UNIVERSITY OF BELGRADE

FACULTY OF TECHNOLOGY AND METALLURGY

Salem Mohamed Embiriekah

FUNCTIONAL PROPERTIES AND POSSIBILITY OF APPLICATION OF WHEY PROTEIN HYDROLYSATES OBTAINED BY BIOTECHNOLOGICAL PROCESSING

Doctoral Dissertation

Belgrade, 2018

UNIVERZITET U BEOGRADU

TEHNOLOŠKO-METALURŠKI FAKULTET

Salem Mohamed Embiriekah

FUNKCIONALNA SVOJSTVA I PRIMENA HIDROLIZATA PROTEINA SURUTKE DOBIJENIH BIOTEHNOLOŠKIM PUTEM

doktorska disertacija

Beograd, 2018

SUPERVISOR:

_____

Dr Marica Rakin, full professor

Faculty of Technology and Metallurgy, University of Belgrade

COMMISSION:

_____

Dr Zorica Knežević-Jugović, full professor

Faculty of Technology and Metallurgy, University of Belgrade

_____

Dr Maja Bulatović, research associate

Faculty of Technology and Metallurgy, University of Belgrade

_____

Dr Danica Zarić, senior research associate

IHIS Technoexperts, Research-Development Center, Belgrade

DATE OF DEFENSE: _____

Greetings

To my family

To my dear supervisor Prof Dr Marica Rakin, full professor at the Faculty of Technology and Metallurgy, University of Belgrade who helps me during my study, who conceived and designed the experiments and whoever helped me and stood with me in this work. Thanks to her immense for help during wrote discussion as well as revised the thesis.

Thanks immensely to Dr Maja Bulatović, scientific associate at the Faculty of Technology and Metallurgy, University of Belgrade, on unselfish help in experimental work, warm cooperation, which has perfectly improved the quality of this doctoral dissertation, patience and encouragement at very important moments during the realization of this doctoral dissertation.

FUNCTIONAL PROPERTIES AND POSSIBILITY OF APPLICATION OF WHEY PROTEIN HYDROLYSATES OBTAINED BY BIOTECHNOLOGICAL PROCESSING

SUMMARY

The amount of whey products on the market is negligible in relation to the amount of unused whey, which leads to the conclusion that developing and improving the process of whey processing must introduce new unconventional products, which would take a more significant place in the range of consumer products. In this sense, the aim of this doctoral dissertation was the production and characterization of bioactive whey proteins hydrolysates, which as such could be applied separately or in combination with other food products.

In order to produce bioactive whey protein hydrolysates, a selection of an optimal biotechnological process for the production of hydrolysates with improved biological properties was performed. In the research, selection of optimal microorganisms and enzymes, optimization of the processes of fermentation and enzymatic hydrolysis were performed in order to produce hydrolysates of high biological activity. The produced hydrolysates are analyzed in both the bioactivity aspect and the aspect of their functional characteristics, after which the optimal production process was adopted. In the last phase of the research, a proposal for the application of the produced bioactive hydrolysate in a confectionery fat filling was made. The fat filling enriched with bioactive hydrolysate of whey proteins has been thoroughly examined in terms of sensory characteristics and texture, and the formulation by which it is possible to obtain a final product with optimal characteristics is proposed.

During the selection of microorganisms and enzymes, biotechnological modification of whey proteins was carried out using *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus reuteri* ATCC 23272 strains, as well as commercial enzymes Pepsin® and Tripsin®. Fermentation and enzymatic hydrolysis processes are optimized with the aim of producing hydrolysates with high antioxidant activity. After that, the selection of optimal bacterial strain and enzyme was

performed, as well as the optimal processes conditions. During the selection, the antioxidant activity, the protein and amino acid content, the degree of hydrolysis, the foam formation ability, emulsifying properties, digestibility and bioavailability were monitored. The obtained bioactive hydrolysates were then dried, analysed and added, in powder form, to the fat filling as a model of the confectionery product. During the characterization, antioxidant, antirheumatic, anti-inflammatory and antimicrobial activity, the ability to inhibit lipid peroxidation, and the stability of the antioxidant activity of the produced hydrolysate powder were monitored. After that, an optimal amount of hydrolysate powders, which can be added to the fat filling without adversely affecting its sensory properties and texture, was tested.

Selection of optimal bacterial strain revealed that increased antioxidant activity has been achieved in all tested combinations of strains and substrates. High levels of antioxidant activity in the range of 29.7-72.2% were achieved in all substrates fermented by strain *Lb. rhamnosus*. In the fermentation of cow's whey, the registered DPPH antioxidant activity was 57.3%, while FRAP antioxidant activity was 0.862 mmol Fe^{2+}/L. Analyzing simultaneously the results related to the functional properties of hydrolysates it can be noticed that cow's whey hydrolysate satisfies this important application criterion. Namely, compared to the goat's whey, cow's whey hydrolysate exhibited significantly better emulsion activity (98.2%), emulsion stability (68.2%), foam activity (307.1%), digestibility (96.9%) and bioaccessibility (120.6%) as crucial parameters for its application. In addition, compared to the goat's whey, cow's whey is much more accessible and voluminous waste product of the dairy industry and its utilization is much more significant. Therefore, by examining all of these facts, it could be suggested that process of microbial fermentation of cow's whey using the strain *Lb. rhamnosus* ATCC 74696 represents the appropriate process for production of bioactive components.

Selection of optimal enzymes revealed that tryptic hydrolysis of whey proteins can enable the release of peptides with high antioxidant activity, originating from parent whey proteins as a complex food matrix. A trypsin hydrolysate, with a maximal total antioxidant activity of 80.0%, can be produced after 4h by hydrolysis at 37 °C and an E/S ratio of 0.5%. The trypsin hydrolysate exhibited 47.9% higher antioxidant activity compared to the nonhydrolysed whey proteins, as well as 25.9% higher antioxidant

activity compared to the pepsin hydrolysate produced under the same conditions. The trypsin hydrolysate, which is mainly composed of hydrophilic (polar aprotic) antioxidant species, exerted an improved digestibility of 95.9% and bioaccessibility of 124.6%, compared to the native whey proteins and peptic hydrolysate. In addition to its good antioxidant properties, the trypsin hydrolysate had excellent functional properties, such as emulsifying and foam-forming abilities. These properties indicate it to be a desirable raw material for the food industry that could replace whey proteins as a traditional additive for product fortification. Based on the improved properties, trypsin hydrolysate can be considered as an excellent carrier for the delivery of antioxidants that contribute to both the health and functional properties of dairy or confectionery products.

Characterization of bioactive hydrolysates revealed that the addition of at least 178.4 mg mL^{-1} of whey protein hydrolysate could inhibits processes of lipid peroxidation for 50% as well as microbial contamination caused by *S. aureus* ATCC25923, *B. cereus* ATCC 11778 and *L. monocytogenes*. In addition, proposed concentration will surely provide antioxidant, anti-arthritic and anti-inflammatory activity greater than 50%. On the other hand, hydrolysate obtained by whey fermentation should be added in concentration of at least 811.5 mg mL^{-1} to achieve all of tested bioactivities, with emphasis on significantly more pronounced antimicrobial activity against all tested strains. Therefore, it could be concluded that enzymatic hydrolysis represents the optimal process for the production of hydrolysate with pronounced bioactive properties that could be considered as very promising natural food supplement.

In the examination of the possibility of use of bioactive hydrolysates, the fat filling was enriched with trypsin hydrolysate at concentrations of 1, 2.5 and 5%. Based on the results presented in this section, fat filling supplemented with 5% of bioactive whey protein hydrolysate powder (WPH-P) expresses for 32% stronger antioxidative power, which corresponds to the IC_{50} of 116.52 mg mL^{-1}, than control sample CFF (171.37 mg mL^{-1}). This fat filling expresses 2.6 times higher hardness than control sample (CFF), which is very important if the fat filling would be used for the filling of biscuits that demands substantially harder fat filling. Confectionery fat filling supplemented with 5% of WPH-P exhibits the highest Casson viscosity values (6,066 Pa·s), surface of the thixotropic loop (2,455 $Pa \cdot s^{-1}$) and hardness (1980.7 g), which are certainly not an

advantage, but can be avoided by a better selection of the emulsifier. This fat filling can be considered as optimal, from the point of view of particle size distribution and sensory characteristics. In addition, it could be suggested that addition of 5%WPH-P could protect and preserve the fat filling during the four months of storage. After 120 days of storage antioxidant activity of fat filling corresponds to the IC_{50} of 137.42 mg mL^{-1}, suggesting that WPH-P still achieves its antioxidant activity and is capable to protect the fat filling from potential free radicals that can be formed during the storage process.

The final result of the dissertation is a functional filled biscuit enriched with bioactive whey protein hydrolysate in an originally designed package, which is as such ready for placement on the market. The results and conclusions presented in this dissertation are the basis for the further development of functional confectionery products.

Key words: whey, fermentation, enzymes, functional fat filling, bioactivity, emulsifying properties, digestibility, bioavailability.

