a sulphur source in the studying of ruminants.

Keywords and phrases: feather, feather meal, poultry, antioxidant, cysteine, sulphurous amino acids

1 Introduction

Feather meal is a feed ingredient derived from the feather of healthy poultry, intended for human consumption, heat treated at a minimum of 145 °C, at a minimum of 0.4–0.5 MPa pressure, and for a minimum of 40 minutes, which is then followed by drying, grinding, sorting, and packaging (*Kovács*, 2017). According to the regulations, it may be used exclusively for the feeding of pets. It has a maximum of 10% water content and 80–90% crude protein content, of which about 55–65% is digestible crude protein (*Csapó & Sarudi*, 1985). (Protein digestibility is around 80% on the average.) Its fat content is between 5 and 7% and its ash content between 1 and 6%, depending on ingredient type and technology. Neither Clostridium perfringens nor Salmonella can be detected, while enterobacteria is less than 10 in 25 g. *Nocek* (1988) measured its energy content at 22–23 MJ/kg, while *Dale* (1992) investigated in detail its true metabolizable energy content.

The feather having 30–50% dry matter content on the average is produced as the by-product of poultry slaughtering and is suitable for producing feather keratin meal. This product was previously used as animal feed, but today is mostly utilized as a fertilizer of high nitrogen content on plough-lands and in horticulture.

Its use as animal feed is also hampered by its very unique amino acid composition that contains such a little amount of essential amino acids, especially lysine, that it can only reduce the biological value of any protein it is admixed with. Many have investigated the nutritional value, first of all, the amino acid composition of feather meal. In the 1950s, *Binley & Vasak* (1951) were the first to study the possibilities of feather meal production, followed by *Naber et al.* (1961), who investigated feather protein composition and the utilization of amino acids in broiler chickens. *Morris & Balloun* (1973a,b) studied the effects of feather meal production technology on nitrogen retention, on net protein value, and on the activity of xanthine dehydrogenase in broiler chickens, while *Baker et al.* (1981) examined the protein content and amino acid composition of feather treated for various periods of time and at different pressures. According to our own investigations, due to its high cystine content, it could be considered as a cystine/cysteine supplement in lamb feed only or perhaps in the feed of angora rabbits (*Csapó & Csapóné*, 1985; *Csapó et al.*, 1986).

Besides the extremely low biological value of feather meal, its utilization as feed is also rendered difficult by the fact that keratin, in its original state and due to its high number of disulphide bridges, is fully resistant to digestive en-

zymes, that is, it passes through the digestive tract without any change. Protein digestibility can be improved by splitting the disulphide bridges, digesting the hydrogen bridge bond stabilizing the protein molecule, and the partial hydrolysis of protein (*Blasi et al.*, 1991; *Latshaw et al.*, 1994; *Cotanch et al.*, 2006, 2007; *Garcia et al.*, 2007); however, the biological value will continue to decrease owing to further essential amino acid decomposition consequent upon technological interventions (*Papadopolous et al.*, 1985, 1986; *Wang*, 1997). Treatment carried out at high pressure in an autoclave dissolves disulphide bridges, protein is denatured, but this treatment does not yield significant protein hydrolysis. Hydrolysis can only be achieved with chemical (alkaline or acidic) methods or the use of enzymes.

Among acidic methods, both hydrochloric and sulphuric acid treatment can be applied to the hydrolysis of keratin. Following hydrochloric hydrolysis, the salt content may increase due to acid neutralization, while during sulphuric acid neutralization the gypsum precipitate is relatively easy to remove from the system. In the course of alkaline hydrolysis, lanthionine is produced from cysteine via dehydroalanine (*Robbins et al.*, 1980; *Moritz*, 2000), which reduces cystine content; but the most relevant problem is the racemization of amino acids, which may even result the racemic mixture of amino acids during the entire procedure leading to hydrolysis (*Pohn et al.*, 1999).

In addition to chemical methods, enzymatic methods have also been developed for keratin hydrolysis, which is a much more gentle procedure considering amino acids. The last twenty years have seen many adopting enzymatic treatment for keratin digestion and to improve its digestibility (*Williams et al.*, 1991; *Hood & Healy*, 1994; *Onifade et al.*, 1998; *Tiwary & Gupta*, 2002; *Yamamura et al.*, 2002; *Bertsch & Coello*, 2005; *Grazziotin et al.*, 2006; *Gupta & Ramnani,* 2006; *Brandelli et al.*, 2010; *Zaghloul*, 2011). Besides the aforementioned, *Bockle & Muller* (1997) successfully used particular enzymes for splitting disulphide bridges to obtain an improved digestibility. Each of the processes is based on the selection of such microorganisms that are able to break the disulphide bonds of the keratin mostly via reduction, whereby they can eliminate protein compactness, thus the protein exerting smaller resistance to digestive enzymes. Changing the technology and optimizing the kinetics of enzymatic reactions make the methods suitable to completely or partially break down the protein and produce free amino acids as well as oligo- and polypeptides.

These procedures, however, do not affect amino acid composition (*Eggum*, 1970). Compared to chemical methods, their only advantage is that the decomposition and transformation of amino acids take place to a lesser degree

under optimal conditions; this is quite insignificant in the case of certain technologies.

In Hungary, 40–50,000 tonne of wet feathers are produced on an annual basis as by-products of poultry slaughterhouses, for which no reassuring placement or utilization has been found yet. The perishable organic material of high protein content causes environmental issues, which is why the destruction or utilization thereof is an economic task. Its destruction is costly, it has high land-use requirements (burying), and the expenses are not compensated by any product.

Summing up the previously mentioned research results, in the utilization of feathers, e.g. in animal feeding, we must consider that the protein, the keratin it contains is insoluble in water and digestive juices, and it resists all substances that do not attack it chemically and digest it. We may conclude from the above that without digestion the meal produced from feathers has a very low feeding value and digestibility, wherefore feather meal, despite its high protein content, cannot be efficiently used for feeding our farm animals. Subsequent to the digestion of feather with chemical or microbiological methods, digestibility is essentially improved, but the biological value of the protein remains unchanged; therefore, the digested feather, the feather protein hydrolysate is difficult to include in the feeding process.

Looking at literature data, we cannot find a single reference – apart from our conference lecture (*Csapó & Albert*, 2015) – to anyone ever using hydrolysed feather meal as antioxidant in feeding. The foregoing prompted us to develop a procedure for the digestion of feather in such a way that the digested and processed material can be suitable for feeding purposes. We examined the feather meal obtained with the application of our technology in terms of its level of digestion, antioxidant effect, and its role played in the rumen fermentation of the ruminants. Making use of the digested feather meal's antioxidant effect, we make a suggestion for the preparation to be used as antioxidant and, due to its high cystine content, for the satisfaction of the sulphurous amino acid needs of ruminants.

2 Materials and methods

Overview of the experiments for feather digestion

We placed 150 kg of air-dried feather into a 500-l alkali-resistant steel container and poured on it 350 l of 1M sodium hydroxide. (14 kg of solid sodium

hydroxide or a corresponding quantity of liquid lye was dissolved in 150 l of water, after which the solution was supplemented to 350 l.) The obtained substance was carefully heated to boiling point, but not too rapidly as that would have led to intensive foaming. After boiling commenced, we boiled it for one hour and then let it cool down. The cooled substance, the protein hydrolysate with a pH around 12.5 was set to pH = 6. The neutralization of the 500 l of hydrolysate requires about 35 l of hydrochloric acid – 37% technical grade. Neutralization must be performed with due care and diligence as foam materials produced during protein precipitation may leave the vessel. A pH meter must be used to carry out and check the setting of the hydrolysate to pH = 6. The hydrolysate produced as described above was desiccated at 60 °C in an exsiccator and then ground.

Assessment of the digested feather meal's antioxidant effect

We set up two experiments for the demonstration of antioxidant properties. In the first experiment, we analysed the correlations between feather meal and the acid, peroxide index variations, while the second experiment looked into the relation between feather meal and the changes occurring in the vitamin A and E concentration of the experimental compound feed.

Over a period of five months, we studied as a function of time the changes in the peroxide and acid number of the following feather meal mixtures: 900 g of complete soybean meal + 100 g of butter, 800 g of complete soybean meal + 100 g of butter + 100 g of feather meal, and 500 g of complete soybean meal + 100 g of butter + 400 g of feather meal. From the samples presented above, we also carried out the determination of vitamins A and E, analysing the changes in the concentration of the two vitamins as a function of time.

Assessing the suitability of hydrolysed feather meal in ruminants

We investigated the effects of feeding the obtained digested feather meal to cows with a rumen fistula. During the experiment, the feed ration consisted of 20 kg of maize silage, 4 kg of pasture hay, and 4 kg of dairy feed. The dairy feed was made up of 21.5% maize, 21.5% wheat, 21.5% wheat bran, 30.1% extracted sunflower meal, 3.2% monocalcium phosphate, and 2.2% feed

lime. When ten days of feeding with the above ration had elapsed, after 2, 4, and 8 hours following the morning feeding on the tenth day, we sampled the rumen fluid, from which we determined the ammonia content, pH value, and acetic acid, propionic acid, and butyric acid content. The obtained results were considered the control data in our experiment.

In the first pilot phase, 1 kg of extracted sunflower meal was replaced by 0.5 kg of feather meal and 0.3 kg of maize meal, and so the experimental feed included 11.2% feather meal. The pilot phase lasted for seven days – after 2, 4, and 8 hours following the morning feeding on the third and seventh day, we sampled the rumen fluid, from which we determined the previously mentioned parameters.

In the second pilot phase, 1.4 kg of extracted sunflower meal was replaced by 0.7 kg of feather meal and 0.4 kg of maize meal, and so the experimental feed included 15.8% digested feather meal. The pilot phase lasted for 14 days – after 2, 4, and 8 hours following the morning feeding on the 3^{rd}, 7^{th}, 10^{th}, and 14^{th} day, we sampled the rumen fluid, from which we measured the previously mentioned parameters.

3 Results

The composition of feather meal obtained following digestion, neutralization, and grinding is included in tables 1 and 2.

Investigation of the digested feather meal's antioxidant effect

Feather as well as the feather meal that has undergone various technological processes contain about 5–7% cystine. From this cystine content, the following are generated: 0.05–0.20% free cysteine due to digestion procedures, the same amount of free cystine, and about 0.2–0.5% cysteic acid due to contact with air and high temperature. Based on theoretical considerations, we may conclude that the hydrolysed feather meal had antioxidant effects due to the cystine-cysteine-cysteic acid system it contains.

Table 1: Effects of digestion time on protein extraction and on the free amino acid content of the hydrolysate

Component	**Digestion time (minutes)**				
	15	30	45	60	120
Feather mass (g)	1,500	1,500	1,500	1,500	1,500
Volume of the hydrolysate (cm^3)	4,850	4,830	4,820	4,800	4,610
Protein content of the hydrolysate (%)	24.1	24.0	24.0	23.9	22.7
Protein mass (g)	1,169	1,159	1,157	1,148	1,047
Protein extraction (%)	94.0	93.2	93.0	92.3	84.1
Protein loss (%)	6.0	6.8	7.0	7.7	15.8
Free amino acid content (%)					
Aspartic acid	0.089	0.130	0.283	0.417	0.823
Threonine	0.027	0.039	0.089	0.102	0.184
Serine	0.154	0.380	0.642	0.129	2.342
Glutamic acid	0.014	0.022	0.044	0.072	0.138
Proline	0.112	0.187	0.415	0.639	1.124
Glycine	0.153	0.301	0.681	0.681	0.143
Alanine	0.172	0.342	0.712	0.960	1.821
Cystine	0.024	0.038	0.064	0.078	0.141
Valine	0.033	0.063	0.099	0.177	0.321
Methionine	0.010	0.015	0.027	0.045	0.083
Isoleucine	0.010	0.019	0.043	0.051	0.092
Leucine	0.041	0.083	0.181	0.222	0.431
Tyrosine	0.038	0.061	0.136	0.180	0.359
Phenylalanine	0.088	0.167	0.302	0.498	0.887
Lysine	0.021	0.023	0.054	0.066	0.119
Histidine	0.011	0.018	0.020	0.027	0.052
Arginine	0.010	0.014	0.030	0.036	0.073
Total	1.007	1.902	3.822	5.721	10.142

Table 2: The amino acid composition of feather meals produced with different digestion times – expressed in units of g amino acid/100 g feather meal (A) and g amino acid/100 g protein (B)

Amino acid	**Digestion time (minutes)**									
	15		30		45		60		120	
	A	B	A	B	A	B	A	B	A	B
Aspartic acid	5.19	6.1	5.20	6.1	5.04	6.0	5.53	6.4	5.12	6.4
Threonine	3.63	4.2	3.59	4.2	3.43	4.1	3.28	4.0	3.14	3.9
Serine	8.54	10.0	8.50	10.0	8.48	10.1	8.32	10.1	8.20	10.2
Glutamic acid	8.89	10.4	8.91	10.5	8.72	10.4	8.54	10.3	8.02	10.0
Proline	7.97	9.3	8.01	9.4	7.79	9.3	7.78	9.5	7.43	9.3
Glycine	6.17	7.2	6.12	7.2	6.05	7.2	6.04	7.3	6.05	7.6
Alanine	5.08	5.9	4.90	5.8	4.87	5.8	4.80	5.8	4.72	5.9
Cystine	**6.14**	**7.2**	**5.89**	**6.9**	**5.72**	**6.8**	**5.64**	**6.8**	**4.98**	**6.2**
Valine	7.32	8.5	7.30	8.6	7.28	8.7	7.21	8.7	7.02	8.8
Methionine	0.69	0.8	0.67	0.8	0.66	0.8	0.60	0.7	0.59	0.7
Isoleucine	3.54	4.1	3.50	4.1	3.48	4.2	3.26	3.9	3.25	4.1
Leucine	7.92	9.2	7.90	9.3	7.94	9.5	7.91	9.6	7.69	9.6
Tyrosine	2.51	2.9	2.48	2.9	2.33	2.8	2.30	2.8	2.14	2.7
Phenylalanine	4.13	4.8	4.10	4.8	4.21	5.0	4.03	4.9	3.99	5.0
Lysine	1.50	1.8	1.50	1.8	1.47	1.8	1.42	1.7	1.42	1.8
Histidine	0.57	0.7	0.58	0.7	0.52	0.6	0.52	0.6	0.51	0.6
Arginine	5.89	6.9	5.91	6.9	5.82	6.9	5.73	6.9	5.75	7.2
Total	85.68	100.0	85.06	100.0	83.81	100.0	82.71	100.0	80.02	100.0

Effects of feather meal on the changes in peroxide and acid number

According to our investigations (*Table 3*), the control sample underwent the following changes: its peroxide number increased from to 2 to 6 in 60 days, to 15 in 120 days, and to 22 in 150 days, while its acid number increased from 4 to 14, 22, and 28 respectively. Samples containing feather meal underwent significantly less changes. The peroxide number doubled, while the acid number barely changed, which is a clear demonstration of the feather meal's antioxidant effect and of that it is able to have the same effect in compound feeds as well.