Scientific field: Technological engineering

Scientific subfield: Biotechnological engineering and biotechnology

UDC number:

FUNKCIONALNA SVOJSTVA I PRIMENA HIDROLIZATA PROTEINA SURUTKE DOBIJENIH BIOTEHNOLOŠKIM PUTEM

REZIME

Količina proizvoda od surutke na tržištu je zanemarljivo mala u odnosu na količinu surutke koja se nepropisno odlaže što navodi na zaključak da bi se razvojem i unapređenjem procesa prerade surutke mogli dobiti novi nekonvencionalni proizvodi koji bi zauzeli značajnije mesto u paleti proizvoda namenjenih širokoj potrošnji. U tom smislu cilj ove doktorske disertacije je bio proizvodnja i karakterisanje bioaktivnih hidrolizata proteina surutke, koji bi kao takvi mogli biti primenjeni zasebno ili u kombinaciji sa drugim prehrambenim proizvodima.

U cilju proizvodnje bioaktivnih hidrolizata proteina surutke izvršena je selekcija optimalnog biotehnološkog procesa pomoću koga se mogu proizvesti hidrolizati proteina surutke unapređenih bioloških svojstava. U okviru istraživanja izvršena je selekcija optimalnih kultura mikroorganizama i enzima, optimizacija uslova procesa fermentacije i enzimske hidrolize u cilju proizvodnje hidrolizata visoke biološke aktivnosti. Proizvedeni hidrolizati su detaljno okarakterisani kako sa stanovišta bioaktivnosti tako i sa stanovišta njihovih funkcionalnih karakteristika, nakon čega je usvojen optimalan proces proizvodnje.. U poslednjoj fazi istraživanja dat je predlog primene proizvedenog bioaktivnog hidrolizata u masnom kremu namenjenom konditorskoj industriji. Konditorski masni krem obogaćen bioaktivnim hidrolizatom proteina surutke je detaljno ispitan po pitanju senzornih karakteristika i teksture na osnovu čega je predložena receptura čijom primenom je moguće dobiti fimalni proizvod optimalnih karakteristika.

Tokom selekcije kultura mikroorganizama i enzima izvršena je biotehnološka modifikacija proteina surutke primenom sojeva *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus reuteri* ATCC 23272, kao i komercijalnih enzima Pepsin® i Tripsin®. Procesi fermentacije i enzimske hidrolize su optimizovani sa ciljem proizvodnje hidrolizata visoke antioksidativne aktivnosti. Nakon toga izvršena je selekcija optimalnog bakterijskog soja i enzima kao i uslova izvođenja

ovih procesa. Tokom selekcije praćena je antioksidativna aktivnost, sadržaj proteina i aminokiselina, stepen hidrolize, svojstvo stvaranje pene, emulgujuća svojstva, svarljivost i bioraspoloživost. Dobijeni bioaktivni hidrolizati su nakon toga sušeni, detaljno okarakterisani i u praškastom obliku dodavani u masni krem kao model konditorskog proizvoda. Tokom karakterizacije praćeni su antioksidativna, antireumatska, anti-inflamatorna i antimikrobna aktivnost, sposobnost inhibicije lipidne peroksidacije kao i stabilnost antioksidativne aktivnosti proizvedenih hidrolizata u prahu. Nakon toga, ispitana je optimalna količina hidrolizata u prahu koja može biti dodata u konditorski masni krem bez negativnog uticaja na njegova senzorna svojstva i teksturu.

Pri selekciji optimalnog bakterijskog soja povećana antioksidativna aktivnost je postignuta u svim testiranim kombinacijama sojeva i supstrata. Visoki nivoi antioksidativne aktivnosti u opsegu 29,7-72,2% su postignuti u svim supstratima fermentisanim sojem *Lb. rhamnosus*. Pri fermentaciji kravlje surutke registrovana DPPH aktivnost iznosila je 57,3%, dok je FRAP antioksidativna aktivnost iznosila 0,862 mmol Fe^{2+}/L. Analizirajući istovremeno rezultate koji se odnose na funkcionalne osobine hidrolizata, uočeno je da hidrolizat kravlje surutke jedini na optimalan način ispunjava ovaj važan kriterijum primene. Naime, u poređenju sa kozjem surutkom, hidrolizat kravlje surutke pokazao je znatno bolja emulgujuća svojstva (98,2%), stabilnost emulzije (68,2%), aktivnost pene (307,1%), svarljivost (96,9%) i bioraspoloživost (120,6%) kao ključne parametre dalje primene. Pored toga, u poređenju sa kozjom surutkom, kravja surutka je mnogo pristupačnija, a obzirom da predstavlja najobimniji otpadni proizvod industrije mleka njena prerada je mnogo značajnija. Stoga, analizom svih ovih činjenica, može se predložiti da proces mikrobne fermentacije kravlje surutke sojem *Lb. rhamnosus* ATCC 74696 predstavlja odgovarajući proces za proizvodnju bioaktivnih hidrolizata proteina surutke.

Pri selekciji optimalnog enzima pokazano je da hidroliza proteina surutke primenom enzima Tripsin® može omogućiti oslobađanje peptida visoke antioksidativne aktivnosti, poreklom iz matičnih proteina surutke kao složene prehrambene matrice. Primenom ovog enzima hidrolizat sa maksimalnom ukupnom antioksidativnom aktivnošću od 80,0%, može biti proizveden nakon 4h hidrolize na 37 °C i pri E/S odnosu od 0,5%. Hidrolizat je pokazao za 47,9% veću antioksidativnu aktivnost u poređenju sa

nehidrolizovanim proteinima surutke, kao i za 25,9% veću antioksidativnu aktivnost u poređenju sa hidrolizatom proizvedenim pod istim uslovima primenom enzima Pepsin®. Hidrolizat proizveden primenom tripsina, koji se uglavnom sastoji od hidrofilnih (polarnih aprotičnih) antioksidativnih vrsta, ispoljava bolju svarljivost od 95,9% i bioraspoloživost od 124,6%, u poređenju sa matičnim proteinima surutke kao i hidrolizatom proizvedenim primenom pepsina. Pored svojih dobrih antioksidativnih svojstava, hidrolizat proizveden primenom tripsina se odlikuje odličnim funkcionalnimkarakteristikama, kao što su sposobnost emulgovanja i stvaranja pene. Ova svojstva pokazuju da je to poželjna sirovina za prehrambenu industriju koja bi mogla zamijeniti proteine surutke kao tradicionalne aditive u proizvodnji prehrambenih proizvoda. Na osnovu poboljšanih osobina, hidrolizat proizveden primenom tripsina se može smatrati odličnim nosačem antioksidanasa koji doprinose kako zdravlju, tako i funkcionalnim svojstvima mlečnih ili konditorskih proizvoda.

Pri karakterizaciji bioaktivnih hidrolizata zaključeno je da dodavanje najmanje 178.4 mg mL^{-1} hidrolizata u prahu proizvedenog primenom tripsina može inhibirati proces peroksidacije lipida za 50%, kao i mikrobiološku kontaminaciju uzrokovanu sojevima *S. aureus* ATCC 25923, *B. cereus* ATCC 11778 i *L. monocitogenes*. Pored toga, predložena koncentracija sigurno će obezbediti antioksidativnu, anti-reumatsku i anti-inflamatornu aktivnost veću od 50%. S druge strane, hidrolizat dobijen fermentacijom surutke treba dodati u koncentraciji od najmanje 811,5 mg mL^{-1}, kako bi se postigao isti nivo testiranih bioaktivnosti, sa naglaskom na znatno izraženiju antimikrobnu aktivnost u odnosu na sve testirane patogene sojeve. Stoga se može zaključiti da enzimska hidroliza predstavlja optimalni proces za proizvodnju hidrolizata sa izraženim bioaktivnim svojstvima koja bi se mogla smatrati veoma promjenljivim prirodnim prehrambenim dodatkom.

U okviru ispitivanja mogućnosti primene bioaktivnih hidrolizata, konditorski masni krem je obogaćen hidrolizatom proizvedenim primenom tripsina u koncentracijama 1, 2.5 i 5%. Na osnovu dobijenih rezultata, krem obogaćen sa 5% bioaktivnog hidrolizata u prahu (WPH-P) izražava za 32% jaču antioksidativnu aktivnost, kojoj odgovara IC_{50} od 116,52 mg mL^{-1}, od kontrolnog uzorka CFF (171,37 mg mL^{-1}). Ovaj krem izražava 2,6 puta veću tvrdoću od kontrolnog uzorka (CFF), što je veoma važno ako se krem koristi za punjenje keksa koji zahtijeva znatno gušće kremove. Konditorski masni krem

dopunjena sa 5% WPH-P pokazuje najveći viskozitet po Cassonu (6,066 Pa • s), površinu tiksotropne petlje (2,455 Pa • s^{-1}) i tvrdoću (1980,7 g), što svakako nije prednost, ali se može se izbeći primenom boljeg emulgatora. Ovaj krem se može smatrati optimalnim, sa stanovišta raspodele veličine čestica i senzornih karakteristika. Pored toga, može se zaključiti da dodavanje 5% WPH-P može zaštititi i sačuvati masni krem tokom četiri meseca skladištenja. Nakon 120 dana skladištenja, antioksidativna aktivnost krema odgovara IC_{50} od 137,42 mg mL^{-1}, što ukazuje na to da WPH-P i dalje postiže svoju antioksidativnu aktivnost i sposoban je da zaštiti krem od potencijalnih slobodnih radikala koji se mogu formirati tokom procesa skladištenja.