Table 3: Effects of feather meal content on the changes in peroxide and acid number

The studied sample	Peroxide number (P) Acid number (S)	Time elapsed from the beginning of the experiment (days)					
		0	30	60	90	120	150
900 g soy + 100 g	P	2	4	6	8	15	22
butter (control)	S	4	8	14	20	22	28
900 g soy + 100 g butter	P	2	2	2	2	4	5
+ 100 g feather meal	S	4	4	4	4	5	6
900 g soy + 100 g butter	P	2	2	2	2	4	4
+ 400 g feather meal	S	4	4	4	4	4	5

Effects of feather meal on the changes in vitamin A and E content

According to our investigations, in the control feed, not including feather meal: 15% of vitamin A and 20% of vitamin E decomposed in 90 days, while 25% of vitamin A and 30% of vitamin E decomposed in 150 days. Upon adding feather meal, the decomposition in 90 days remained under 10% in both vitamins, and neither of them reached a 15% decomposition in 150 days.

In the light of all the foregoing, we can say that feather meal obtained with the procedure described above has antioxidant properties, wherefore it can partially or fully replace the artificial antioxidants annually imported to Hungary, worth several million dollars. Beyond its economic benefits, it allows us to have antioxidants with not fully explored effects on the animal organism replaced by a natural substance completely harmless to animals and their environment.

Examination of the hydrolysed feather meal's suitability in ruminant feed to replace sulphurous amino acids

Effects of the digested feather meal feeding on the relevant rumen parameters

The free ammonia content of the rumen fluid had a moderate decrease in the first experimental phase (*Table 4*) and a more substantial decrease in the second experimental phase (*Table 5*). This is presumably the consequence of the fact that we have removed from the feed a relatively rapidly and well-degradable nitrogen source, the extracted sunflower meal, and replaced it with digested feather meal. Based on our assumptions, the rumen's microorganisms directly utilize the free amino acids and part of the small-size peptides without the occurrence of a significant protein decomposition in the rumen. The pH of the rumen fluid is the same as that of the control in the first experimental phase, and it shows a certain increase in the second experimental phase only. By increasing the feather meal portion, the acetic acid content of the rumen fluid also increases while exhibiting high variability, and the propionic acid content shows a slight increase in the first experimental phase, whereas in the second experimental phase it has similar values to that of the control. Butyric acid content decreased upon increasing feather meal quantity.

Analysing the parameters pointing at rumen fermentation, we may conclude that regarding ammonia and propionic acid content a greater proportion of feather meal may act depressively. Based on our results, the maximum ration of the digested feather meal in the case of lactating cows should be 10% of the dairy feed or not exceeding 0.5 kg per day.

Effects of feeding the various feather meal rations on rumen fermentation and on the break-down of the feeds used

The break-down of the silage-maize silage's and the dairy feed's dry matter content slightly decreases, whereas their crude protein content decreases minimally upon the increasing feather meal feeding. The cellulolytic activity of the rumen is slightly reduced due to feather meal feeding despite that the rumen pH fell within the range of the cellulose-decomposing bacteria's activity optimum. There is a likelihood that for lack of a satisfactory nitrogen source, the number of bacteria decomposing structural carbohydrates slightly decreased. Based on the foregoing, we suggest feeding 0.5 kg of digested feather meal and the use of a well- and rapidly degradable nitrogen source in the feed ration.

Table 4: Changes in the ammonia, pH, and volatile fatty acid content of the rumen in the first experimental phase

Examined parameters	Control phase			First experimental phase					
	Sampling on the 10th day of the preliminary feeding			Sampling on the 3rd day of the experiment			Sampling on the 7th day of the experiment		
	Time elapsed after feeding (hours)								
	2	4	8	2	4	8	2	4	8
Ammonia	295	218	198	231	73	104	188	68	113
pH	6.36	6.38	6.35	6.39	6.37	6.53	6.27	6.31	6.15
Acetic acid	0.65	0.64	0.65	0.62	0.65	0.65	0.63	0.66	0.67
Propionic acid	0.18	0.18	0.17	0.21	0.19	0.20	0.20	0.19	0.19
Butyric acid	0.15	0.12	0.13	0.13	0.12	0.11	0.12	0.11	0.10

* Ammonia, acetic acid, propionic acid, butyric acid: (mg/100g)

Effects of the digested feather meal feeding on the dry matter and protein content of milk as well as on its protein fractions

Upon hydrolysed feather meal feeding, the dry matter and crude ash content of milk as well as its protein fractions (total protein, true protein, whey protein, true whey protein, casein, non-protein nitrogen) remain unchanged compared to control specimens not consuming feather meal.

Discussion of the results

Unfortunately, we cannot evaluate our results in the light of the literature as we have not found any studies on hydrolysed feather meal used as antioxidant or investigating the changes of the concentration of volatile fatty acids produced in the rumen as a function of feather meal feeding rate. Our description of the procedure and the suggested utilization open up new avenues for using feather meal as feeding stuff. Our aim was the processing and utilization of a polluting by-product high in protein content, that is, the several tens of thousands of tonnes of wet feather produced annually, in such a way that over and above the elimination of adverse effects (protein degradation, soil contamination, air pollution) a useful end-product is produced in livestock-farming, whose price can partially or entirely cover the processing costs.

Table 5: Changes in the ammonia, pH, and volatile fatty acid content of the rumen in the main experiment

Examined parameters	Main experiment – Phase 1						Main experiment – Phase 2					
	Sampling on the 3rd day			Sampling on the 6th day			Sampling on the 9th day			Sampling on the 12th day		
	Time elapsed after feeding (hours)											
	2	4	8	2	4	8	2	4	8	2	4	8
Ammonia	203	57	48	127	46	73	203	69	44	97	40	73
pH	6.23	6.30	6.39	6.51	6.47	6.82	6.39	6.44	6.29	6.41	6.37	6.43
Acetic acid	0.67	0.69	0.71	0.66	0.67	0.69	0.65	0.67	0.68	0.70	0.71	0.71
Propionic acid	0.19	0.18	0.17	0.20	0.19	0.18	0.19	0.19	0.18	0.17	0.17	0.17
Butyric acid	0.09	0.08	0.08	0.10	0.10	0.10	0.11	0.10	0.10	0.09	0.009	0.09

* Ammonia, acetic acid, propionic acid, butyric acid: (mg/100g)

The high cystine content of the digested feather meal creates a low-priced solution to meet the sulphurous amino acid needs of the ruminants, whereas the cystine-cysteine-cysteic acid system is able to protect the oxidation-sensitive components of premixtures and feeds due to its antioxidant properties. The antioxidant properties of the obtained digested feather meal can be clearly demonstrated, which is why its application can replace the use of artificial antioxidants. Its natural quality speaks for the utilization of feather meal since we cannot yet fully and accurately assess the physiological effects of artificial antioxidants.

Our cattle feeding experiments showed that applying a sufficient dose of digested feather meal: has beneficial effects on rumen fermentation; decreases the break-down of protein feed in the rumen; in rations of 0.5 kg per day, it increases propionic acid production; does not make significant changes to the utilization of dry matter, cellulose, and hemicellulose content of the feeding stuffs.

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Production of prebiotics via reactions involving lactose as well as malic acid and citric acid

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Abstract. Prebiotics are such indigestible food ingredients that enter the colon and serve as nutrient for bifidobacteria and lactobacilli. Since fibres and oligosaccharides are the typical prebiotics, we produced prebiotics in our experiments with the reaction of lactose and malic acid as well as citric acid, where these reactions made use of an appropriate concentration of these substances, had an adequate duration, and were carried out under optimal temperature conditions. We determined the optimal parameters of the reaction, measured the loss of the starting materials as well as the increase in concentration of the end-product, and analysed the total sugar content of the hydrolysed prebiotics after hydrolysis by hydrochloric acid. In vitro experiments were performed to demonstrate our end-product's resistance to carbohydrate-degrading enzymes, which is a fundamental requirement for a prebiotic so that upon reaching the colon it can serve as nutrient for the probiotic bacteria found there.

Keywords and phrases: lactose, malic acid, citric acid, prebiotics, determination of sugar, enzymatic breakdown

1 Introduction

The nomenclature of probiotics (probiotics, prebiotics, and synbiotics) developed in the last two decades of the 20th century, and they became internationally standardized both in their designations and contents. We call probiotics all those human-friendly enteric bacteria that have multiple beneficial effects on the host organism's state of health. Prebiotics are all those natural nutrients that are typically the exclusive nutrients of probiotics, wherefore they facilitate the latter's multiplication and prevalence. Synbiotics mean the joint presence of pro- and prebiotics, that is, the effects of the two beneficial factors become cumulative, often synergistic. Subsequently, those dairy products have synbiotic qualities, for example, whose production process involves not only probiotics but one or more prebiotics as well. Prebiotics, previously termed bifidus or bifidogenic factors, are oligosaccharides built up from 2–9 simple sugars (monosaccharides). They are not metabolized in the organism, which is why they are intact (indigested) upon reaching the colon. These are dietary fibres – the finest of them due to their solubility in water. Besides functioning as dietary fibres, their true usefulness lies in serving as exclusive nutrients for probiotics. Since there is a small amount of digestible nutrients in the colon, a relative food shortage characterizes it, the consumed prebiotics thus setting the ground for the multiplication of human-friendly probiotics (*Csapó et al.*, 2016).

Prebiotics occur naturally in a number of foods. They are abundantly present in e.g. the roots of the Jerusalem artichoke and chicory, but they are also traceable in red onion, garlic, leek, artichoke, oatmeal, wheat, banana, milk, and matured cheese. Alimentary practices typically include industrially prepared pure products, which may be liquid concentrates and powders, the concentration of their active substance ranging between 40% and 95%. Natural industrial concentrates – depending on their constituent monosaccharides – may be e.g. galacto-, fructo-, malto-, or xylo-oligosaccharides. In the year 1995, they were already producing an annual amount of 80,000 tonnes of prebiotics, but production has increased to approx. 200,000 tonnes by today, marking the worldwide success of this special "elixir of life". About 40% of the amount produced is galacto-oligosaccharide (e.g. lactulose), whose basic ingredient is lactose (*Csapó et al.*, 2014a,b,c).

2 Literature review

Prebiotics are non-digestible polysaccharides and oligosaccharides, which, upon reaching the colon, inhibit Salmonella and Escherichia coli bacteria and promote the growth of bifidobacteria and lactic acid bacteria. The term prebiotic was originally coined by *Gibson* and *Roberfroid* in 1995. In 2004, *Gibson et al.* outlined the conditions required for a nutrient to have prebiotic qualities. These are as follows: they should resist gastric acid and the pepsin found therein, mammalian enzymes should not break them down in the gastrointestinal tract, and they should serve as nutrients for those bacteria in the gastrointestinal tract that contribute with their metabolites to humans' well-being and health. A great number of food ingredients meet these criteria – in 2007, *Stowel* grouped these ingredients as follows: inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, and polydextrose, whereas isomalto-oligosaccharides, xylo-oligosaccharides (XOS), and lactitol were grouped in the category of emerging, potential prebiotics.

Prebiotics occur in plenty of foods. Chicory root, for example, contains fructo-oligosaccharides derived from inulin, while wheat bran contains arabinoxylo-oligosaccharides (AXOS) and xylo-oligosaccharides (XOS), widely adopted in nutrition (*Sabater-Molina et al.*, 2009; *Femia et al.*, 2010; *Xu et al.*, 2009). Mannitol, maltodextrin, raffinose, lactulose, and sorbitol are also prebiotics with health-protective effects (*Yeo* & *Liong*, 2010; *Vamanu* & *Vamanu*, 2010; *Mandal et al.*, 2009). Resistant, starchy seeds are also considered to be prebiotics, giving proof of several beneficial effects during their consumption. These are not digested and absorbed in the small intestine, but, upon reaching the colon, intestinal microflora can make use of them during fermentation, while short-chain fatty acids (SCHFA, propionic acid, butyric acid, valeric acid, and caproic acid) are formed, which, while reducing pH values, suppress the reproduction of putrefactive bacteria producing toxins (*Vaidya* & *Sheth*, 2010).

Such fermentable dietary fibres as the beta-glucan from rye, the rubbery polysaccharides of linseed and fenugreek may also be considered prebiotics, which are able to serve as basic ingredients for short-chain fatty acids, thus disposing of health-protective effects. Apart from the aforementioned, mannans can be found in large amounts in the wall of yeast cells, which are again prebiotics (*Lin et al.*, 2011).

Also, as a result of malnutrition, smoking, and alcoholism in modern age, there is a growing disease and mortality rate. Typical diseases of our age are chronic obesity, stomach and intestinal problems, diabetes, cardiovascular

diseases, cancer, and degenerative changes, whose numbers have been on a significant rise in the past few years. With a view to prevent or contain these diseases, a growing number of people have turned to foods containing health-protective prebiotics too, expecting substantial improvement from these with a view to their state of health.

Consumers are increasingly looking for low-carbohydrate, high-fibre and -protein foods, while there is a growing interest in prebiotic foods as well. A fine example for this are foods containing blackcurrant leaf extract in the form of powder, lactoferrin, and lutein, produced by several companies in large quantities worldwide. These products have significantly increased the number of bifidobacteria and lactobacilli in the colon, while substantially reducing the number of bacteroides and clostridia. Further, they have reduced beta-glucuronidase (GUS) and enhanced beta-galactosidase enzymatic activity in the small intestine, thus aiding the digestion of e.g. lactose in lactase-deficient humans. Therefore, these products can be truly considered prebiotics (*Molan et al.*, 2010).

As a result of wheat germ supplement applied for 20 days, the colon pH was significantly reduced, just as a strong decrease could be observed in the number of the clostridium population, whereas the amount of lactobacilli and bifidobacteria has significantly increased, indicating a considerable improvement in the quality of life of individuals consuming such products (*Matteuzzi et al.*, 2004). *Glover et al.* (2009) established that gum arabic has a beneficial effect on individuals' suffering from systolic blood pressure and diabetic renal failure. *Phillips* and *Phillips* (2011) found that administering 25 g of gum arabic preparations throughout a period of 8–12 weeks was highly beneficial to the condition of diabetic patients and significantly reduced systolic blood pressure.

Previously, we (*Csapó et al.*, 2014a,b,c) have performed the structural and quantitative analysis of exopolysaccharides and oligosaccharides produced by lactic acid bacteria, whereas our present work's ambition is to report on the results of our experiments where we produced prebiotics via the reaction of lactose as well as malic acid and citric acid. In our work, we have considered *Gaertner* and *Daytoni's* (1956) as well as *Antrim et al.'s* (2003) patent specification as reference material, who aimed at producing surfactants by creating ester linkages between carbohydrates and dicarboxylic acids and studied the mechanism of these reactions. Their methods were successfully tested with sugar alcohols, sugars, oligosaccharides, and polysaccharides as well.

3 Experimental objectives

Relying on literature results and our own previous research, we aimed at producing prebiotics, during which we established such linkages between lactose and malic acid as well as between lactose and citric acid that can resist acidic medium and the attack of carbohydrate-degrading enzymes in the human stomach and the forward section of the gastrointestinal tract and that get into the colon, where they function as nutrients for the probiotic microorganisms settled therein. Our goal was to determine the optimal reaction parameters, temperature, time, and reactant concentration, to measure the loss of the starting materials as well as the increase in concentration of the end-product, and to analyse the total sugar content of the hydrolysed prebiotics after hydrolysis by hydrochloric acid. We further aimed at performing in vitro experiments in order to demonstrate our end-product's resistance to carbohydrate-degrading enzymes, which is a fundamental requirement for a prebiotic.

4 Materials and methods

4.1 The materials used

Our experiments were performed with pharmaceutical-grade lactose, citric acid, and malic acid. The malic acid we used had a purity of 95%, containing less than 1% of fumaric acid and less than 0.05% of malonic acid. The quality certificate of the malic acid no E296 E can be downloaded from the following link: http://bbbb.hu/spec/almasav.jpg. The material that we used was in compliance with the pharmacopoeia of the USA, the EU, and Hungary as well as with the Hungarian Food Codex standards nos 1-2-89/107.

The citric acid used for our experiments was also food-grade, or, even better, pharmaceutical-grade citric acid monohydrate (E330), whose quality certificate can be downloaded from the following link: http://www.bbbb.hu/spec/Citrom.jpg, while its safety data sheet can be accessed from here: http://www.bbbb.hu/spec/citrombizt.jpg. Its CAS number is 5949-29-1, and its EU number is 201-069-1. As per the quality certificate, its citric acid monohydrate content is nearly 100%, it has a maximum water content of 8.8% and an oxalic acid content of less than 100 mg/kg. All of its parameters comply with the EU and the Hungarian Food Codex standards.

The lactose used in our experiments was a 95% pure, food-grade, finely pulverized D(+)-lactose 1-hydrate, isolated from bovine milk and spray-dried produced. Its quality was in compliance with the Ph.Eur 8.0 quality standards.

4.2 The applied analytical methods

As a method for monitoring the reaction of the lactose and malic acid as well as citric acid, we measured the lactose content as this seemed the easiest way to do it. The decrease in lactose content was indicative of the reaction since if the free glycosidic hydroxyl group of the lactose forms a bond, it will no longer manifest a reaction typical of reducing sugars. Lactose belongs to the group of reducing disaccharides, and it manifests Fehling's reaction. During the reaction, due to the aldehyde group of sugar, Cu^{+} ions are formed from Cu^{2+} ions – determining the amount of Cu^{+} ions lets us determine the exact sugar content. During the examination procedure, we measured out and introduced 2 g of sample material into a 100-cm^3 volumetric flask, added to it 50 cm3 of water, and subjected it to a one-hour-long shaking operation in a shaking apparatus. For the removal of substances interfering with the determination of sugar, we added 20–20 cm^3 of Carrez I and II solution. Afterwards, we filled it up to volume mark with 80% ethanol, shook it up, and filtered it. An amount of 20 cm^3 was separated from the filtrate, the bulk of the ethanol was evaporated, and the evaporation residue was flushed with distilled water into a 20-cm^3 volumetric flask, and, after cooling down, filled up to volume mark. Subsequently, this solution was used for the determination of the reducing sugar content. From the solution prepared this way, we removed an amount of 5 cm^3, put it into a 100-cm^3 Erlenmeyer flask, added to it 5 cm^3 of Luff-Schoorl reagent as well as a few pieces of pumice, brought it to boiling point within 2 minutes over open flames and by shaking it, boiled it for 10 minutes, and cooled it down immediately afterwards. The resulting copper(I)-oxide was iodometrically titrated using 0.1 mole of sodium thiosulphate solution, and lactose content was calculated from the amount decreased.

4.3 Engineering the reactions between malic acid and citric acid as well as lactose

In the first step, we added 20% of citric acid, while in the second step the same percentage of malic acid to the pharmacy-quality lactose. Following a thorough examination of the literature and the patents at our disposal, we ascertained that most reactions were performed at a temperature between 130 and 180 °C – therefore, we opted for 170 °C. The samples were blended in a mortar, ensuring their maximum homogeneity. Following this, the samples were distributed in quantities of approx. 10 g into glass vessels and heat treated for 5-10-20-30-40-50-60 minutes; after cooling down, the lactose content of the

samples was determined.

The following experiment investigated into the effect of temperature on the reaction between citric acid and malic acid as well as lactose. In the first stage, the samples containing 20% citric acid, 20% malic acid, and 80% lactose were treated at 130 °C for 30 minutes, in the second stage at 140 °C, in the third stage at 150 °C, in the fourth stage at 160 °C, while in the fifth stage at 170 °C. After cooling down, the lactose content of the samples was determined.

5 Results and conclusions

5.1 Effects of the duration of heat treatment at 170 °C on the reaction between lactose and carboxylic acids

Table 1 shows the effects of the duration of heat treatment at 170 °C on the reaction between lactose and citric acid.

Table 1: Effects of the duration of heat treatment at 170 °C on the reaction between lactose and citric acid

Sample designation	Sample	Heat treatment duration (min)	Lactose (%)
P1	80 g lactose + 20 g citric acid	5	73.6
P2	80 g lactose + 20 g citric acid	10	63.4
P3	80 g lactose + 20 g citric acid	20	48.4
P4	80 g lactose + 20 g citric acid	30	35.4
P5	80 g lactose + 20 g citric acid	40	21.9
P6	80 g lactose + 20 g citric acid	50	12.3
P7	80 g lactose + 20 g citric acid	60	7.1

Heat treatment temperature: 170 °C

By adding 20% of citric acid to the lactose and performing a heat treatment of 170 °C for various times, we established that upon heat treatment the initial white mixture changed to yellow in 5 minutes and turned to a brownish colour in 10 minutes, after which only its colour became increasingly darker, but its volume remained practically unaltered.

Table 2 shows the effects of the duration of heat treatment at 170 °C on the reaction between lactose and malic acid.

By adding 20% of malic acid to the lactose and performing heat treatment for various times, similarly to the case of citric acid, we found sample colour

almost unchanged in 5 minutes' time, within ten minutes, it took on a somewhat yellowish colour, in 20 minutes, it turned yellowish brown, and then it became increasingly swollen, while the last sample changed to dark brown.

Table 2: Effects of the duration of heat treatment at 170 °C on the reaction between lactose and malic acid

Sample designation	Sample	Heat treatment duration (min)	Lactose (%)
P8	80 g lactose + 20 g malic acid	5	70.6
P9	80 g lactose + 20 g malic acid	10	68.3
P10	80 g lactose + 20 g malic acid	20	52.1
P11	80 g lactose + 20 g malic acid	30	33.4
P12	80 g lactose + 20 g malic acid	40	25.3
P13	80 g lactose + 20 g malic acid	50	19.2
P14	80 g lactose + 20 g malic acid	60	16.4

Heat treatment temperature: 170 °C

5.2 Effects of heat treatment performed at various temperatures and for the same duration on the reaction between lactose and carboxylic acids

Table 3 shows the effects of heat treatment performed at various temperatures and for the same duration on the reaction between lactose and citric acid as well as between lactose and malic acid.

Upon a 30-minute heat treatment at 130 °C, the samples practically maintained their white colour; at 140 °C, both the samples containing citric acid and those containing malic acid changed to yellow; at 150 °C, this yellowing further intensified in both carboxyl acids; at 160 °C, the sample with citric acid as well as the one with malic acid took on a deeply brown colour; at 170 °C, the sample with citric acid formed a brown-coloured mass, just as the sample containing malic acid, which, however showed a less brownish discolouration.

Table 3: Effects of heat treatment performed at various temperatures and for the same duration on the reaction between lactose and citric acid as well as between lactose and malic acid

Sample designation	Sample	Heat treatment duration (min)	Lactose (%)
P15	80 g lactose + 20 g citric acid	130	78.2
P16	80 g lactose + 20 g malic acid	130	56.8
P17	80 g lactose + 20 g citric acid	140	48.1
P18	80 g lactose + 20 g malic acid	140	55.4
P19	80 g lactose + 20 g citric acid	150	33.4
P20	80 g lactose + 20 g malic acid	150	38.3
P21	80 g lactose + 20 g citric acid	160	25.6
P22	80 g lactose + 20 g malic acid	160	29.7
P23	80 g lactose + 20 g citric acid	170	16.4
P24	80 g lactose + 20 g malic acid	170	25.5

Treatment: 30 minutes

5.3 Discussion of results, conclusions

5.3.1 Time dependence, temperature dependence

The determination of lactose content allowed us to model what percentage of the originally 80% lactose turned into some kind of oligomer or polymer. If the lactose concentration significantly decreases during the reaction, it inevitably means that the applied hydroxycarboxylic acids reacted with the lactose in some way, creating molecules of various sizes during heat treatment.

The determination of lactose content performed with the use of 24 samples yielded the following results. In the first experiment, 20 g of citric acid was added to 80 g of lactose, while in the second experiment 20 g of malic acid was admixed with 80 g of lactose, whereafter heat treatment was carried out at 170 °C for a duration of 5, 10, 20, 30, 40, 50, and 60 minutes. A subsequent experiment investigated heat dependency, treating the above listed samples (citric acid, malic acid) at 130, 140, 150, 160, and 170 °C for 30 minutes. This time, we tried to identify the optimal temperature at which an adequate reaction can be produced between lactose and the various added carboxylic acids.

In the first case, we measured 79.1% from the control sample (a heat-treated mixture of erythrite and lactose), which in fact gives us the theoretical value.

Exposing the lactose with citric acid to heat treatment for 5 minutes, its quantity decreased to 73.6%, while upon a 60-minute heat treatment it decreased to 7.1%. So, it seems that with the application of citric acid 93% of the lactose transformed into some kind of oligomeric or polymeric compound.

In the second experiment, we measured a 70.6% lactose content for the sample containing malic acid and heat treated for 5 minutes, whereas the sample heat treated for 60 minutes had a lactose content of 16.4%, meaning that 83–84% of the lactose transformed into some sort of product during those 60 minutes of heat treatment. Therefore, we may conclude that both malic acid and citric acid are suitable for forming oligomers or polymers with lactose.

Regarding the temperature dependency of the reaction, we obtained the following results. In the sample with citric acid, only about 1.2% of the lactose was transformed upon a 30-minute heat treatment at 130 °C, whereas from the same sample we could only retrieve 16.4% of lactose upon a 170 °C heat treatment; so, more than 80% of the lactose transformed into oligomer or polymer. When repeating the experiment with malic acid, approximately 30% of the lactose was transformed at a temperature of 130 °C and in a period of 30 minutes, while at 170 °C this value increased to 70%.

The second experiment leads us to the conclusion that citric acid and malic acid alike proved to be 'eligible partners' in the formation of lactose oligomers or polymers at elevated temperature. 130 °C appears to be a temperature too low – a heat treatment performed at 160–170 °C and for 30 minutes (or perhaps at 150–160 °C for 1 hour) is what we consider optimal since it allows the bulk of the lactose to transform into a product. The experiment performed with erythrite clearly demonstrates that the applied thermal conditions and duration did not lead to lactose degradation as this experiment, where no reaction whatsoever was expected to take place between the erythrite and the lactose, resulted in almost the full retrieval of the lactose.

5.3.2 Determining the sugar content of the obtained prebiotic after hydrolysis by hydrochloric acid

Hydrolysis by hydrochloric acid was applied in an attempt to release lactose from its bonds in the obtained samples, which was followed by sugar content determination, allowing us to establish whether the lactose had integrated into some kind of non-reducing polymer, or perhaps something else occurred during the reaction. Following hydrolysis by hydrochloric acid, all sugar content determinations brought along positive results. The sample heat treated at 170 °C for 60 minutes in the presence of 20% citric acid had a lactose content of 7.1%,

which, subsequent to hydrolysis by hydrochloric acid, increased to 46.3% when measured for total sugar content. Following a heat treatment at 170 °C for 60 minutes in the presence of 20% malic acid, lactose content increased from 16.4% to 51.2% expressed in total sugar after hydrolysis by hydrochloric acid. Upon heat treatment at 170 °C for 30 minutes, lactose content increased from 16.4% to 54.9% in the presence of citric acid, whereas it increased from 25.5% to 53.8% in the presence of malic acid, expressed in total sugar content.

What conclusions can be drawn from these investigations? First of all, we can point out that most of the lactose did neither disappear nor degrade nor get damaged but got transformed into an oligomer or polymer that manifests Fehling's reaction to a minimum extent. However, when we used hydrochloric acid to transform the oligomers and polymers into mono- or disaccharides, the resulting sugar-like substances (most probably, glucose and galactose for the most part and lactose to a lesser extent) did manifest Fehling's reaction and could be determined as total sugar.