Finalni rezultat disertacije je funkcionalni punjeni keks obogaćen bioaktivnim hidrolizatom proteina surutke u originalno dizajniranom pakovanju, koji je kao takav spreman za plasman na tržište. Rezultati i zaključci izneti u ovoj disertaciji predstavljaju bazu za dalji razvoj funkcionalnih konditorskih proizvoda.

Ključne reči: surutka, fermentacija, enzimi, funkcionalni masni krem, bioaktivnost, emulgujuća svojstva, svarljivost, bioraspoloživost.

Naučna oblast: Tehnološko inženjerstvo

Uža naučna oblast: Biohemijsko inženjerstvo i biotehnologija

UDK broj:

CONTENT

1. INTRODUCTION

In recent years, consumer knowledge about health implications relating to food consumption has increased. Today's consumers express a clear tendency towards a healthy lifestyle, by choosing healthy, safe and well-designed innovative products[1]. Although the use of chemical ingredients included in food preservation represents a comfortable solution for the food industry, recognition of chemical or synthetic ingredients as potential hazards, has led to an increase in consumer interest for a completely natural food. Therefore, research of natural antioxidants as alternatives to synthetic ones, and development of new healthy products that respond to the both food industry expectations and consumer demands for the healthy lifestyle, in recent years becoming the main objective of the food industry development with great interest among researchers[2].

The main focus of this issue is a free radical chain reaction that causes auto-oxidation of lipids and other molecules like proteins, pigments, and vitamins, by releasing highly reactive molecules known as free radicals that causing the degradation of food and generally biological systems, including the human body[3]. Free radicals are involved in the oxidative decomposition of unsaturated fatty acids, modification of DNA, proteins and other cellular molecules. Thus, they are responsible for the development of rancidity and reduction of food shelf-life, as well as the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus, neurological disorders, and even the Alzheimer's disease[4]. On the other hand, nature is an unlimited source of a wide range of active compounds that are able to prevent or break these undesirable reactions. These compounds are known as antioxidants and it can be defined as low molecular weight substances that, even at low concentrations, could significantly prolong or suppress the oxidation of the food and biological systems[5]. Antioxidants are enough stable molecules that donate an electron to a free radical, neutralize it and reduce its capacity to damage. They safely interact with free radicals and terminate the chain reaction before vital molecules are damaged, and thus, delay or inhibit cellular damage[6].

Due to the health benefits and potential food preservative applications, natural antioxidants were recently attracted great attention of many food manufacturers, which

tend to produce healthy foods. Antioxidants are classified into two broad divisions, hydrophilic (soluble in polar solvents) and lipophilic (soluble in nonpolar solvents). In general, hydrophilic antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipophilic antioxidants protect cell membranes from lipid peroxidation[7].

Several food products are oil-in-water emulsions such as milk, dressing, yogurt, mayonnaise, etc. An emulsion consists of an aqueous phase, an oil phase, and an oil-water interface. The aqueous phase in an emulsion contains both antioxidants and pro-oxidants, and the water-oil interface influences interactions between water-soluble and oil-soluble components. Therefore, lipid oxidation in emulsions is initiated at this interface, which facilitates interactions between hydrophilic and lipophilic components, i.e., water-soluble pro-oxidants and lipids[8].

The oxidative stability of dairy and confectionary products is the result of a delicate balance between the anti- and pro-oxidative processes influenced by factors such as degree of fatty acid unsaturation or content of transition metal ions. Oxidation processes can result in the appearance of strong off-flavours and deterioration, thus, the natural antioxidants could be excellent preservative ingredients that can contribute to the product stability[9].

Dairy substrates possess antioxidant activity resulting from the presence of such components as bioactive peptides derived from casein, whey proteins, lactoferrin, urate, ascorbate, a-tocopherol, ß-carotene, coenzyme Q10, and various enzymatic systems (superoxide dismutase, catalase, and glutathione peroxidase)[10,11]. In addition, soy substrates are potent antioxidative ingredients due to the presence of phenolic components and isoflavones. All of these compounds possess metal-chelating capabilities and radicalscavenging properties[12]. Antioxidant activity of these substrates could be enhanced by fermentation that tends to enhance targeted property by releasing different by-products[13] or by presence of starter microorganisms that also possess some antioxidant potential[10,11]. During fermentation, soy and dairy proteins could be hydrolysed by the extracellular proteinases of the LAB, resulting in an increase of peptides that can contribute to the antioxidant activity of fermented substrates. The amount of peptides formed during fermentation depends on the used strain, specifically on it proteolytic activity, as well as the used substrate[14]. Optimal strain-substrate

combination is crucial point in the production of high potent antioxidative formulation that can be easily used in different food products.

Except that treatment with antioxidants offers a valuable preservation technique that can prolong the shelf life of food, application of natural antioxidants as dietary supplements and natural health products attracts much greater attention based on their therapeutic properties. In recent years, whey proteins have been marked as a rich source of bioactive peptides with high antioxidant activity[15]. Enzymatic hydrolysis of milk and whey proteins leads to the release of several of bioactive peptides, encrypted within the native sequence of the proteins that have great antioxidant properties[16], related to their high tendency to chelate metals[17], and the ability to donate electrons and atoms[18].

The special challenge in this field is production of antioxidants on food matrixes that can be suitably used for a wide range of products. Although skim milk powder is mostly used in the standardization of the nonfat dry matter content, in recent years, caseinates and whey protein concentrates have been preferred in order to improve both the texture and the functional properties of dairy products[19]. In that sense, whey protein-based ingredients are good carriers for delivery of antioxidants into the dairy and confectionery products.

There are a small number of papers that comparatively analyze the contribution of lipophilic and hydrophilic antioxidants to the total antioxidant capacity of whey based substrates hydrolyzed using enzymes and the functional properties of hydrolysates.

The main objective of the study was to evaluate total antioxidant activity of enzymatic hydrolyzed whey based substrate. Paper analyzes the nature (hydrophilic/lipophilic) of produced antioxidant species. In addition, the paper proposes optimal enzyme-substrate combination as well as hydrolysis parameters that allow the production of the substrate with high antioxidant activity, which can be comfortably used as the ingredient with improved functional properties that can contribute to the both health and sensorial properties of dairy or confectionery products.

2. THEORETICAL PART

2.1. Whey

Whey is a major by-product of the dairy industry, which occurs in the processes of cheese and casein production. About 6% of the total annually produced whey is obtained directly as a by-product during the production of casein from skimmed milk, while a much greater quantity of whey (about 94%) is obtained in the form of cheese whey remaining after the production of various types of cheese. Production of 1 kg of cheese generates approximately 9 kg (or 8-12L) of whey, depending on the type of cheese produced[20]. Its exploitation is becoming more and more important, primarily due to its valuable composition, but also due to increasingly emphasized environmental issues. The amount of whey in the production of cheese is high, and due to its non-use, the whey becomes a very large pollutant. One liter of whey has high HPK values (chemical consumption of oxygen) of 57-75 g/L and BPK5 (biological oxygen consumption over 5 days) of 35-40 g/L and the release of whey into watercourses implies a major ecological problems[20].

According to the Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations, that publish the report with an assessment of trends and perspectives in the main agricultural commodity markets, there is a steady increase in production of cheese projected until the 2020 (Figure 1) which leads to increased production of whey[21].

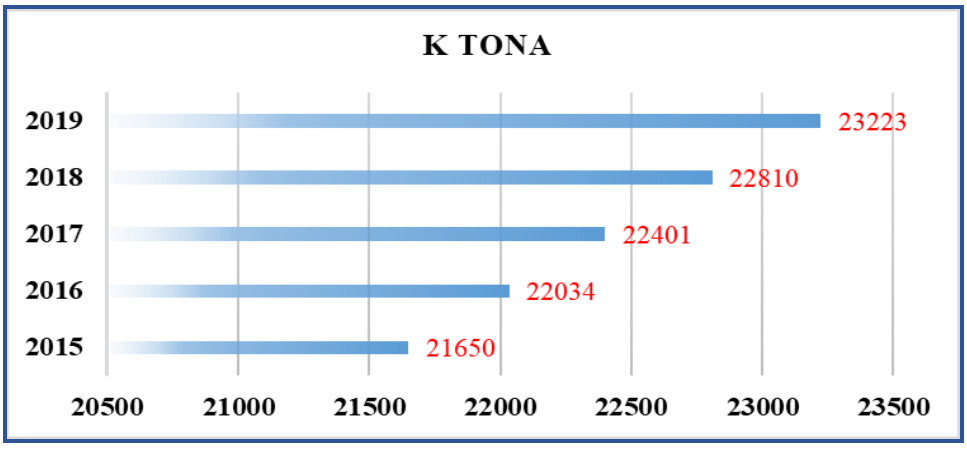

Figure 1. Perspective of global cheese production projected until 2020 year[21]

Over the past decade, due to environmental protection, there has been rapid development of separation methods, and nowadays there are more and more perfect systems of ultrafiltration, reverse osmosis, gelling, electrodialysis, dehydration, etc., which provide concentrated forms of certain whey ingredients or its separate fractions.