5.3.3 Enzymatic treatment of the 'obtained' prebiotic with *amylase*

The same samples were hydrolysed with *amylase* as well, thus modelling the reactions taking place in the forward section of the gastrointestinal tract. Following hydrolysis with *amylase*, total sugar content remained practically unchanged, meaning that *amylase* did not react naturally with disaccharide lactose and was not able to split the oligo- and polysaccharide derivatives. This way, the not yet identified, presumably oligomer or polymer product has all the conditions to become a prebiotic, that is, it is not degraded in the forward section of the gastrointestinal tract and most probably gets into the colon, where it can serve as a nutrient for the probiotics found there.

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Production of protected amino acids using the reaction between hydroxycarboxylic acids and amino acids as well as binding on the bentonite

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Abstract. We have developed methods for the production of protected methionine and protected lysine, making use of the reaction between citric acid and malic acid as well as methionine and lysine, on the one hand, and of the interaction between swollen bentonite and the two amino acids, on the other hand. Our in vivo and in vitro experiments have demonstrated that one part of the amino acids transformed during the reaction, while another part bound on the bentonite's surface to a significant degree. Assisted by the reaction between hydroxycarboxylic acids

Keywords and phrases: methionine, lysine, essential amino acids, protected amino acids, rumen degradability, microbial protein synthesis, limiting amino acids, protein quality, biological value of protein

and amino acids, we achieved a protection of about 75% for methionine and 60% for lysine, that is, 25% of the methionine and 40% of the lysine appeared in the free amino acid fraction. The swollen bentonite bound 75% of the added methionine and 60% of the added lysine. Our chemical analyses have demonstrated that through the time–temperature combinations applied by us the methionine and lysine do not undergo significant degradation and can be fully released from the protected form. Further, our in vitro experiments using rumen fluid from fistulated cattle showed that during the average retention time of the fodder in the rumen the protected amino acids will resist microbial enzymes and maintain their protected status during their presence in the rumen.

1 Introduction

Our ruminant domestic animals can provide us with an adequate amount and quality of food products of animal origin if, on the one hand, we ensure for them degradable nitrogenous substances, first of all, protein, necessary for the development of the ruminal microorganisms, and, on the other hand, if we provide the animals with proteins and amino acids that do not degrade in the rumen but, at the same time, can optimally complement the amino acid composition of the proteins produced by bacteria. Once these two requirements are met, the ruminant animal (cattle) will be able to make maximum use of its genetic make-up to produce a great amount of good-quality milk and meat. Thus, rumen-degradable (RDP) and rumen-undegradable dietary protein (UDP) must achieve an optimal quantitative balance in ruminants' fodder (*Schwab*, 1995; *Rode* & *Kung*, 1996).

Proteins are the most valuable components of the fodder, wherefore their optimal utilization and their availability in the animal are indispensable. Optimal protein supply and proteins with appropriate amino acid composition make possible the production of a great amount of good-quality milk and meat protein.

A potential solution for this is the use of rumen-undegradable, protected proteins and amino acids (*Arambel et al.*, 1987; *Ayoade et al.*, 1982; *Buttery et al.*, 1977; *Kamalak et al.*, 2005; *Schwab*, 1995).

In dairy cattle, especially in the first trimester of lactation, protein is the limiting component in cattle fodder since the protein content of the dry matter in the fodder intake cannot satisfy the protein needs of dairy production (*Robinson et al.*, 1992; *Olmos Colmenero* & *Broderick*, 2006). The least efficient solution is fodder supplementation with more and more proteins as most

part of them is degraded by the ruminal microorganisms that build up their own proteins from the ingredients obtained this way. According to examinations (*Rode & Kung*, 1996), a mere 25–35% of dietary intake protein reaches the small intestine, wherefrom amino acids degraded by digestive enzymes may be absorbed. A much more effective way is to use so-called bypass proteins (rumen-undegradable intake protein, UIP), which ruminal microorganisms can not degrade, and so they can get to the small intestine (*Calsamiglia & Stern*, 1993). Yet another expedient solution may be the application of protected amino acids (rumen-protected amino acids, RPAA), which, among others, can counterpoise the ill-balanced amino acid composition of the fodder (*Chalupa*, 1975).

When it comes to animals, we cannot talk of protein needs but rather of essential and non-essential amino acid needs as these are the building blocks of their body proteins and contribute to producing foods of animal origin such as milk, meat, or eggs. In terms of dairy production, methionine and lysine enter the category of limiting amino acids as the protein produced by ruminal microorganisms cannot meet dairy production needs regarding these two amino acids, and thus it cannot satisfy the lysine and methionine needs of milk protein synthesis (*Broderick et al.*, 1974; *Lee et al.*, 2012, 2015).

This statement holds true for the amino acid content of microbial protein too, which is well-balanced for cattle needs and which covers approximately half of the amino acid needs of the cattle. Therefore, microbial protein is of fine quality for cattle, but in cases of high milk production lysine and methionine deficiency may develop (*Chalupa*, 1975; *Mepham*, 1982).

Protozoa's protein contains more lysine and less methionine compared to bacteria, but this difference is not significant as their contribution to meeting the amino acid needs of the cattle is not a substantial one (*Harrison et al.*, 1979). Microbial protein synthesis is limited by the energy necessary for fermentation and by the proportion of feed protein degradable in the rumen, which is why in many cases microbial protein cannot meet the amino acid requirements of animals with high milk production (*Doepel et al.*, 2004).

2 Literature review

Recently, a great many types of rumen-ungradable proteins have been applied in an effort to meet animals' amino acid needs. Through various technological treatments, these proteins were made more resistant to the microorganisms' enzymes (*Waltz & Stern*, 1989). However, the composition of these amino

acids leaves much to be desired as there are very few proteins with an optimal methionine and lysine content (*de Boer et al.*, 1978). First of all, heat treatment is applied to these protein resources, during which indigestible Maillard reaction products are created, while such cross-links are formed between the proteins that can resist the proteolytic enzyme of the microbes (*Ashes et al.*, 1984; *Broderick & Craig*, 1980). Nevertheless, heat treatment must be approached very cautiously as it may cause losses in the case of heat-sensitive amino acids such as lysine, methionine, or cystine.

Since there is no ingredient to be used for the production of microbial proteins, rumen-undegradable proteins can reduce the microorganisms' protein synthesis as well. Not any rumen-ungradable ingredient has been found that could fully satisfy the amino acid needs of dairy production; what is more, the majority of such proteins turned out to be deficient in several essential amino acids (*Wang et al.*, 2016). The best way to address this issue is to apply a combination of several proteins of this kind, which will complement one another. In addition, some experiments performed with rumen-undegradable proteins showed a reduced amount of milk and milk protein content; therefore, it is extremely difficult to assess the outcomes of feeding such protein (*Rossi et al.*, 2003).

Among essential amino acids, lysine is the limiting element when animals are fed grain-based fodder, whereas methionine becomes limiting when animals are fed leguminous plants or, formerly, fodder of animal origin. *Schwab et al.* (1992a,b) established that in terms of dairy production protected lysine is of greater importance at the culmination of lactation production when compared to protected methionine, while in mid-lactation period both of them are equally significant in this perspective. Perhaps, this can account for the differences found when cows in various lactation stages were fed exclusively protected methionine (*Vyas & Erdman*, 2009). *Schwab et al.* (1992a) observed that feeding with protected methionine and protected lysine increased dairy production and milk protein content, which was a more striking increase regarding casein, a rather essential element in terms of cheese production.

In some experiments, the amount of milk and milk protein content increased due to protected methionine and protected lysine, some other experiments found no changes in this respect (*Trinacty et al.*, 2009), while in most experiments amino acid supplementation did not influence milk fat matter (*Buttery et al.*, 1977). In a few cases, protected methionine increased fibre digestibility and the amount of milk fat; the methionine may have even played a role in milk fat synthesis and metabolism (*Giallongo et al.*, 2015). The aforementioned let us conclude that the effect of methionine depends on lactation conditions, fod-

der quality, and the animal's physical condition, wherefore we cannot clearly outline the actual effect (*Weber et al.*, 1992; *Donkin et al.*, 1989; *Patton*, 2010).

The amount of amino acid supplementation necessary for cattle is yet another factor that is hard to define sometimes. According to *Schwab* (1995), requirements can be expressed in absolute terms (g/day) or can be given in relative values, expressed as a percentage in comparison with the amino acid composition of the fodder. About lysine content, for instance, it is being maintained that it has to cover 15% of all absorbable essential amino acids, while for methionine this value is only 5.3%. These values are considerably higher than what is available for cattle under normal feeding conditions. Applying an indirect approach, *Socha* and *Schwab* (1995) found that lysine supplementation had a much greater impact on the amount of milk and milk protein content than methionine supplementation. When the amount of lysine was below 14% of the essential amino acids, methionine supplementation reduced both milk protein content and the amount of the produced milk protein. Therefore, assessing the rate of the optimal lysine and methionine supplementation for traditionally fed cows becomes particularly difficult (*Loerch* & *Oke*, 1989).

Free amino acids are quickly broken down by the enzymes of ruminal microorganisms, wherefore these are not suitable either for mitigating the amino acid deficiency of the cattle or for supplementing the protein having an inadequate amino acid composition. However, protected amino acids are not degraded in the rumen but produce their effect via absorption in the forward section of the small intestine. Several procedures have been worked out to produce protected amino acids, which need to meet the following criteria: resistance to microbial degradation, being released in the abomasum or the forward section of the small intestine, absorption in the small intestine, and contribution to meeting the animal's essential amino acid needs. Additionally, they should also withstand technological interventions and low silage pH and make their way safely to the small intestine.

Plenty of methods have been elaborated for the production of both the protected proteins and protected amino acids. The best known of these are heat treatment and chemical modification, but their combination is not a rare thing to come across either. An outstanding compilation of these methods can be found in *Schwab's* (1995) publication.

Studies on free amino acids quickly pointed out that in the growth stage of ruminants the two limiting amino acids of protein formation are lysine and methionine. Nitrogen retention experiments carried out on lambs found methionine to be the first and lysine the second limiting amino acid. Thus, in accordance with the foregoing findings, ruminants' primary needs are protected

lysine and protected methionine supplementation in order for their optimal growth and then milk production capacity can be made full use of (*Schwab* & *Broderick*, 2012).

A great number of procedures have been put together to produce protected amino acids. Most important of these are as follows: the application of amino acid analogues, or amino acid derivatives in feeding (*Amos et al.*, 1974; *Ayoade et al.*, 1982); lipid coating (*Neudoeeffer et al.*, 1971); encapsulation with pH-sensitive materials that withstand ruminal conditions but are decomposed in the abomasum and the small intestine, thus releasing the amino acids (*Schwab*, 1995).

In the early 1970s, the amino group or the carboxyl group was modified or such derivatives were created where the amino group was replaced by some other (e.g. hydroxyl) group, this way protecting the amino acid from rumen degradation (*Griel et al.*, 1968; *Jones et al.*, 1988). It is highly essential that such a protected derivative should transform in the small intestine into the original amino acid, be absorbed, and contribute to meeting the animal's amino acid needs (*Mir et al.*, 1984). Particularly in the case of methionine were such analogue compounds created and tested in the context of in vivo experiments. Most researchers investigated those derivatives where long-chain fatty acids were linked to the amino group, protecting it from rumen degradation (*Langar et al.*, 1978). Many studied the hydroxy analogue of methionine, the N-hydroxymethyl-DL-methionine-Ca and the di-hydroxymethyl-L-lysine-Ca. Since in the abomasum only a small amount of these derivatives reverted to the amino acid under discussion, they cannot be considered protected amino acids (*Weber et al.*, 1992; *Kenna* & *Schwab*, 1981).

Another possible method for developing the ruminal protection of amino acids is coating them with materials that can resist the microorganisms' enzymes and that are degraded in the abomasum alone or, further on, in the small intestine, during which amino acids are released from the coating. Fats and oils were frequently utilized to this end, often in combination with inorganic materials and carbohydrates, such as stabilizing agents, but plasticizers and fillers were also used to ensure protection. Since protection ensured their safe use, these materials were widely applied in practice, although many of them proved to be inadequate for the protection of methionine (*Loerch*, 1989).

The very first protected methionine was made up of 20% of DL-methionine, 20% of kaolin, and 60% of tristearin, of which 65% of the methionine was absorbed from the small intestine. From most similarly protected preparations, 70–80% of the methionine was absorbed and 20–30% of it was eliminated along with the faecal matter (*Lapierre et al.*, 2006, 2012). In the next step, such

coating materials were also developed on which amino acids are released in smaller quantities, making thus possible that an optimal amount of them be available at all times, bringing about a significant increase in their utilization.

Such pH-sensitive polymers have also been applied that maintain their stability in ruminal conditions but are degraded due to low abomasum pH, releasing the amino acids protected this way. Various polymers and copolymers were developed to suit this purpose, enabling the amino acids' protection against digestive enzymes during their presence in the rumen up to 48 hours.

In summary, it has become clear that the two limiting amino acids of meat and milk production are lysine and methionine, as the otherwise good-quality microbial protein does not contain a sufficient amount of any of them to satisfy the needs of animals with high milk production. This supplementation cannot be carried out with free amino acids as they are subjected to degradation by the microorganisms' enzymes, and microbial protein is re-created from degradation products. The only solution is the production and application of protected amino acids, which are able to withstand ruminal conditions prevailing in the rumen as well as microbial enzymes and which make their way to the abomasum and the small intestine to be released and become available for the animal to facilitate the production of large amounts of good-quality food of animal origin.

3 Justification and objectives of the experiment

The aim of our research was to create protected amino acids by applying a novel method, never used before and yet unknown to specialized literature. We tried to address the issue in two different ways. We attempted to produce protected amino acids via the chemical reaction between di- and trihydroxycarboxylic acids, on the one hand, and by exploiting the connections existing between clay minerals and amino acids, on the other. Our intention was to test amino acid 'protection' with in vitro and in vivo experiments.