In the last five years, the dietary supplements market has been overflowed with products containing whey proteins, or fragments of proteins, because many scientific papers have demonstrated their positive effects on the human health related to the antimicrobial, anti-carcinogenic, antihypertensive, antiviral, imunomodulatory activity. New products change the image of whey which from the category of an unwanted by-product becomes an important raw material for the production of quality functional food and diet products.

Production and export of whey in the Republic of Serbia are almost non-existent. Annually, whey is exported in the amount of several tens of thousands of dollars, while imports are more significant and range from 1.9 to 4.7 million USD. It is mostly imported from Croatia, Belgium, the Netherlands and Hungary[22].

2.2. Composition of whey

Whey is a liquid phase obtained in the production of protein dairy products (cheese, casein). It is a fluid rich in protein that separates from the cluster after the coagulation of milk using enzymes, acids and heat. The whey produced in the acidic treatment of milk is called acid whey, while whey produced through the enzymatic treatment of milk represents sweet whey.

Acid Milk Treatment is a method milk acidification using lactic acid bacteria at 25 °C, or using the food grade hydrochloric acid or sulfuric acid at 45 °C, where casein and cheese are obtained. Casein is precipitated at pH ≈4.5. The precipitated casein is separated from the whey using centrifuging technique.

Enzymatic Milk Treatment is the technological process of cheese production which is characterized by the production of large amount of supernatant - called sweet whey. In this process milk is first standardized, to fat content of 2.5 to 3.5% (using a fat separator), after which it goes to pasteurization. In the pasteurizer, the milk is kept at 72 °C for 15s, where vegetative cells of pathogenic microorganisms are destroyed. After cooling at 30 °C it is seeded with a desired mixture of lactic acid bacteria, depending on

the type of cheese, and the mixture of enzymes: renin (himozin) and pepsin, which results in milk clotting.

Irrespective of the way it is obtained, whey is a fairly light product, with low dry matter content of about 6.5%. The chemical composition of whey is presented in Table 1. The solids consist essentially of lactose, whey proteins, ash, lactic acid and fat[23]. The composition and properties of whey, green-yellow liquid, depends on several factors: from the cheese production technology, the quality and type of milk used, the period of the year, the lactation period, and the type of food used in animal nutrition.

The composition of whey depends on the way it is obtained, and the average whey contains about 93% water and 7% dry matter. It can be said that whey contains approximately 7% of lactose, small amounts of protein, salt and fat. Water-soluble vitamins in whey are in the amount in which they are present in milk[24]. Whey contains about 50% of the dry matter originated from milk: mainly lactose and whey proteins, soluble mineral substances and vitamin B groups, while vitamin C decomposes during the process of cheese production. It is thought that one liter of whey can satisfy the daily requirement of riboflavin (vitamin B2), which gives the yellow-green color of whey. The amount of riboflavin in whey can be higher than in milk, which is the result of the activity of lactic acid bacteria in the production of cheese, so the whey can also be used to obtain the concentrate of this vitamin. Cobalamin (vitamin B12) and folic acid are bound with whey proteins, while riboflavin is up to 95% in free form. According to the nutritional value, 3 kg of whey, it is equivalent to the approximately 1 kg of milk[25].

Table 1. Average chemical composition of sweet and acid whey (%)

Ingredient (%)	Sweet whey	Acid whey
Water	93-94	94-95
Dry matter	6.0-7.0	5.0-6.0
Lactose	4.5-5.0	3.8-4.3
Lactic acid	≤ 0.8	≤ 0.8
Total protein	0.8-1.0	0.8-1.0
Whey protein	0.6-0.65	0.6-0.65
Citric acid	0.8-1.0	0.8-1.0
Minerals	0.5-0.7	0.5-0.7
pH	6.4-6.2	5.0-4.6

Whey mainly contains the water soluble vitamins, while the amount of fat soluble vitamins depends on the amount of fat that is lagging behind in the production of cheese. Due to its composition, whey is a very suitable substrate for the growth and development of microorganisms present in cheese or milk, so it should be processed as soon as possible in the desired product.

The amount of mineral matter of whey is very variable due to significantly different biochemical processes in cheese production technology. Whey is very rich in soluble mineral substances, especially acid whey, which composition can be seen in Table 2. There is mainly a difference in the amount of calcium and phosphorus, which are mostly in the form of salt (Ca-phosphate). During the heat treatment, the solubility of mineral matter decreases, which reduces the nutritional value of whey. In addition, a high amount of mineral matter as well as a high amount of water is the main reasons for technological problems in the processing of whey, which reduces the economy of products[25].

Table 2. Mineral matter content in sweet and acid whey

Minerals	Recommended daily intake (mg) - WHO	Sweet whey (mg/100g)	Acid whey (mg/100g)
Calcium	500	796	2054
Zinc	22	1.97	6.31
Magnesium	300	176	199
Phosphorus	1000	932	1348

Lactose is a disaccharide made of glucose and galactose and is the largest part of the dry matter of whey. In sweet whey, it content ranges from 74% to 75%, while less than 1% is found in the isolated whey protein. The chemical hydrolysis of lactose is very difficult, while it can be easily hydrolyzed by β galactosidase and lactase, present in the mammary small intestine. Slow hydrolysis of lactose to glucose and galactose during food digestion generates prolonged energy supply, thus representing an efficient energy source that contributes to the value of whey. This reaction increases the sweetening effect, since lactose itself is less sweet than both hexoses and 6 times less than sucrose. Nutritional value of lactose is reflected in the fact that, together with amino-sugar in the gastro-intestinal tract, it facilitates the absorption of calcium and other alkaline earth metals, creating helates and preventing their precipitation. Lactose is important for the

human body because it: stimulates peristalsis of the intestine, facilitates the absorption of calcium and phosphorus, fixing calcium in bones - skeleton construction, creates the mildly acidic environment in the intestines and thus prevents the growth and reproduction of harmful bacteria.

The protein content, which makes it a high-grade raw material, is very similar in acid and sweet whey. In traditional cheese production, regardless the milk coagulation process, whey proteins migrate completely into whey, because they are insensitive to the action of acids and enzymes. Whey proteins are one of the most valuable proteins due to its composition, characterized by a high proportion of essential amino acids (mostly lysine, cysteine and methionine). The amino acid composition of whey proteins is given in Table 3.

Table 3. Amino acid profile of whey proteins

Aminoacid (AA)	mg AA/g protein
Isoleucine	49.7 – 57.3
Leucine	79.8 – 106.6
Valin	18.4 – 59.3
Lysine	76.1 – 88.1
Methionine and cysteine (combination)	≈ 79.7
Phenylalanine and tyrosine (combination)	≈ 58.2
Treonin	61.1 – 68.7
Tryptophan	≈ 17.3
Histidine	7.8 – 18.7
Alanine	42.1 – 55.5
Arginine	22.0 – 27.1
Glutamine	141.4 – 158.4
Glycine	13.8 – 53.2
Prolin	46.7 – 66.6
Serin	38.8 – 53.0
Aspartate	≈ 94.1

The biological value of the protein depends on the amount and type of amino acids contained therein. It is larger that the amino acid composition of proteins from foods and similar to the amino acid composition of proteins in the human body. The amino acid composition of a protein product is comparable with aminoacid composition of milk or egg proteins, which biological value is 100. Protein of vegetable origin has a

lower biological value because they lack some essential amino acids, or they do not have enough essential amino acids. Due to this amino acid composition, whey proteins have a much higher biological value than casein, or even than reference egg protein. The usefulness of whey proteins in the organism is closely related to the cysteine/methionine ratio that is about 10 times higher than in case of casein[26].

However, the basic deficiency of whey proteins is their thermolability. Proteins are generally irreversibly denaturated in heat treatment, but their denaturation can also be reversible depending on the temperature and duration of heat treatment.

2.3. Whey proteins

Whey proteins are: α-lactalbumin, β-lactoglobulin, blood serum albumin and immunoglobulins. Less commonly are glycomacropeptide, lactoferrin, lactoperoxidase, and lactostatin. The composition of whey proteins is shown in Table 4.

Table 4. Distribution of fractions in whey proteins

Protein	% of whey proteins	Benefits
β-lactoglobulin	50-55%	Source of essential amino acids and branched chain amino acids
α-lactalbumin	20-25%	Primary protein found in human milk
		Source of essential amino acids and branched chain amino acids
Immunoglobulins	10-15%	Primary protein found in colostrum
		Immuno-modular benefit
Lactoferrin	1-2%	Antioxidants
		Antibacterial, antiviral and anaphylactic
		Promote the growth of useful bacteria
		It occurs naturally in mother's milk, tears, mucus, bile, blood
Lactoperoxidase	0.50%	It inhibits bacterial growth
Bovin serum albumin	5-10%	The source of essential amino acids
		Large protein
Glycomacropeptide	10-15%	The source of the branched chain amino acid
		Lack of aromatic amino acids

2.3.1. β-lactoglobulin

β-lactoglobulin is the main whey protein. It makes 50 - 55% of whey proteins and 12% of total milk proteins. It is a dimer composed of two peptide chains that are firmly bound by non-covalent bonds, and one monomer consists of 162 amino acids with five cysteine residues[27]. The concentration of β-lactoglobulin in whey is 1290 - 2280 mg/L[28], and in milk 2 - 4 g/L[29]. It has the ability to bind fat-soluble molecules and improve the absorption of fat soluble nutrients.[29]

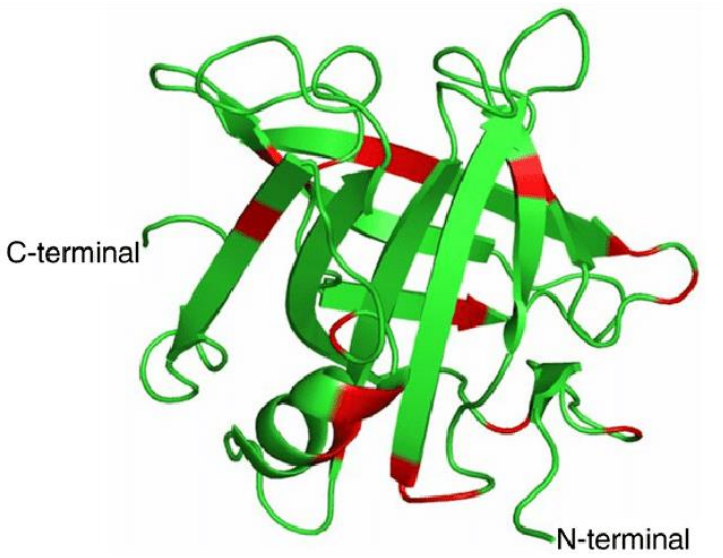

Figure 2. Molecular structure of β-lactoglobulin

According to the literature data[30,31,32] the health effect of β-lactoglobulin is reflected in facilitating digestion of milk fat by binding free fatty acids liberated by lipases in the mouth and reducing cholesterol levels. Certain modifications of β-LG give potential HIV-1 inhibitors, which inhibit the type 1 and 2 herpes simplex virus.

2.3.2. α-lactalbumin

α-lactalbumin consists of a single chain with approximately 123 amino acids and represents about 20-25% of total whey proteins. The concentration in whole milk is 0.6-1.7 g/L, and in whey it varies from1.2 to 1.5 g/L. The share of cysteine residues is 6.5%[29]. It represents a good nutritional source for newborns, since the similarity with human α-lactalbumin from breast milk is 72%[33].

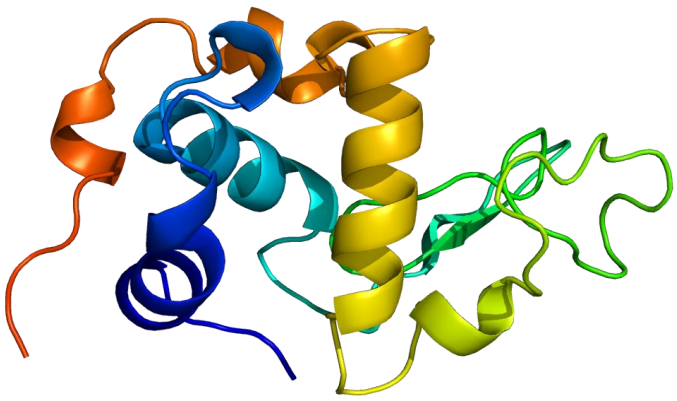

Figure 3. Molecular structure of α-lactalbumin

The health effect of α-lactalbumin[34,35,31] is that it carries fragments that possess antimicrobial activity. It is also prebiotic-proven and is capable to stimulate the growth of certain "good" bacteria in the intestines. The most important health effect is that it contributes to reducing the risk of some types of cancers[34,35]. α-lactalbumin positively affects stress due to the high concentration of tryptophan, the amino acid involved in the synthesis of serotonin (the happiness hormone), and is used in the treatment of chronic fatigue treatment.

2.3.3. Bovine serum albumin

Bovine serum albumin (BSA) contains 583 amino acids. Its concentration in milk is 0.4 g/L, and in whey it is approximately 5-10%[29.] BSA allow binding to hydrophobic molecules and potentially increase the intake of hydrophobic fat soluble molecules[36], and also act as antioxidants, protecting the fat from oxidation. This protein also has an ACE inhibitory activity.

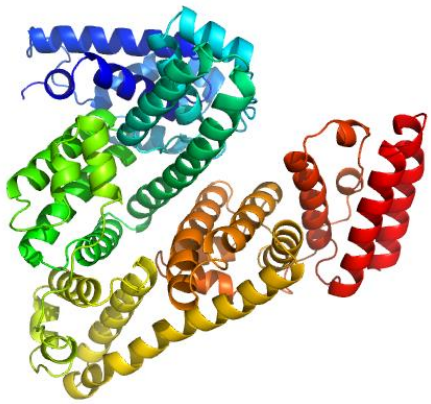

Figure 4. Molecular structure of bovine serum albumin

2.3.4. Immunoglobulins

Whey immunoglobulins have various forms, and its concentrations account for up to 8% of total whey proteins. Immunoglobulins are small molecular weight proteins, and some of them contain disulfide bridges in their structure and represent the richest source of amino acid cysteine, which stimulates the synthesis of glutathione - the main antioxidant in human body. Bioactive peptides of whey have a tendency to act on the immune system much more than proteins from other sources, which is exactly attributed to whey immunoglobulins[29]. Immunoglobulins are monomers or polymers composed of 4 chains. They are grouped into three groups according to their function in the human organism. IGG, IGA, IGM. Even 80% IG in whey belongs to the IGG group[37].
Immunoglobulins from whey showed a certain level of protection of the tooth from the appearance of caries. They also positively affect the mucous membrane of the gastrointestinal tract and protect it from the pathogen. Currently, the possibility of using immunoglobulins from milk in the treatment of patients with AIDS[38] is being examined.

2.3.5. Lactoferrin

Lactoferrin (LF) is an iron chelating glycoprotein monomer. Human lactoferrin contains 691 amino acid residues, while bovine contains 989 amino acid residues[37]. In the human organism it is found in milk, tears, sputum and other secretions. The amount of lactoferrin in milk depends on the lactation period and the animal species. Human milk contains up to 4.0 mg/mL of lactoferrin, while cow milk it is significantly less than 0.02 to 0.35 mg/ mL of lactoferrin[38].

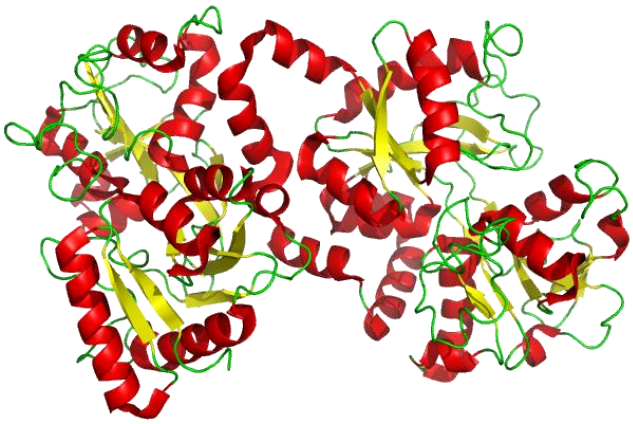

Figure 5. Molecular structure of lactoferrin

Lactoferrin exhibits a highly bactericidal and antiviral effect. It works on a series of viruses, and its effect on the HIV virus is also examined. It has an anti-cancer effect attributed to its ability to bind iron, which has potentially mutagenic effects on cells by causing oxidative damage to the nucleic acid chain[38].

2.3.6. Casein macropeptide

Casein macropeptide (CMP) is a heterogeneous polypeptide fraction formed by cleavage of casein. During cheese production, the milk is hydrolysed by renin, so casein is hydrolyzed into two parts; one is para-casein that remains in cheese and another CMP that goes into whey. CMP stimulates the growth of good bacteria in the intestine and shows a strong anti-inflammatory effect[39,40] and a potent ACE inhibitory activity. Consuming whey stimulates the release of satiety hormones, and CMP is responsible for this.

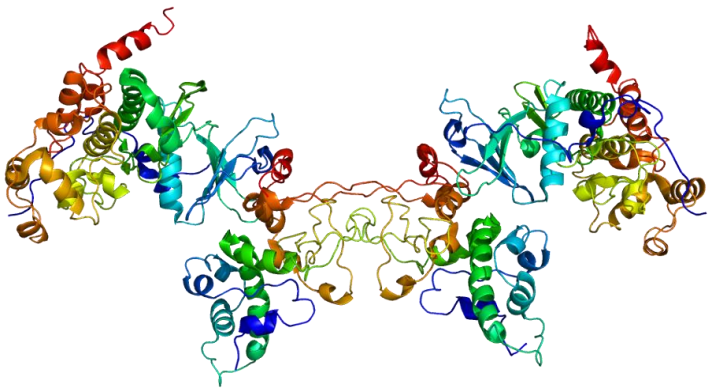

Figure 6. Molecular structure of casein

2.4. Importance of whey proteins

Whey proteins are not only important because of their health characteristics, which we have described in the previous chapter, but also due to their technological characteristics: solubility, water retention ability, foam production and emulsifying properties.