4 Materials and methods

4.1 Experiments performed with citric acid and malic acid for the production of protected methionine and protected lysine

Dry heat treatment of amino acids and hydroxycarboxylic acids

1 g of citric acid was added to 9 g of lysine and the same amount of citric acid was added to 9 g of methionine, while in the subsequent experiment citric acid was replaced by malic acid. Heat treatment was performed at a temperature range of 140–170 °C for 30–60 minutes. Afterwards, the amount of citric acid and malic acid was increased to 50%, and heat treatment was repeated at 170 °C for 30–60 minutes.

Aqueous heat treatment of amino acids and hydroxycarboxylic acids

Following experiments performed under dry conditions, the mixture of citric acid, malic acid, and amino acids was admixed with an amount of distilled water sufficient to obtain a slurry, whereafter heat treatment was performed at a temperature range of 140–170 °C for 30–60 minutes followed by measuring the amount of free lysine and free methionine.

4.2 Production of protected methionine and protected lysine with the use of bentonite

Dry mixing of bentonite and amino acids

At first, we tried to link lysine and methionine to bentonite in a dry mixture for various durations. 20 g of lysine hydrochloride was mixed with 80 g of bentonite, ground, and then treated at 100 °C in an exsiccator for 5-10-15-20-30-40-50-60-120, and 180 minutes. The same experiment applying the same temperature and the same duration was repeated with methionine. Finally, the free methionine and free lysine content of the treated samples was determined.

Aqueous mixing of bentonite and amino acids

In the next series of experiments, bentonite was activated via swelling and suspension by distilled water so that the active groups can bind the amino acids under discussion. In the first stage of the wet experiment, the bentonite was swollen in distilled water overnight, and then a concentrated solution of methionine and lysine was added to the swollen bentonite. Thereafter, the mixture of bentonite and amino acids was suspended in an amount of distilled water sufficient to obtain a low-viscosity mixture, the resulting suspension was left to stand overnight, and the next morning it was subjected to heat treatment at 100, 150, and 200 °C for 5-10-15-20-30-40-50-60-120, and 180 minutes. In the course of heat treatment, particular attention had to be paid to choosing the moment of measurement carefully: the measuring process had to

be initiated once the water had been completely vaporized as the temperature of the wet sample can hardly be over 100 °C. Then, an analysis was carried out on the composition of the bentonite–amino acid complexes heat treated at various temperatures and created via wet procedure. Parallel to the distilled swelling experiments, we attempted to swell the bentonite–amino acid mixture in 0.1 mol as well as 1 mol hydrochloric acid, whereafter an analysis of amino acids was performed.

During our subsequent experiments, we added 40 g of lysine-hydrochloride and 300 cm^3 of water to 160 g of bentonite, and then we repeated the experiment with the same amount of methionine instead of the lysine-hydrochloride. With both amino acids, we obtained a slurry-like liquid mixture. We waited until the liquid had evaporated, and then the remainder was heat treated at 200 °C in an exsiccator for one hour. The obtained samples were utilized in two types of experiments.

4.3 In vitro experiment with living ruminal flora and fauna

1 g of lysine was added to 20 cm^3 of rumen fluid, containing living microorganisms, of a Holstein-Friesian cow found in its mid-lactation period and having a ruminal fistula. In a further experiment, 1 g of methionine was added to the same amount of rumen fluid, which we considered as a control sample. Following this, we repeated the above two experiments, this time using 'protected' methionine and lysine of the same amount as previously specified. The samples were incubated at 38–39 °C in an exsiccator for 16 hours, whereafter they were hermetically sealed and stored in a freezer until determination of the amino acids.

During the analysis of the rumen fluid samples, we added 2.5 cm^3 of 6% sulphosalicylic acid to 5 cm^3 of rumen fluid for the purpose of protein precipitation. After shaking it up and leaving it to stand for 10 minutes, we centrifuged it for ten minutes at 6,000 rotation/minute. Following centrifugation, we set pH to 2.2, but there was actually no need for us to set the pH because the pH of the ruminal fluid decreased exactly to this value upon addition of the sulphosalicylic acid. After protein precipitation and centrifugation, the solution remained turbid; therefore, the samples were filtered prior to determination of amino acids, and then we applied decimal dilution to determine the free methionine and free lysine content of the rumen microflora.

4.4 In vivo experiments with fistulated cows

The in vitro experiment was also performed with fistulated cows under in vivo conditions. The in vivo experiments were carried out on four fistulated cattle, applying the nylon bag technique, during which we added 10 g of 'protected methionine' as well as 'protected' lysine to the nylon bag. Following a 16-hour-long storage in the rumen and upon drying the bags, we measured their free methionine and lysine content. Throughout the experiment, we made use of the same samples that were used with the ruminal fluid.

4.5 Determination of the samples' amino acid composition with ion-exchange column chromatography

Amino acid analyser type INGOS AAA400 was used to determine the free lysine and free methionine content of the samples. The ion-exchange column was a 35 × 0.37 cm OSTION Lg ANB, containing sulphonated polystyrene synthetic resin cross-linked with divinylbenzene. The pH and concentration of the applied sodium citrate buffers varied as follows: 1: pH 2.7, 0.2 M Na^+ 0–29 minutes; 2: pH 4.25, 0.5 M Na^+ 29–44 minutes; 3: pH 6.9, 1.12 M Na^+ 44–66 minutes; 4: 0.2 M NaOH 66–71 minutes, equilibration with buffer 1: 71–101 minutes. The temperature programme was as follows: 0–29 minutes: 50 °C, 29–44 minutes: 60 °C, 44–66 minutes: 74 °C, 66–74 minutes: 60 °C, and 74–101 minutes : 50 °C. A detailed description of the method can be found in the work of *Csapó et al.* (2008).

5 Results and conclusions

5.1 Experiments performed with citric acid and malic acid for the production of protected methionine and protected lysine

Malic acid is such a hydroxydicarboxylic acid that may be capable of reactions with amino acids via both its carboxyl groups and hydroxyl group. Citric acid is a hydroxytricarboxylic acid with three carboxyl groups and one hydroxyl group, which may likewise be suitable for reactions with both lysine and methionine. Ester linkages might also be formed between the carboxyl group of the amino acid and the hydroxyl group of the hydroxy acids, but acid anhydride bond is also possible to take place between the carboxyl group of the amino acids and the malic acid as well as the carboxyl groups of the citric acids. In addition to the above, we may also consider a reaction of the

two and three carboxyl groups of the malic acid and citric acid, respectively, with lysine and methionine. Furthermore, we cannot exclude either that the carboxyl group of the hydroxy acids will form a quasi-peptide bond with the a-amino group of the amino acids or that one of the carboxyl groups of the carboxylic acids will react with the carboxyl group of the amino acid and the other one with the amino group of the amino acid, creating condensation polymers of higher molecular weight. In the case of lysine, a new possibility arises due to the e-amino group, which can also react with the carboxyl group of the carboxylic acids.

Dry mixing of amino acids and hydroxycarboxylic acids

Since the melting point of the malic acid is between 129 and 133 °C and that of the citric acid is 153 °C, we considered as a first step to melt a proper proportion of the mixture of the two amino acids and the two carboxylic acids at a temperature of 160–170 °C in the hope that a reaction would take place between the carboxylic acids and the amino acids. After a half-an-hour-long heat treatment at 140–170 °C, no reactions were observed whatsoever with either any of the amino acids or any of the carboxylic acids, meaning that the free amino acids were assayed by weighing with the help of amino acid analysis, in the same amount as the sample that was not subjected to heat treatment. Thereafter, the amount of citric acid and malic acid was increased to 50% and heat treatment was repeated at 170 °C for half an hour. Again, the dry heat treatment yielded no results, leading us to the conclusion that in solid phase, at 170 °C no significant reaction took place between the carboxylic acids and the amino acids despite that both carboxylic acids had melted.

Aqueous mixing of amino acids and hydroxycarboxylic acids

Following experiments performed under dry conditions, the mixture of citric acid, malic acid, and amino acids was admixed with an amount of distilled water sufficient to obtain a slurry, whereafter heat treatment was performed at a temperature of 170 °C for one hour followed by measuring the amount of free lysine and free methionine.

We analysed the following: the lysine content of the mixture dry treated (170 °C, one hour) with citric acid and malic acid; the methionine and lysine content of the samples treated (170 °C, one hour) with citric acid and malic acid, suspended in water; the composition of the samples treated with citric acid and malic acid; ran a full analysis of the free lysine content of the sample heat treated under aqueous conditions and of the lysine content after

hydrolysis by hydrochloric acid. Chromatograms reveal that in the case of the sample treated with citric acid there is no ninhydrin positive compound on the chromatogram other than lysine and that, apart from lysine, there is no degradation product present in a significant concentration even after hydrolysis by hydrochloric acid. The same treatments and analyses were performed with methionine as with lysine, while the amount of methionine also underwent analysis as to how it changed as a result of the various treatments.

During the experiment performed with methionine, increasing the concentration of the citric acid and malic acid was not particularly successful in the case of wet treatment because in both hydroxycarboxylic acids 75% of the methionine transformed into some kind of compound, it was not detectable from the free amino acid fraction, and 25% of it remained free in the case of both amino acids. The same was the case with lysine, except that approx. 60% of it transformed and 40% remained in the free amino acid fraction.

We performed 6M hydrochloric acid hydrolysis of the obtained compound at a temperature of 110 °C for 24 hours, and methionine as well as lysine concentration was measured following hydrolysis. Results looked promising as, subsequent upon hydrolysis, we have retrieved nearly 100% of the amino acids in question as compared to the control, meaning that amino acids did not break down during the reactions performed with carboxylic acids but merely transformed into such a product that could be transformed back into the amino acid under discussion by the hydrochloric acid hydrolysis applied in the process of determining the amino acid composition of the protein.

5.2 Production of protected methionine and protected lysine with the use of bentonite

In our experiments, amino acid analyser was applied to determine the lysine and methionine content of the utilized 'lysine-hydrochloride' as well as 'methionine'. The lysine content of the 'lysine-hydrochloride' was measured to be 79.48%, whereas the methionine content of the 'methionine' was 99.77%. Upon adding 20 g of lysine-hydrochloride to 80 g of bentonite, we obtained a lysine content of 15.91%, while upon adding 20 g of methionine to 80 g of bentonite resulted a methionine content of 19.97%.

Following a heat treatment performed at 200 °C for one hour, we could assay by weighing 9.73% of the lysine content of approx. 16%. Therefore, it appears that 40% of the lysine was bound by the bentonite under these experimental conditions, while 60% of it was still present in its free form in the sample. In the case of methionine, we could assay by weighing 4.93 g of the methionine

content of 20%, which means that nearly 75% of the sample was bound to the bentonite and only 25% of it remained in its free form.

After heat treatment at 200 °C, a full-scale analysis was performed with both the lysine and the methionine, during which we were interested in knowing whether the amount of amino acids would decrease while under heat treatment and whether any kind of by-product would be produced. In the course of the analysis, we established that lysine content remained practically unchanged throughout the heat treatment, while in the case of methionine we could detect in the sample a minimum amount of methionine sulphone, the oxidation derivative of methionine, which, however, did not reach 5% in any of the cases.

As we observed a protection of 40–80% in the case of both lysine and methionine, we performed the experiments with the use of ruminal fluid as well, where in the case of methionine and upon adding the control (non-protected) methionine, we could assay by weighing 0.87 g of methionine, whereas upon addition of protected methionine to the ruminal fluid the assay by weighing yielded 0.30 g. This means that approx. 65% of the methionine remained in protected form during the experiment with the ruminal fluid, that is, nearly 80% of the protected methionine preserved its protected form following the experiment with the ruminal fluid. This is due to the fact that only 75% of the material used was protected, 25% of it was in free form, which probably dissolved right away into the ruminal fluid, while in what followed a further 10% would dissolve from the 75% protected quantity, whereupon we assessed the protected status of the obtained material to be at 65% after exiting the rumen.

In the case of lysine, upon treatment with ruminal fluid, the lysine content of the control sample (pure synthetic lysine) was assayed by weighing 0.44 g, while the lysine content of the protected lysine was 0.35 g, which means that a total of 21% of the sample's lysine content remained protected after the treatment with ruminal fluid; thus, 80% of it dissolved into the ruminal fluid.

In terms of protection, the in vivo experiment yielded results that were 50% worse as compared to the in vitro experiment performed with ruminal fluid, an outcome most probably due to the fact that part of the methionine and lysine linked through the pores of the nylon bag to the fine, powdery bentonite fell out of the nylon bags mechanically, which reduced the amounts of protected methionine as well as protected lysine by 50%.

In conclusion, if the bentonite undergoes a minimum of 8-hour-long swelling process in (distilled) water, then, according to chemical analyses, it will become capable of binding 40% of the lysine and about 80% of the methionine

following heat treatment and upon 20% of added lysine and the same amount of added methionine. From this amount of lysine, 25% and from the methionine 65% remain bound to the bentonite even after treatment with ruminal fluid containing live rumen bacteria. When the experiment was performed with fistulated cows, protection decreased by 50%, which was first of all not owing to the different physiological conditions but rather to the fine, granular, protected, and bentonite-bound amino acids falling out of the bags.

6 Discussion of the results

An important question is whether the 40% of protected lysine and the 80% of protected methionine are sufficient amounts under conditions applied as previously described. We could possibly enhance the amino-acid-binding effect of the bentonite by increasing the concentration of the amino acid solution as well as the applied temperature. Heat should not be a concern as the examinations carried out have demonstrated that none of the amino acids undergoes relevant changes at 200 °C.

We could carry on the experiments with the rest of the essential amino acids. Leucine, isoleucine, and valine are highly resistant to all external effects, but certain problems may arise with threonine and tryptophan as both of them, especially the latter one, are extremely sensitive to acidic conditions.

Besides in vitro experiments, others could be performed too with the already protected amino acids and with the use of duodenal-fistulated cows, as all we know about these amino acids is that they are protected in the rumen, but it would also be an outstanding achievement if we could obtain some evidence of their absorption and availability in the small intestine.