Solubility is one of the most important properties of polydispersed whey proteins. It is lost by heat denaturation. Immunoglobulins are denatured at 70 °C, while β-lactoglobulin, α-lactalbumin, albumin of the bovine serum and the pepton fractions are denaturated at 100 °C. The solubility of the protein is the balance between

hydrophobicity, i.e. the tendency of the molecules to interact with each other and the formation of a protein-protein bond that reduces the solubility of proteins and hydrophilicity, and the ability of proteins to build protein-water relationships, which increases the solubility of the protein. External factors such as pH, temperature, as well as the presence and concentration of different agents in the system, have a great influence on the solubility of the protein. Large proteins have less solubility than small proteins, which is the reason for the positive effect of hydrolysis on the solubility of whey proteins.

Water binding is one of the most interesting properties of whey proteins. It depends on the relationship between free and bound water and the method of water binding, which affects the consistency of the food and its stability. This is especially important for maintaining the freshness of the product. The capability of water binding native and denaturated whey proteins ranges from 0.5-1.2 g H_2O/1 g of dry matter[41]. Molecules with different lengths and structures react differently with water and fats. Large molecules like long proteins have the ability to retain water and oil in two ways: chemical bonding and "capturing" molecules between proteins. Short peptide chains do not have this ability to physically "capture" the water and oil molecules.

Creation of foam is a characteristic feature of whey proteins. Foam is created when the whey proteins are mixed with water with the bubble or air incorporation. In order to create foam it is necessary to introduce a large amount of energy into the system, but in spite of this, the foam as a form is unstable.

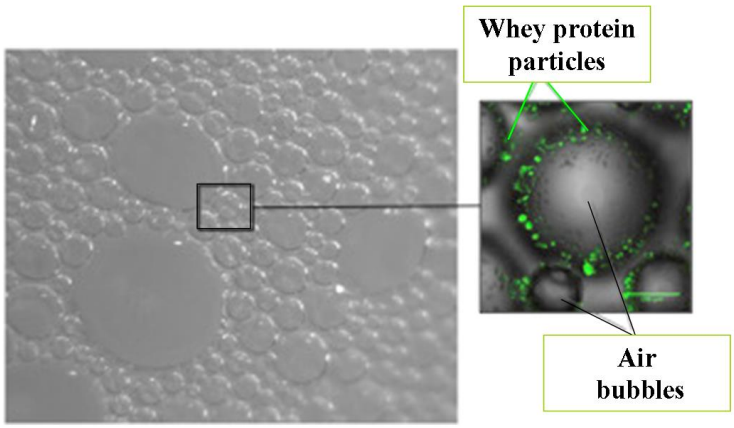

Figure 7. Influence of whey protein particles on foam forming

The instability of the foam lying in the fact that the interactions between the water molecules are considerably stronger than the interaction between water and air, which leads to the integration of smaller molecules into larger and to the reduction of the liquid layer between the bubble thus the disappearance of the created foam. Foam stability is a key parameter in the production of various fat fillings in the confectionery industry.

Emulsion is an essential property of whey proteins, and is especially important for the production of creams, fillings and chocolate in the confectionery industry. Emulsions are the dispersions of liquids that do not interfere with each other, which are stabilized by the emulsifier, the surfactant that form the film on the contact surface of the two phases. The oil phase in the emulsion tends to separate to the surface of the aqueous phase. Continuous-phase proteins have the ability to act more or less as surfactants and reduce surface stress, which contributes to the stability of the emulsion.

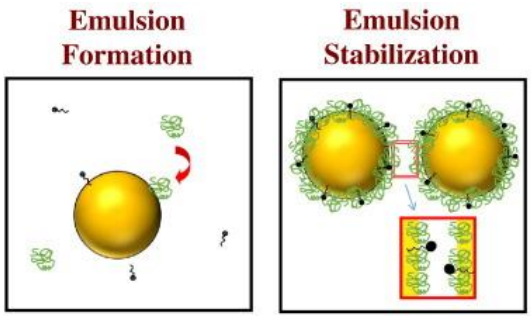

Figure 8. Influence of whey protein particles on emulsion formation and stability

Enzymatic hydrolysis can significantly affect the ability of the protein to stabilize the emulsion, while a high degree of hydrolysis does not lead to better emulsifying properties. The reason for this is that very small peptides do not have the capacity to form a stable peptide film at the boundary surface of water and oil[42].

2.5. Biotechnological modification of whey proteins

Proteins are composed of amino acid chains, and amino acids are compounds containing carboxylic and amino groups. The formation of a peptide bond allows the association of two amino acids, and the formation of a chain of amino acids. By hydrolysis of such chains, the protein is divided into smaller parts of the peptide, which can contribute to the increase in the nutritional value of protein foods.

By protein hydrolysis, and enriching food with given peptides, the nutritional properties of food, texture, solubility, degree of coagulation and emulsion can be significantly

enhanced. Also, hydrolysis slows down the process of food spoilage, prevents unwanted interactions of food ingredients, the appearance of foreign odors, flavors, toxic and inhibitory substances.

Hydrolyzed whey proteins can be used as an anti-allergic agent because they lose the ability to induce an allergic reaction. They are easy to digest, because they are partially processed, provide additional antioxidants, strengthen the immune system and help repair muscle. However, the digestion speed that is significantly higher than in non-hydrolysed proteins makes the hydrolyzed proteins much favorable whey ingredients. For the application of proteins and hydrolysates in the food industry, their behavior as well as the technological properties in production and storage conditions needs to be well defined.

2.5.1. Enzymatic modification of whey proteins

Controlled enzymatic hydrolysis implies enzymatic cleavage of proteins into smaller fractions of different molecular weights, under controlled enzyme conditions. Hydrolysis is mainly interrupted by thermal or pH inactivation of the enzyme after a certain degree of hydrolysis has been reached. The advantage of enzymatic hydrolysis in relation to chemical reactions is that enzymes are active at moderate temperatures, pH, and pressure in aqueous media, which allows the enzymatic processes to be carried out under gentle reaction conditions, whereby products of better quality and in higher yield are obtained. The choice of enzymes that will be applied in the process of enzymatic hydrolysis of proteins is of great importance. The properties of hydrolysates are also largely dependent on the control of hydrolytic processes by adjusting the temperature, pH and duration of hydrolysis. Hydrolysis is carried out in buffered solutions or in the pH-stat system.

In the first 60-90 minutes, protein degradation intensifies, and then, depending on the enzymes and substrates, there is stagnation and deceleration of process. Digestive enzymes such as pepsine, trypsin and chymotrypsin are the most commonly used enzymes in the case of controlled enzymatic hydrolysis of whey proteins.

2.5.1.1. Proteolytic enzymes

Proteolytic enzymes or proteases represent a large group of enzymes that catalyze hydrolysis of the peptide bond in proteins and peptide molecules, whereby they gave

protein products with less molecular weight. They belong to the third group of enzymes so-called hydrolases.

The largest amounts of proteases are produced by microorganisms, but certain types of plant and animal origin are still used. Classification can be done on the basis of several criteria:

1. The position of the peptide bond in the protein of the protein on which they act
• exopeptidase
• endopeptidase
2. Structure of the active center
3. Mechanism of action
4. pH value at which they work

2.5.1.1.1. Trypsin

Tripsin is an enzyme that is synthesized in the pancreas in the form of an inactive form of trypsinogen. Due to the fact that activation of trypsin demands the environment conditions with pH value 8, it is excreted together with the alkaline pancreatic juice. Tripsinogen is activated autocatalytically in trypsin by the action of the trypsin itself. The tripsinogen is constructed from one polypeptide chain and the activation process comprises hydrolysing a peptide bond, in which one hexapeptide with the NH_2 end of the trypsinogen is formed. The alone trypsin carries out the hydrolysis of the peptide bond between the carboxyl groups of the basic amino acids and the aminogroup of the adjacent amino acid, thereby obtaining less form-peptides. Using other proteases, the peptides are further hydrolysed into amino acids that are absorbed into the bloodstream. It is of great importance to trypsin digestion because proteins are hydrolysed into smaller forms that are now available, and which can be absorbed through the small intestine mucosa, making the reaction itself one of the essential in our body[43].

In addition to pancreas, this enzyme is also obtained through food. Pancreatic enzymes are of animal origin and only function in the small intestine. If pancreatic enzymes are brought together with food, they will be destroyed by stomach acid, so they are not as effective. However, plant enzymes are significantly more effective because they have the ability to function in acidic and in the base environment. As we age, our body loses

the ability to produce its own enzymes, so we have to feed them through food. One way is to eat raw organic food, and the other is to take enzymatic supplements[44].

2.5.1.1.2. Pepsin

Pepsin is an enzyme that breaks down proteins into smaller peptides (that is, a protease). It is produced in the stomach and is one of the main digestive enzymes in the digestive systems of humans and many other animals, where it helps digest the proteins in food. Pepsin has three dimensional structure, of which one or more polypeptide chains twist and fold, bringing together a small number of amino acids to form the active site, or the location on the enzyme where the substrate binds and the reaction takes place. It is one of three principal proteases in the human digestive system, the other two being chymotrypsin and trypsin. During the process of digestion, these enzymes, each of which is specialized in severing links between particular types of amino acids, collaborate to break down dietary proteins into their components, i.e., peptides and amino acids, which can be readily absorbed by the small intestine. Pepsin is most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine The purification, molecular properties, kinetics and mechanism of action of pepsinogen and pepsin have been reviewed[45].