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The role of selenium in nutrition – A review

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Abstract. The role of selenium has been changed over the last decade. The element that was previously considered to be toxic turned out to be present in the human body in amounts of 10–15 mg, and almost every cell of our body contains it. Selenium contributes to growth, supports healthy muscle activity, reproductive organs, reduces the toxicity of certain elements such as mercury, supports the immune system, and even delays the spread of certain viruses (influenza, Ebola, HIV). Selenium-deficient areas of Europe could be a risk for their populations. The recommended daily intake (RDA) of selenium is 55 µg/day, while WHO and FAO have set up the daily tolerable dose at 400 µg/day. We must count with the harmful effects of selenium overdose, but it is almost impossible to introduce this amount into our body solely with food. Our selenium sources can be refilled with food supplements or selenium-enriched functional foods. In the review article, we report about the role of selenium in the environment, selenium-enriched plants, selenium-enriched yeast, the role of selenium in animal feed and in the human body, the opportunities of selenium restoration, selenium-enriched animal products, and the selenium content of milk.

Keywords and phrases: selenium, selenium supplementations, seleno-enzymes, seleno-amino acids, selenocysteine, selenomethionine, nutrition, enriched animal products, functional foods, milk

1 Introduction

The role of selenium has been considerably appreciated thanks to recent years' research. Selenium was discovered in 1817 by *Berzelius* and *Gahn* (*Széles et al.*, 2007). In the 1930s, it was considered that selenium is a toxic heavy metal, which in higher doses leads to the destruction of the living organism (*Vernie*, 1984). In 1943, its carcinogenic effect has been described (*Nelson et al.*, 1943). Some years later, *Clayton* and *Baumann* (1949) found out that selenium supplementation decreases the number of tumorous cases. The essential role of selenium was first published in 1957, when rat experiments proved that selenium added to food prevented the necrosis of the liver (*Schwarz* & *Foltz*, 1957). From 1966, we can read about the anti-cancer effects (*Shamberger* & *Rudolph*, 1966). The activity of selenium-dependent enzyme proteins was studied in 1973 (*Turner* & *Stadtman*, 1973). Glycine reductase and glutathione peroxide is found in bacteria and mammals (*Rotruck et al.*, 1973). In 1976, the chemical characterization of the selenoprotein component of *glycine reductase* was studied, and selenocysteine as the organoselenium moiety was identified (*Cone et al.*, 1976).

Researchers have found that if so many selenium forms are known, no selenium content of the organism can be deduced from the selenium yield. According to *Thomassen* and *Nieboer* (1995), we must count with toxicity, accessibility, and the study of absorption in the organism. The oxidation state and the complex training with other substances must be investigated, and thus the distribution and volume of the selenic alterations can be determined by means of speciation analysis (*Ebdon et al.*, 2001).

2 Selenium in the environment

Selenium is rarely found in its elemental form in the environment (*Craig*, 1986). Soil, water, and all living organisms contain –2 (selenide), +4 (selenite), and +6 (selenate) oxidation status (*Gómez-Ariza et al.*, 1998), but these forms depend on the environmental effects (*Skinner*, 1999). Selenates and selenites are water-soluble, so they occur most often in these forms in water (*Gómez-Ariza et al.*, 1999).

Besides the inorganic alterations, there are so many forms of organic bindings in which selenium is present as selenide (*McSheehy et al.*, 2000; *Michalke et al.*, 2001). They are mostly seleno-amino acids or their derivatives. Foods of plant origin include selenomethionine and those of animal origin contain selenomethionine and selenocysteine. Selenomethionine is essential to humans

and animals, but they can produce selenocysteine from selenomethionine in the organism (*Beilstein & Whanger*, 1986).

3 The role of selenium in the human body

Selenium plays a significant role in many physiological processes in a direct or indirect way. Our foods contain selenomethionine and selenite. Selenite is reacting in our organism with the thiols in the effect of glutathione, and then it forms H_2Se. Selenocysteine is formed from selenomethionine through various processes. Selenocysteine decomposes into hydrogen selenide as the result of the β-*lyase* enzyme. Approximately 90% of selenocysteine integrates into the proteins of our organism (*Mitchell et al.*, 1976). The amount of these selenoproteins decreases when the diet is incomplete. Most of them have been identified in the 20th century, such as the *iodotyrosine deiodinase*, which is responsible for the activation of thyroid hormones (*Allan et al.*, 1999), or selenoprotein P (*Ungvári*, 2015).

With the average selenium intake, the excess is excreted in the urine as a seleno-amino sugar in large quantities, it is excreted through the respiratory processes as dimethyl-selenide, while the urine may excrete trimethylselenium ions (*Suzuki & Ogra*, 2002; *Kobayashi et al.*, 2002; *Bendhal & Gammelgaard*, 2004).

The most important role of selenium is its antioxidant effect. This is expressed by the interaction with various enzymes (*Awashti et al.*, 1975; *Molnár*, 2013; *Rigó*, 2002). It plays a key role in the function of the *glutathione peroxidase* enzyme, which responds to hydrogen peroxide and other harmful lipids and phospholipid hydroxides to prevent harmful free radicals, inhibit DNA damage and the development of metabolic active carcinogens (*Karag et al.*, 1998). The amount is determined by the amount of selenium and reduced glutathione in our body (*Meister & Anderson*, 1983). Selenium is incorporated into the enzyme as selenocysteine, where the sulphur is located. In the body's antioxidant defence system, the biochemical property provides the importance of selenium to reduce the amount of sulphur more easily (*Cser & Sziklai-László*, 1998).

The *iodotyrosine deiodinase* enzyme assists in the production and function of T_3 and thyroxin hormones, wherefore selenium is essential for growth and normal thyroid function (*Wilson et al.*, 1992; *Holben & Smith*, 1999). *Thioredoxin reductase* regulates cell growth (*Mustacich & Powis*, 2000), while selenoprotein N is responsible for normal muscle development (*Zhang et al.*, 2012).

The antioxidant effect of selenium can prevent the oxidation of LDL cholesterol (*Gey*, 1998), reduce inflammation, strengthen the immune system, help protect the body against oxidative stress, and thereby indirectly reduce HIV virulence (*Dworkin*, 1994; *Stone et al.*, 1997; *Weeks et al.*, 2012). Selenium reduces some toxic elements, such as mercury toxicity, via inhibiting their absorption by forming insoluble compounds (*Feroci et al.*, 2005).

In the case of people on a healthy diet, the risk of selenium deficiency is small, but in selenium-deficient areas, such as Hungary or Germany (*Gondi et al.*, 1992), there may be a health risk of persistent selenium nutrition (*Ellis & Salt*, 2003).

Selenium deficiency affects about half a billion people annually (*Combs*, 2001). Many diseases may develop and exacerbate, for example, depression (*Finley & Penland*, 1998), cardiovascular disasters, tumour disorders, thyroid dysfunction, or spread of viruses (influenza, HIV, Ebola) (*Tamás*, 2000); it weakens the viability of sperms (*Reilly*, 1998), but some studies also write about the selenium's capability of delaying ageing (*Bankhofer*, 1988).

Selenium deficiency may cause Keshan disease, which has been discovered in China – it mainly affects children and causes cardiopulmonary dysfunction, leading to myocardial infarction. The Kashin–Beck syndrome (degenerative joint disease) can also be linked to selenium deficiency (*Burke & Opeskin*, 2002). Recent studies have shown that inadequate selenium supply can be associated with Down's syndrome and the development of infant cretinism (*Ani et al.*, 2007, *Chanoine*, 2003).

The normal human body (60–70 kg) contains 10 to 15 mg of selenium. Almost every cell of our body contains it, but most of the selenium accumulates in the kidneys, liver, spleen, pancreas, and testes. According to Codex Alimentarius Hungaricus (*152/2009 (XI. 12) FVM*), the recommended daily intake (RDA) of adults is 55 μg/day. According to the Institute of Medicine, Food and Nutrition Board (2000), the maximum limit of Se is 400 μg/day over which negative selenium effects are expected (*Arthur*, 1991). The first symptoms of selenium poisoning are metallic mouth taste, garlic smell breath, in chronic cases, hair loss, the loss of nails, skin rashes, discolouration of the teeth, and ultimately neurological disorders. Acute selenium toxicity only rarely causes death, and the lethal dose of selenium is 5–10 mg/kg (*Olson*, 1986). This quantity cannot be taken in with food (*Ungvári*, 2015).

Table 1: Comparison of Recommended Sun Intake (RDA) and Maximum Tolerable Volume (UL) by age

Age (year)	RDA (μg Se/day)	UL (μg Se/day)
1–3	20	90
4–8	30	150
9–13	40	280
14–18	55	400
19–	55	400

Source: Institute of Medicine, 2000

4 Opportunities of selenium restoration

The selenium content of foods is highly varied. The South American Brazilian walnut (*Bertholletia excelsa*) has the highest selenium content, more than 100 μg per piece (*Chang*, 1995). The richest selenium source among our foods is animal organs (animal meat) and seafood. Since the content of selenium in foods consumed during a daily meal is not significant, increasing the amount of food cannot increase selenium intake. Our selenium needs can be covered with dietary supplements and foods enriched with selenium. Dietary supplements can be used to satisfy our needs of nutrients and physiological substances since the 1980s. Nowadays, excellent products are available in capsules or tablets for this purpose (*Horacsek et al.*, 2006). Selenium-encapsulated food supplements have already been produced at the University of Debrecen (*Eszenyi et al.*, 2011). These products mainly contain selenium, selenate, selenomethionine, or selenium-enriched yeast. Selenium occurs naturally or in near natural form in selenium-enriched functional foods (*Csapó et al.*, 2016). When preparing foods with this technology, the plant or animal is supplied with selenium as a nutritional supplement that undergoes several transformations and reaches its natural form. During the transformations, the oxidation state of the selenium may change, and so it is important to track what form the plant or animal product contains. The cancer-preventing effect of selenium-enriched garlic has been reported (*Clement* & *Lisk*, 1995), but selenium-enriched bread, pastry, eggs, and margarine had already been on the market by then in Hungary.

5 Selenium-enriched plants

Plants can transform selenite into organic selenium form by fertilization or spraying. It is safe for animals and humans because the chances of overdose with selenium consumed with plant foods are low (*Terry et al.*, 2000). Even in selenium-rich soils, the selenium content of plants does not reach 10 mg/kg in dry matter. Most plants contain only 1–2 mg/kg of Se, but there are some that can accumulate a larger amount. Plants belonging to the family of *Brassicaceae* and *Fabaceae* can produce up to several thousand milligrams of selenium in a kilogram of dry matter (*Ellis & Salt*, 2003). This can be explained by the fact that the plants mainly synthesize methylselenocysteine, which is stored for a long time (*Brown & Arthur*, 2001), but it is not incorporated into plant tissues; they can also be used for the purification of toxic soils (*Bañuelos et al.*, 2011). In the case of normal Se content, the element is incorporated into the plant proteins as selenocysteine and selenomethionine. Selenium-enriched garlic and green onion, chive and broccoli contain selenium in the form of methylselenocysteines. Wheat, maize, rice, and soybean selenium forms (*Beilstein et al.*, 1991; *Tamás & Csapó*, 2015) were investigated, and it was found that selenium is mostly present as selenomethionine in these plants.

In the case of plants, fertilizers or foliar fertilizers with different selenium contents may be used to increase the selenium content. In the case of animals, inorganic selenium formulations can be used to increase the selenium content of animal tissues, but this is better if the feed contains organically bound selenium such as selenium-enriched fodder (*Hidiroglou & Jenkins*, 1975).

6 The role of selenium in animal feed

How selenium deficiency does affect the function of animal organisms? *Florian* and his colleagues (2010) found that lesion of the colon has occurred in mice raised with selenium-poor feed. In the case of animals, selenium shortage can lead to muscular dystrophy anaemia, growth disorders, infertility, heart diseases, and increasing taint of diseases (*Dredge*, 2005). As for cattle, the shortage of selenium negatively influences the production of milk, the risk of udder inflammation increases, and fertility decreases.

The supplement of the minerals can be done by salt lick in addition to forage. Before the appearance of salt lick, grinded salt was given to the animals. Since 1920, there are salt licks that are manufactured especially for animals. The researchers noticed that animals' needs of minerals are more efficiently

satisfied by blocks rather than grinded salt added to their forage (*Sampson*, 1923). Moreover, the NaCl makes animals drink more water, which promotes the milk production and the health of the livestock. On the market, many salt licks of different ingredients are available. In the USA, the colours of the salt licks indicate the different types in the following way:

- The white only contains NaCl.
- The yellow contains Sulphur.
- The red has iron and iodine added to it.
- The blue contains cobalt and iodine.
- The brown contains cobalt, iodine, copper, molybdenum, magnesium, and potassium.
- The black one contains the ingredients of the brown and selenium (*Keyes*, 2012).

It is customary in Hungary that cattle farms and bull nurseries have 60 mg/kg of selenium-containing salt blocks for animals. By adding a small amount of selenium (and partially vitamin E), lambs can be protected from white muscular disease (WMD) and pigs can be treated with vitamin E deficiency (VESD syndrome). Its effect is similar to the human mechanism through selenium-containing enzymes. In the case of selenium deficiency, the absence of thyroid hormones T3 and T4 in animals may also occur, which may also affect the animal weight benefits as well.

7 Selenium-enriched animal products

By feeding with selenium feed additives, we could produce selenium-enriched meat, eggs, or cow's milk (*Csapó & Albert*, 2018). As most of our foods contain little selenium, it would be necessary to develop functional products in order to increase the Hungarian population's selenium intake. The production of foods with an increased selenium level is relatively complicated. One of the forms of selenium is added to animal feed, whereafter it undergoes transformation in the body until it finally reaches a natural form. Since the oxidation state of selenium changes during the transformation, it is necessary to monitor what form of selenium is present in animal products.

As for pigs, feed supplementation uses organic and inorganic selenium-containing substances to increase the amount of Se in meat. In piglets and sows, the selenium content of the offal and the muscles increases with the addition of an appropriate quantity and quality of selenium (*Surai*, 2006). In

laying hens, they act in the same way so as to increase the selenium content of the egg, whereas in cattle this is a common method of selenium-enriching meat and milk.

Selenium supplementation increases the activity of selenium-containing enzymes such as *glutathione peroxidase*. Selenomethionine is incorporated into the body's protein, thereby providing the body's selenium supply.