The pepsin consists of a peptide series consisting of 321 amino acids and has a molecular weight of 35 kDa. Pepsin is most active in acidic environments between 37 °C and 42 °C. Accordingly, its primary site of synthesis and activity is in the stomach (pH 1.5 to 2.0). Pepsin will digest up to 20% of ingested amide bonds by cleaving preferentially at the N-terminal side of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine. Increased susceptibility to hydrolysis occurs if there is a sulfur-containing amino acid close to the peptide bond, which has an aromatic amino acid. Pepsin cleaves Phe1Val, Gln4His, Glu13Ala, Ala14Leu, Leu15Tyr, Tyr16Leu, Gly23Phe, and Phe24 in the insulin B chain. Pepsin exhibits maximal activity at pH 2.0 and is inactive at pH 6.5 and above, however pepsin is not fully denatured or irreversibly inactivated until pH 8.0. Therefore, pepsin in solution of up to pH 8.0 can be reactivated upon re-acidification[46,47,48].

2.5.2. Microbiological modification of whey proteins

It is known that certain lactic acid bacteria have proteolytic activity. Bioactive peptides during the milk processing can be formed by the action of the native microorganism enzymes or enzymes of the starter cultures used in the processing of milk. *Lactobacillus* species are most commonly used industrial microorganisms. Most of they are probiotic because of their beneficial effect on human and animal health. They inhabit the residences that are rich in amino acids, peptides, nucleotides, fatty acids, vitamin B groups, minerals, and other necessary growth factors (digestive tract). These bacteria mostly use a lot of complex growth media because they can not synthesize all the necessary nutrients themselves. In industrial conditions, additional enrichment of the media with sources of nitrogen and vitamins is necessary for achieving higher yields, which is reflected in the total production costs[49].

2.5.2.1. Proteolytic bacterial strains (*Lactobacillus rhamnosus* ATCC 7469)

The important property of BMK, given their ability to grow in milk and other protein substrates, is the possession of a proteolytic enzyme system. The effectiveness of proteases, peptidases and the transport system is of great importance for the activity and propagation of BMK in the substrate in which the proteins represents the basic source of nitrogen. For their normal development, BMK requires free amino acids that are not enough in whey. Therefore, they produce protease responsible for the decomposition of whey proteins whose final degradation products, amino acids and peptides, remain in the fermented product as significant carriers of bioactivity.

One of the most frequently used proteolytic cultures is *Lactobacillus helveticus*. Many studies have shown that fermentation of milk with this culture results in the release of ACE inhibitory peptides. Terzić-Vidojević et al. (2013)[50] found that as much as 45% of lactobacilli and 54% of lactococci have proteolytic activity. Strains *Lactobacillus delbrueckii* ssp. *bulgaricus* CRL 454, *Lactobacillus acidophilus* CRL 636 and *Streptococcus thermophilus* CRL individually and in combination show good proteolytic abilities and represent a good choice in the production of functional whey based beverages[51]. A good proteolytic activity was also demonstrated by commercial yogurt culture ABY 6 composed of 4 cultures: *Streptococcus* salivarius ssp.

thermophilus (80%), *Lactobacillus acidophilus* (13%), *Bifdobacterium bifidum* (6%), *Lactobacillus delbrueckii* ssp. *bulgaricus* (1%), and combination of ABY 6 and *Lactobacillus rhamnosus* ATCC 7469 in the fermentation of substrate that contains 70% whey and 30% milk[52]. Good proteolytic activity also shows the bacterial culture *Streptococcus thermophilus*, giving very good results related to the bioactivity of the resulting peptides in the form of antioxidant activity and ACE inhibitory activity[53]. *Lactobacillus rhamnosus* ATCC 7469 is used in many fermented dairy products to improve the tissue characteristics of the fermented food product because it produces exopolysaccharides (EPS). EPS are polymeric compounds that are considered natural thickeners that contribute to the structure of fermented dairy products, texture in synergy with taste. *Lactobacillus rhamnosus* produces from 931.0 to 1275.0 mg/L exopolysaccharides from glucose or lactose medium at temperatures of 32 or 37 °C. The produced exopolysaccharides possess 72% glucose equivalent and 1.1% protein content. At a maximum concentration (about 20 g/L), the viscosity of the solution of this exopolysaccharide is about 17.7 mPas. A fermented beverage with improved antioxidant activity of 49.2% could be produced using the mixture of ABY-6: *L. rhamnosus* culture[49].

2.6. Bioactive peptides

Recently, there is a growing consumer interest in food that is not only a source of basic nutrients, but has a positive impact on health and quality of life. Whey is a rich source of proteins and peptides that can be used to produce functional foods. Enrichment of fermented products with selected bioactive proteins and whey peptides represents a practical solution for the formulation of a functional product that meets the demands of today's consumers.

Bioactive peptides are defined as specific protein fragments that have a positive effect on human health, reduction of hypertension, prevention of carcinoma, increase in pure muscle mass, stimulation of the immune system, and inhibiting the pathogenic microorganisms. Depending on their function, they can be divided into those with sensory, antioxidant, surface-active and physiological function. The arrangement of the associated amino acids affects the biological activity of the peptides such as: binding of

minerals, metals, optical abilities, antioxidant (5-16 amino acid residues), antimicrobial, and anticoagulant, hypocholesteremic, immune and antihypertensive.

These peptides are inactive in the original protein, but by separation they become biologically active. One peptide may contain between two to twenty amino acid residues. They can be components of plant origin: pulses, soybeans, cereals and mushrooms or animal, so that they are separated from milk, dairy products, eggs, animal blood.

2.6.1. Production of bioactive peptides

Biologically active peptides are typically composed of 2-20 amino acids and can be formed in the following ways:

- during the process of milk fermentation, the activity of proteolytic enzymes from the starter cultures
- during the enzymatic hydrolysis in the gastrointestinal tract under the action of digestive enzymes or microflora of the intestinal tract
- during the milk processing process using commercial enzymes of microbiological or plant origin

Bioactive peptides can be formed by the action of enzymes of microorganisms or enzymes of starter cultures used. Proteolytic system of lactic acid bacteria of the genus *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* is very well characterized and consists of cellular intracellular proteases including endopeptidases, aminopeptidases, tripeptidases, and dipeptidases. Recent studies have shown that besides a large number of different bioactive peptides, such a proteolysis produces a significant number of peptides with immunomodulatory function[54]. Bioactive peptides formed during the digestion process are usually products of casein degradation by the action of proteases: pepsin, trypsin or chymotrypsin. In the first phase of digestion, precursors of bioactive proteins are subjected to pancreatic enzymes; they pass through the stomach where they can spend up to several hours, broken down by gastric juice (HCL and pepsin). After that, the activity of the intestinal tract enzyme is followed. Microbiological enzymes of the microflora of the digestive tract act only on those protein fractions that reach the colon. In comparison with microbiological enzymes of microflora or starter culture during the fermentation

processes, human digestive enzymes have different active sites included in protein hydrolysis. The resulting bioactive peptides can therefore significantly differ in both their function and absorption[54]. A large number of starter cultures that are commercially used in the production of yoghurt and other milk fermented and probiotic beverages have proteolytic activity. Thus, in many fermented products based on milk and whey, in addition to lactic acid, various bioactive peptides are released. This process is very complex and poorly controlled precisely because of a series of accompanying biochemical processes that take place during fermentation along with protein hydrolysis. This method for the production of bioactive peptides is most often used for the commercial production of peptides with a particular biological function, such as antihypertensive, immunomodulatory, and antioxidative. Proteolytic enzymes isolated from BMK have been successfully used to produce bioactive peptides with ACE inhibitory activity. These enzymes are the most commonly calcium-binding enzymes[54]. One of the most frequently used proteolytic cultures is *Lactobacillus helveticus*. Many studies have shown that fermentation of milk with this culture results in the release of ACE inhibitory peptides.

2.6.2. Significance of bioactive peptides

A large number of studies have shown the positive effect of the peptides in regulation of the nervous system, gastrointestinal tract, modulation of digestive enzymes, absorption of nutrients, cardiovascular and immune system. Peptides derived from ß-lactoglobulin show antihypertensive, anticoagulant, opioid, antimicrobial, immunomodulatory, and hypocholesteremic characteristics. In addition, all of these peptides have significant antioxidant activity and the ability to capture free radicals. In addition to these, there are many multifunctional features shown by casomorphins, lactorphins, immunopeptides, and carotenoids[55]. The functions and properties of bioactive peptides depend on the activity of endogenous (proteolytic) enzymes as well as the proteolytic capabilities of the microorganism used. The advantage of using bioactive peptides produced by microbial fermentation is that in addition to proteolytic activity, BMK also provides additional health benefits[55].