Despite the decrease in milk consumption, it is also a main source of selenium, and so it is advisable to increase its selenium content. Knowing the selenium concentration, the health status of the stock and the udder is measurable. The addition of selenium to the feed of dairy cows allows the production of selenium-enriched milk.

8 Selenium-enriched yeast

The most commonly marketed selenium source is selenium yeast (*Schrauzer*, 2000). Selenium-enriched yeast is produced by fermenting *Saccharomyces cerevisiae* in high sodium selenite, sodium selenate, and selenomethionine medium. Yeast cells are destroyed by heat treatment, then spray dried, and finally checked for the organic and inorganic selenium content of the product. Selenomethionine can integrate into the body proteins and serve as a selenium source (*Thomson*, 2004 a,b). The total selenium content can be up to 3,000 mg/kg, which is found as selenomethionine in yeast proteins (*Polatajko et al.*, 2004), while selenocysteine is only present in small amounts (*Kotrebai et al.*, 2000). The inorganic selenium content of selenium yeast is also useful in the formation of proteins but does not become a selenium reserve (*Varo et al.*, 1988). In some European countries, selenized yeast has already been authorized as a feed additive to produce functional food. The absorption of selenium is influenced using yeast strain, the technology of production, and the selenium form (*Fox et al.*, 2005). They also found that selenium-enriched yeast is more efficiently incorporated into the body than the inorganic form, and so it is available for a longer period.

In 1993, a research group studied the use of selenium yeast and selenomethionine in breast-feeding and non-breast-feeding mothers. It was found that the levels of selenium in blood increased because of treatments. Each of the groups receiving selenomethionine increased the blood plasma selenium level; however, this was only observed in non-lactating mothers consuming selenized yeast. The amount of selenium in the milk of selenium-treated mothers increased (*Mcguire et al.*, 1993).

From the above examples, you can see that the addition of selenium yeast activates the selenium enzymes in the body and selenomethionine builds on the protein of the body to provide continuous selenium replacement, and it is slowly excreted. The absorption of selenized yeast is slower than that of selenomethionine because the former has to be dissected before the valuable substances can be released. Slower absorption is also caused by the presence of inorganic salts in addition to organic selenium formulations.

9 Milk as selenium source

In Hungary, according to the latest figures of the Central Statistical Office, milk consumption per capita was 161.0 litres in 2015. This shows a decreasing tendency compared to previous years; nevertheless, milk is a selenium source for humans as part of our basic diet. The average selenium content of milk is 25 μg/l – milk and dairy products amount to 6–10% of the daily selenium intake (*Csapó* & *Csapóné*, 2002). Selenium supplementation in cattle feed provides an opportunity to increase the Se content of milk. Se supplementation can be accomplished by feeding with selenium-enriched plants or inorganic sodium selenite or by addition of organic selenomethionine, selenocysteine, or selenium-enriched yeast. However, when selecting the additive, it is important not to ignore that selenium in the rumen of ruminant animals may be reduced to insoluble selenide or elemental selenium. Hydrogen selenide is released as rumen and intestinal gases, and the elemental selenium leaves the faeces. It is practical to favour the organic form because its absorption is more satisfying than that of inorganic form (*Bokori et al.*, 2003). Several scientists carried out experiments to measure the change of milk selenium level in selenium supplementation. *Surai* (2006) published that organic and inorganic selenium treatments increase plasma selenium level.

In 2010, Australian researchers investigated the elimination of selenium in cattle. They found that 66% of selenium intake left the body with urine, faeces, and gases, 17% of which were excreted in the milk, and 17% were incorporated into animal tissues (*Walker et al.*, 2010). An Italian research team published their results in 2010 and 2011, in which the effects of sodium selenite and selenized yeast were investigated on selenium status and milk selenium content. The incorporation of SeCys and SeMet, or selenium yeast, is more effective than inorganic forms and reduces heat stress in dairy cattle (*Calamari et al.*, 2010, 2011).

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Development of fortified bakery products based on *kokoro*, a traditional Nigerian snack

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Abstract. Variants of kokoro snack samples were produced by fortification of maize flour-Fibersol 2-whey protein blends at 1% each with functional ingredients (ginger, fenugreek, turmeric, spirulina, red paprika) and a final blend containing all the functional ingredients at 1% level each. The resultant kokoro snack samples produced were evaluated for proximate composition and sensory qualities. The results of proximate analysis showed a significant ($P < 0.05$) difference in moisture, protein, ash content, crude fat, crude fibre, carbohydrate content, and energy values in all the blends of the kokoro snack sample and ranged from 51.20% to 36.80%, from 4.46% to 3.85%, from 1.15% to 0.98%, from 0.13% to 0.00%, from 4.93% to 3.94%, from 53.57% to 39.2, and from

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232.30 kcal/100 g to 172.99 kcal/100 g respectively. There was also a significant ($P < 0.05$) difference in the sensory attributes of all kokoro samples in terms of appearance, aroma, taste, texture, and overall acceptability. The kokoro snack blend R 97:1:1:1 (Maize: Fibersol 2: Whey protein: Red paprika) was most preferred by the panellists, having the highest mean sensory score of 8.97. The results of the evaluation of the kokoro snack samples showed that an acceptable fortified bakery product based on kokoro can be produced by the addition of maize flour-Fibersol 2-whey protein blends to red paprika and ginger at 1% level of fortification. This will further encourage the cultivation and utilization of these spices in food formulation and hence provide health-promoting benefits to target consumers.

1 Introduction

The major risk factors which have been implicated in the prevalence of cardiovascular disease and other non-communicable diseases are poor diet and morbid lifestyle. In Africa, statistics showed that one in four people are hungry and numbers have increased from 175 to 220 million in the past few years. According to *FAO* (2012), about 16 million undernourished people live in developed countries. Paradoxically, obesity and overweight are associated with more deaths than underweight worldwide. There seems to be a global shift in diet from food of plant origin rich in fibre towards a diet dominated by higher intakes of animal and partially hydrogenated fats (*Popkin*, 2002). The consumption of these calorie-rich foods is attributed to the increasing prevalence of obesity, a major risk factor for several non-communicable diseases (*Popkin*, 2006).

According to *Hoffmeister et al.* (2005), *Labadarios et al.* (2005), and *Mostert et al.* (2005), carbohydrate foods constitute a range of staples in sub-Saharan Africa. In Tanzania, Ugali, a stiff porridge of maize is the most common carbohydrate as opposed to South Africa, where maize and bread constitute the major carbohydrate sources. However, roots and tubers constitute a major source of carbohydrate in Nigeria (*Oguntona et al.*, 1998; *Oguntona-Akinyele*, 2002; *Afolabi et a*l., 2004).

In the last decades, there has been a shift regarding consumers' demand in food production. Consumers are more informed about their health in relation to their diet (*Mollet-Rowland*, 2002; *Young*, 2000). Nowadays, foods are consumed for their health-promoting effects beyond basic nutrition and hunger satisfaction (*Menrad*, 2003; *Roberfroid*, 2000b). Conventional foods are developed to meet consumer requirements of sensory attributes and convenience.

The new trend is the development of functional foods as a nutritional therapeutic approach in combating metabolic syndrome (*Guthman*, 2003; *Grunert*, 2002; *Stephen*, 1998). It has been documented that bioactive compounds from our dietary intake can help provide additional protective mechanisms against the excessive generation of reactive oxygen species due to the deficiency in the human body's internal protection mechanisms (*Huang et al.*, 2005; *Pietta*, 2000).

In Nigeria, garlic, hot red paprika, and ginger constitute an important part of Nigerian cuisine (*Oloyede et al.*, 2013). These spices added to food at the various stages of food preparations help in disease prevention and have several proven functional and medicinal properties, which include but are not limited to anti-cancer, anti-diabetes, anti-inflammatory, and anti-tuberculosis effects and are due to the presence of bioactive compounds in them (*Otunola*, 2010, 2014). Several biological activities of these spices, such as antioxidants, anti-diabetes, anti-hyperlipidemic, or anti-hypertension, have been reported and validated (*Banerjee et al.*, 2002; *Al-Amin et al.*, 2006; *Mahmoodhi et al.*, 2006; *Nazam Ansari et al.*, 2006; *Benavides et al.*, 2007; *Ghayur et al.*, 2007; *Otunola et al.*, 2010, 2014).

Health-conscious consumers are eating snacks for health-promoting reasons rather than for refreshment. Hence their demand for snacks, which not only meet their organoleptic requirements but have health-promoting characteristics (*Norazmir et al.*, 2014). There has been a considerable increase in the number of consumers who are conscious of the relationship between their health, well-being, and dietary intake (*Nazzaro et al.*, 2014; *Norazmir et al.*, 2014; *Manzoni et al.*, 2012; *Taverna et al.*, 2012). Since grazing is a new trend for food consumers, snacking is an integral part of such phenomenon (*Costa et al.*, 2010; *Datamonitor*, 2007), thus constituting an essential potential market for snacks that fulfil the requirements of health-conscious snackers.

Maize-based snacks, such as *kokoro*, are principally carbohydrates and are popular amongst inhabitants of south-western Nigeria. According to *FAO* (2015), maize and other cereal crops, such as rice and wheat, constitute an integral part of the human dietary intake. With more than 1,000 million tons of maize harvested in 2014, it is the most popular cereal crop globally (*FAO*, 2015). Hence, fortifying maize-based snack with functional ingredients, such as spices and prebiotics with proven health-promoting properties, will enhance its application in combating disease conditions such as obesity, cardiovascular diseases (CVD), hyperlipidaemia, etc. The aims of the research were to develop a novel fortified bakery product based on a traditional Nigerian snack (kokoro) and to determine the proximate composition and sensory attributes

of the product for overall acceptability and potential application as a complementary solution in the treatment of metabolic syndrome.

2 Materials and methods

2.1 Sample collection and processing

The maize flour (yellow variety) was obtained from the Institute of Food Technology Food Processing Factory of the University of Debrecen, Hungary. Similarly, the supplementary ingredients, such as Fibersol 2, whey protein powder, spices, and spirulina, were also obtained from the food processing factory.

2.2 Formulation and production of kokoro samples

Table 1: Percentage of blend formulation

Blends	Maize Flour (%)	Whey Protein (%)	Fibersol 2 (%)	Ginger (%)	Spirulina (%)	Fenugreek (%)	Red paprika (%)	Turmeric (%)
Control	98	1	1	-	-	-	-	-
G	97	1	1	1		-	-	-
S	97	1	1	-	1	-	-	-
F	97	1	1	-	-	1	-	-
R	97	1	1	-	-	-	1	-
T	97	1	1	-	-	-	-	1
A	93	1	1	1	1	1	1	1

A blend of maize flour containing Fibersol 2 and whey protein powder was produced and marked as the control sample (98% maize flour: 1% Fibersol 2: 1% whey protein powder). Various blends were thereafter produced by fortification of maize flour-Fibersol 2-whey protein blend with 1% each of the functional ingredients (ginger, fenugreek, turmeric, spirulina, red paprika) and a final blend containing all the functional ingredients at 1% level each.

Kokoro was produced by modifying the method adopted by *Uzo-Peters et al.* (2008). 196 g of maize flour was added to 2 g each of Fibersol 2 and whey protein powder respectively. 2.5 g of table salt was added and then mixed thoroughly using a hand-held mixer (model: CNHR2, Robert Bosch Housgerate GmbH, Munich, Germany) at low speed. The resultant mixture was then added to 450 ml of boiling water, cooked and stirred continuously until a homogenous dough was formed. This was cooled down to a temperature of about

40 °C and kneaded manually by hand for about 4 mins. The kneaded dough was formed into uniform shapes on a chopping board, baked in a preheated hot air oven (model: RXB-610-SMART 3.5, Valladolid, Spain) at 180 °C for 17 mins, cooled and packed in Ziploc bags, and stored at refrigerated temperature for subsequent analysis.

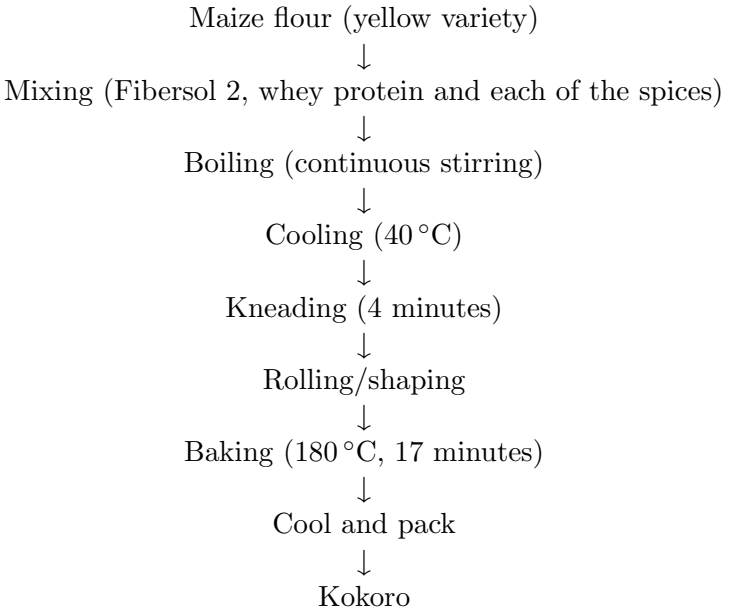

Figure 1: Flowchart of kokoro production

2.3 Proximate analysis of kokoro from maize-fibre-whey protein-spices blends

The moisture, ash, fat, protein, and fibre contents were analysed using the methods described by the official Hungarian National Standards MSZ 6943-1:1979, MSZ 6943-2:1980, MSZ EN ISO 11085:2015, MSZ EN ISO 20483:2013, and MSZ EN ISO 16472:2006 respectively.

Carbohydrate was calculated by difference (100- [sum of moisture, ash, fat, protein, and fibre contents]). Energy values were estimated using the Atwater factors (4×% carbohydrate + 4×% protein + 9×% fat) in kcal/100 g.

2.4 Organoleptic/sensory assessment of kokoro from maize flour-fibre-whey protein-spices blends

The acceptability of kokoro samples was carried out using a 9-point hedonic preference scale and multiple comparison tests. This was achieved by evaluating the samples with 30 panellists, comprising 12 males and 18 females, who were staff members, students, and members of the Faculty of Agriculture, Food Sciences and Environmental Management, University of Debrecen, Hungary.

Following the approval of the panellists to participate and screening for their sensory acuity, the panellists were presented with randomly coded samples each and were asked to score each attribute based on appearance, taste, aroma, texture, and overall consumer acceptability using a 9-point hedonic scale, where 9 was assigned to like extremely and 1 assigned to dislike extremely.

2.5 Statistical analysis

The data obtained in duplicates from proximate analysis and sensory assessment were subject to one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, version 19.0. Armonk, NY: IBM Corp).

Means obtained from the data were compared at $p < 0.05$ for significant differences using Duncan's Multiple Range Test.

3 Results and discussion

3.1 Results of proximate analysis of kokoro from maize-fibre-whey protein-spices blends

The results of proximate analysis of the different blends of the kokoro snacks samples are shown in *Table 2*. Moisture content represents an essential quality parameter in food product, and it maintains quality. The moisture contents of the samples were high and ranged from 51.20% to 36.80%. The control sample C 98:1:1 (maize: Fibersol 2: whey protein) had the lowest (36.80%) while sample **S** 97:1:1:1 (maize: Fibersol 2: whey protein: spirulina) had the highest moisture content.

Table 2: Results of proximate analysis of kokoro snack samples from maize flour-fibre-whey protein-spices blends

Parameters	**Moisture** (%)	**Protein** (%)	**Ash** (%)	**Fat** (%)	**Fibre** (%)	**CH0** (%)	**EV** (kcal/100g)
C	$36.80^{f}\pm0.29$	$4.46^{a}\pm 0.01$	$1.15^{a}\pm0.02$	$0.02^{c}\pm0.01$	$3.94^{e}\pm0.02$	$53.57^{a}\pm0.06$	232.30^{a}
G	$46.00^{d}\pm0.19$	$4.17^{ab}\pm0.16$	$1.12^{a}\pm0.02$	$0.00^{c}\pm0.00$	$4.20^{d}\pm0.03$	$44.44^{c}\pm0.30$	194.49^{abc}
S	$51.20^{a}\pm0.67$	$3.85^{b}\pm 0.05$	$1.02^{b}\pm0.04$	$0.09^{b}\pm0.01$	$4.61^{b}\pm0.02$	$39.21^{f}\pm0.61$	172.99^{e}
T	48.15c±0.64	$4.24^{ab}\pm0.23$	$0.98^{b}\pm0.01$	0.00c±0.00	$4.26^{cd}\pm0.06$	$42.33^{d}\pm0.80$	186.26^{d}
F	$45.90^{d}\pm0.72$	$4.12^{ab}\pm0.14$	$1.08^{ab}\pm0.05$	$0.13^{a}\pm0.01$	$4.36^{c}\pm0.04$	$44.34^{c}\pm0.84$	195.00^{c}
R	$43.15^{e}\pm0.60$	$4.09^{ab}\pm0.07$	$1.12^{a}\pm 0.00$	$0.08^{b}\pm0.01$	$4.22^{d}\pm0.04$	$47.30^{b}\pm0.58$	206.24^{b}
A	$49.55^{b}\pm0.60$	$3.95^{b}\pm 0.10$	$1.09^{ab}\pm0.04$	$0.08^{b}\pm0.00$	$4.93^{a}\pm 0.06$	$40.34^{f}\pm0.60$	177.89^{e}

This is in sharp contrast with results obtained from the moisture content of some kokoro snacks made from maize with different flour blends of defatted groundnut (*Uzor-Peters et al.*, 2008), soybean (*Adelakun et al.*, 2004), beniseed (*Ayinde et al.*, 2012), protein hydrolysate from pigeon pea (*Akoja et al.*, 2016), distillers' spent grain (*Awoyale et al.*, 2011), and defatted coconut paste (*Adebowale* & Komolafe, 2018). The higher moisture content may be attributed to the adopted cooking (baking) method rather than frying since oils/fats are able to reach higher temperatures than water. This is over and above the optimum safe moisture level ($\leq 12\%$) for the longer shelf stability of food products.

Therefore, the kokoro snacks sample under study may likely experience shorter shelf life.

The crude protein content ranged from 4.46% to 3.85% with sample **S** 97:1:1:1 (maize: Fibersol 2: whey protein: spirulina) having the least value (3.85%) while the control sample **C** 98:1:1 (maize: Fibersol 2: whey protein) having the highest value (4.46%). These values, however, are not in agreement with crude protein values recorded from previous results of kokoro snacks produced from varying substitution levels of defatted groundnut (*Uzor-Peters et al.*, 2008), beniseed cake (*Ayinde et al.*, 2012), soybean (*Adelakun et al.*, 2004), protein hydrolysate of pigeon pea (*Akoja et al.*, 2016), defatted groundnut paste (*Otunola et al.*, 2012), pigeon pea (*Adegunwa et al.*, 2015), African yam bean (*Idowu*, 2015), and defatted coconut paste (*Adebowale & Komolafe*, 2018). The moderately low crude protein values could be attributed to the fact that the bulk of the ingredients were very low in protein.

Ash content is a measure of the mineral composition of the kokoro snacks blends, and it ranged from 1.15% to 0.98%. The kokoro snacks sample **T** 97:1:1:1 (maize: Fibersol 2: whey protein: turmeric) had the lowest value (0.98%), while the control sample **C** 98:1:1 (maize: Fibersol 2: whey protein) had the highest value (1.15%). These values were lower than those recorded from previous results of kokoro 27 supplemented with varying proportions of protein hydrolysate of pigeon pea (*Akoja et al.*, 2016), defatted coconut paste (*Adebowale & Komolafe*, 2018) but higher than ash content documented for defatted groundnut (*Uzor-Peters et al.*, 2008). However, it is close to that reported for defatted groundnut paste (*Otunola et al.*, 2012).

The crude fat of the kokoro samples was very low and ranged from 0.13% to 0.00%. Kokoro snack samples **G** 97:1:1:1 (maize: Fibersol 2: whey protein: ginger) and **T** 97:1:1:1 (maize: Fibersol 2: whey protein: turmeric) had the lowest values while sample **F** 97:1:1:1 (maize: Fibersol 2: whey protein: Fenugreek) had the highest. These values, however, are in sharp contrast to the

previous results of kokoro substituted with different proportions of soybean (*Adelakun et al.*, 2004), African yam bean (*Idowu*, 2015), defatted groundnut (*Uzor-Peters et al.*, 2008), protein hydrolysate from Pigeon pea (*Akoja et al.*, 2016), distillers' spent grain (*Awoyale et al.*, 2011), beniseed (Ayinde et al., 2012), and defatted coconut paste (*Adebowale & Komolafe*, 2018). The shelf life of the kokoro sample blends may be increased due to their low fat contents because all fats and fat-containing foods contain some unsaturated fatty acids and hence are potentially susceptible to oxidative rancidity (*Ihekoronye & Ngoddy*, 1985). The low crude fat content recorded may be attributed to the negligible fat contents of the ingredients used. Thus, this low fat content of the kokoro snack may be desirable as high fat consumption has been implicated in the incidence of metabolic syndrome such as obesity, cardiovascular diseases, etc.

The crude fibre content ranged from 4.93% to 3.94%. Control sample **C** 98:1:1 (maize: Fibersol 2: whey protein) had the lowest value (3.94%), while kokoro snack sample **A** 93:1:1:1:1:1:1:1 (maize: Fibersol 2: whey protein: ginger: spirulina: turmeric: fenugreek: red paprika) had the highest crude fibre content (4.93%). These values were higher than those documented for kokoro made from maize flour blends with different proportions of soybean (*Adelakun et al.*, 2004), (*Uzor-Peters et al.*, 2008), distillers' spent grain (*Awoyale et al.*, 2011), African yam bean (*Idowu*, 2015), pigeon pea (*Adegunwa et al.*, 2015), beniseed (*Ayinde et al.*, 2012), defatted groundnut, soybean protein hydrolysate from pigeon pea (*Akoja et al.*, 2016), and defatted coconut paste (*Adebowale & Komolafe*, 2018). The high crude fibre content obtained may be attributed to the high fibre contributions from the ingredients.

The carbohydrate content ranged from 53.57% to 39.21%. Kokoro control sample **C** 98:1:1 (maize: Fibersol 2: whey protein) had the highest value (53.57%), while kokoro snack sample **S** 97:1:1:1 (maize: Fibersol 2: whey protein: spirulina) had the least value (39.21%). These values were about the same as the results obtained from kokoro made from maize flour blends with different substitution levels of soybean (*Uzor-Peters et al.*, 2008) but lower than that documented for soybean (*Adelakun et al.*, 2004), beniseed (*Ayinde et a*l., 2012), African 28 yam bean (*Idowu*, 2015), pigeon pea (*Adegunwa et al.*, 2015), protein hydrolysate from pigeon pea (*Akoja et al.*, 2016), defatted groundnut (*Uzor-Peters et al.*, 2008), distillers' spent grain (*Awoyale et al.*, 2011), and defatted coconut paste (*Adebowale & Komolafe*, 2018). The low values of carbohydrate may be connected to the relatively low carbohydrate contents of the ingredients.

There was a significant ($p < 0.05$) difference in the energy values of the

kokoro snacks samples. The energy values were low and ranged from 232.30 kcal/100 g to 172.99 kcal/100g. Control sample **C** 98:1:1 (maize: Fibersol 2: whey protein) had the highest energy value (232.30 kcal/100g), while kokoro snack sample **S** 97:1:1:1 (maize: Fibersol 2: whey protein: spirulina) had the lowest value (172.99 kcal/100g). These values were lower than the previous results of kokoro made from maize flour with varying proportions of pigeon pea (*Adegunwa et al.*, 2015), soybean (*Adelakun et al.*, 2004), beniseed cake (*Ayinde et al.*, 2012), defatted groundnut paste (*Otunola et al.*, 2012), African yam bean (*Idowu*, 2015), protein hydrolysate of pigeon pea (*Akoja et al.*, 2016), defatted groundnut (*Uzor-Peters et al.*, 2008), and defatted coconut paste (*Adebowale & Komolafe*, 2018). The low energy values obtained may be attributed to the low values of crude fat, carbohydrate, and protein contents of the kokoro snacks. This is desirable as the kokoro snacks will not be energy laden since studies have proven the relationship between the consumption of energy-laden foods and the occurrence of metabolic diseases such as obesity, cardiovascular disorders, etc.

Values are means of duplicate determinations. Means with different superscripts within the same column are significantly ($p < 0.05$) different.

– **CHO**: Carbohydrate, **EV**: Energy Values (kcal/100 g).

Mean values with different superscripts within the same column are significantly ($p < 0.05$) different. **C** 98:1:1 (maize: Fibersol 2: whey protein); **G** 97:1:1:1 (Maize: Fibersol 2: whey protein: Ginger); **S** 97:1:1:1 (maize: Fibersol 2: whey protein: Spirulina); **T** 97:1:1:1 (maize: Fibersol 2: whey protein: turmeric); **F** 97:1:1:1 (maize: Fibersol 2: whey protein: fenugreek); **R** 97:1:1:1 (maize: Fibersol 2: whey protein: red paprika); **A** 93:1:1:1:1:1:1:1 (maize: Fibersol 2: whey protein: ginger: spirulina: turmeric: fenugreek: red paprika).

3.2 Results of the sensory evaluation of kokoro from maize flour-fibre-whey protein-spices blends

Table 3 shows the results obtained for the nine-point hedonic and multiple comparison tests of the different blends of the kokoro snacks samples. There were significant differences ($p < 0.05$) in the appearance, taste, aroma, texture, and the overall acceptability of the control (98:2 maize-fibre-whey protein) and the maize-fibre-whey protein-spices-based kokoro snacks.

However, there was no significant difference ($p < 0.05$) between samples **T** and **F** (turmeric- and fenugreek-spiced kokoro blends) in terms of all the sensory parameters evaluated. The red-paprika-spiced kokoro blend (R) was

the most preferred one by the panellists, as reflected in the highest mean sensory score of 8.97, followed by sample **G** (ginger-spiced) with a sensory score of 8.10 and **F** and **T** formulations (turmeric- and fenugreek-spiced kokoro blends) with mean sensory scores of 7.37 and 7.40 respectively.

The **A** formulation (all ingredients-based blend) was the least preferred with a mean sensory score of 4.27 for all the sensory attributes evaluated. The highest mean sensory score and overall acceptability of **R** formulation (red 29 paprika spiced kokoro blend) could be attributed to the incorporation of red paprika as an important and basic ingredient in Hungarian dishes and often the most utilized spice (*Szűcs & Szabó*, 2014).

Table 3: Mean scores of sensory evaluations of kokoro from maize flour-fibre-whey protein-spices blends

Sample	**Appearance**	**Aroma**	**Taste**	**Texture**	**Overall acceptability**
C	6.07^{d}	6.07^{d}	6.07^{d}	6.07^{d}	6.07^{d}
G	8.10^{b}	8.10^{b}	8.10^{b}	8.10^{b}	8.10^{b}
S	5.10^{e}	5.10^{e}	5.10^{e}	5.10^{e}	5.10^{e}
T	7.37^{c}	7.37^{c}	7.37^{c}	7.37^{c}	7.37^{c}
F	7.40^{c}	7.40^{c}	7.40^{c}	7.40^{c}	7.40^{c}
R	8.97^{a}	8.97^{a}	8.97^{a}	8.97^{a}	8.97^{a}
A	4.27^{f}	4.27^{f}	4.27^{f}	4.27^{f}	4.27^{f}

4 Conclusions

This research has shown that maize-based fortified bakery products based on kokoro, a traditional Nigerian snack with better nutritional value and consumer acceptance, can be developed with the addition of Fibersol 2, whey protein, and spices at 1% level of fortification with maize flour. This will therefore promote the utilization of all these ingredients in bakery food formulations.

However, the moisture content of the snacks was higher than the optimum safe moisture level (12%) and thus may affect the storability of the snacks. Nevertheless, further research should be done to investigate the effects of packaging materials and storage conditions on the shelf stability of the snacks. In addition, further studies should be carried out to determine the effects of

higher levels of fortification on the proximate composition and sensorial qualities of the kokoro snacks. This will further help in the evaluation of the optimum level of fortification to achieve optimum results.

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