2.6.2.1. Effects of bioactive peptides on the gastrointestinal system

Whey is often used as a support for gastrointestinal health. Its role in the protection of mucous membranes has been documented in several animal studies and is presumed to be associated with its GSH-stimulating property339. In addition to its role in the synthesis of GSH, glutamate can play an additional role when converted to glutamine, an amino acid used as a fuel by the intestinal mucosa. Formulas based on whey peptides cause less diarrhea accidents than casein based formulas due to the better and faster absorption (unpublished data). Since the digestive function is significantly impaired in severely affected patients, and diarrhea is one of the more serious problems they face, whey proteins and peptides play an important role in the protection against diarrhea, which can also lead to hydration.

2.6.2.2. Effects of bioactive peptides on the nervous system

Peptides separated from whey proteins showed positive effects on the nervous system. These peptides are known as opioid peptides and they represent short amino acid sequences that bind to opioid receptors in the brain. The first detected neuropeptides were beta-cacomorphins, isolated as fragments of beta-casein. When they are absorbed in the blood, they are transported to the brain and other organs of the nervous system and exhibit a effect similar to that of opium. This may have a calming and sleeping effect[56].

2.6.2.3. Effects of bioactive peptides on the immune system

Bioactive peptides play an important role in maintaining the immune system. Proteins of whey and peptides isolated from whey proteins have an immunomodulatory role. Whey peptides can reduce allergic reactions and increase mucosal immunity in the gastrointestinal tract. Also, some studies have shown that peptides have an anti-tumor effect. Therefore, we can conclude that bioactive peptides play a major role in the formation of the immune system of the newborn[57].

2.6.2.4. Effects of bioactive peptides on the cardiovascular system

Hypertension is one of the major risk factors for cardiovascular disease. Eating habits are very important in the prevention and treatment of hypertension, special importance

is attached to foods containing substances with antihypertensive activity. Antigensin-converting enzyme-ACE is an enzyme that is found in many tissues and plays an important role in increasing blood pressure. As ACE inhibitors, fragments derived from alpha-lactalbumine and beta-lactoglobulin are used mainly. Thrombosis is defined as the appearance of blood clots in a blood vessel that can cause many cardiovascular diseases. Fibrinogen is a protein that converts into fibrin in the formation of blood clots. In order to aggregate the platelets, binding of fibrinogen to platelets is necessary. This binding can be inhibited by the action of peptides derived from whey such as glycomacropeptide and lactoferrin[58].

2.6.2.5. The role of whey in the treatment of sarcopenia and muscle wasting

Whey proteins and essential amino acids promote the reduction of fat tissue and increase the synthesis of muscle proteins during low calorie diets in elderly and obese individuals. Sarkopenia is associated with reduced muscle protein synthesis as a response to food intake. The difference in digestion and the absorption kinetics of dietary proteins or their amino acids, or both, modulates the accumulation of proteins in the muscles. Pennings et al.[59] compared protein digestion, absorption kinetics, and accumulation of muscle proteins after taking whey, casein, and hydrolyzate casein in healthy elderly people, and concluded that whey proteins stimulate the accumulation of muscle proteins more efficiently than casein and casein hydrolyzate in elderly men. This effect is attributed to a combination of faster digestion and whey absorption and increased leucine content, which contributes to the prevention of sarcopenia and muscle wasting.

In addition to all of the above, several studies emphasize that whey can be indicated as a therapy for allergies, diabetes, amyotrophic lateral sclerosis and burns. However, evidence of clinical efficacy in these conditions is limited, in particular with regard to doses and duration of therapy.

2.7. Antioxidative activity of whey protein hydrolysates

Pathological conditions of the organism (aging, cancerogenesis, cardiovascular, neurological diseases, diabetes, infertility) are caused by uncontrolled and intermediate production of free radicals in the human body, usually reactive oxygen species which

cause degradation of cell proteins, enzymes, lipid membranes, DNA and thereby reduce cellular resorption[54,60]. Pathological changes in the organism are most often the result of diet, food quality and the environment, as well as habits and physical activity. Even 2-5% of the molecular oxygen that is introduced into the body remains unmanaged in the respiratory cycle or cells and represents a toxic form. Under normal conditions, the formation of free radicals is in balance with the antioxidant defense system of the organism. If the balance is disturbed, there is an increased production of free radicals or a decrease in the concentration of antioxidants. This condition is called oxidative stress.

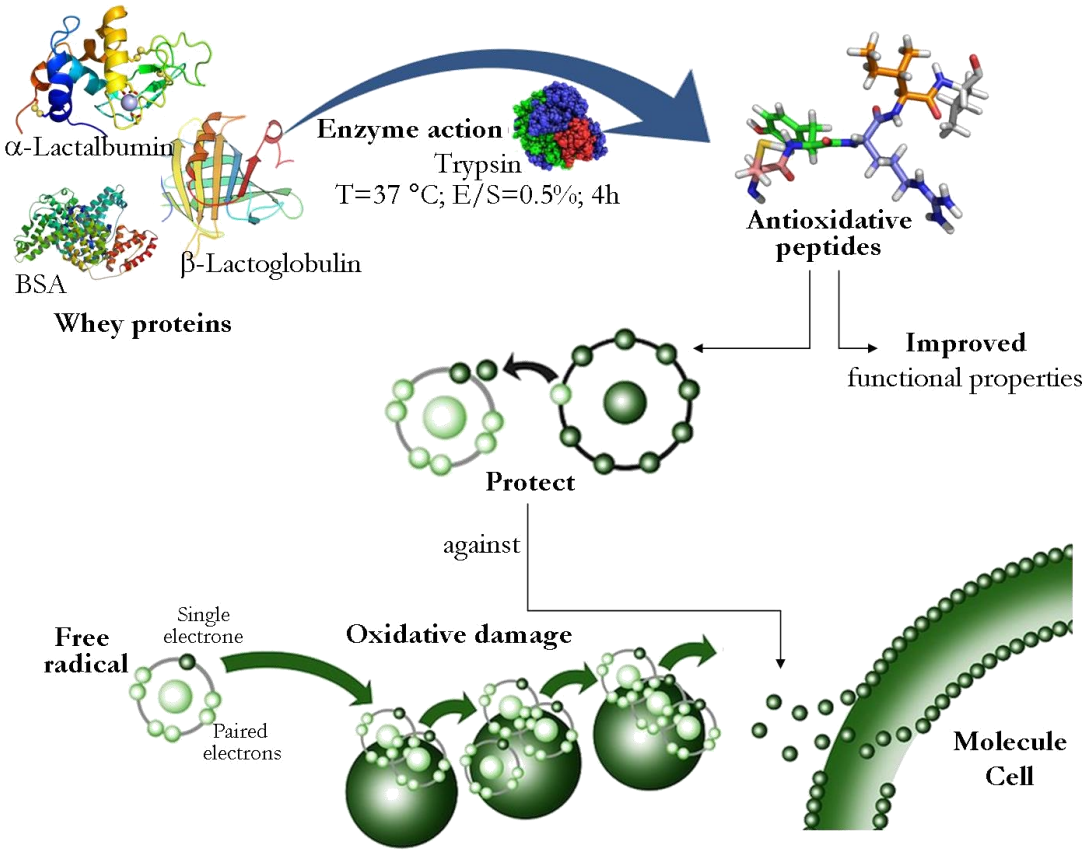

Figure 9. Mechanism of action of antioxidative peptides

Whey, fermented and hydrolysed whey products are extremely rich in antioxidant substances. The antioxidant activity depends primarily on the content of antioxidant species, the fat content, and the additives used in the production process such as fruits, or prebiotics. The mechanism of antioxidant activity of fermented dairy products is attributed to the bioactivity of certain protein fractions of milk as well as to direct activity, the ability of starter cultures to inhibit peroxidases and to decompose the

superoxide anion and hydrogen peroxide[54]. The amino acids Tyr, Trp, Met, Lys, Cys and His possess active chemical groups that give peptides an antioxidant property. On the other hand, amino acids with aromatic side residues are not able to affect oxidative processes. This proves that the antioxidant properties of hydrolysates directly depend on the amino acids contained therein. Also, the position of amino acids in the peptide sequence has an effect on the antioxidant activity of bioactive peptides. Removal of terminal He in the Leu-Leu-Pro-His-His peptide sequence leads to a significant decline in the antioxidant activity of the same. The Pro-His-His sequence independently gives the highest degree of antioxidant activity[54].

2.8. Possibility of using whey protein hydrolysate

More recently, many studies have focused on the ability to use these active molecules in the diet. Whey proteins have found a great application in the nutrition of bodybuilders and other professional athletes, their application is based on the ability of proteins and whey peptides to transfer directly through the small intestine, blood and lymphoma directly to the muscle in which they are involved. In this way, frequent exercise muscles gain more weight on the mass than the consumption of some other proteins.

Whey protein can be used as a supplement to cassava flour. This flour was used for the production of two types of pastry: muffin and biscuit. Also, the addition of whey protein significantly enhances the protein composition of cassava flour products[61]. The addition of whey proteins into bakery products from wheat, as well as in wheat and rice products significantly improves their nutritional content, and does not adversely affect other properties of products[62].

The addition of whey protein hydrolysate is somewhat more complicated compared to the addition of non-hydrolysed proteins due to the large differences in the technological and other physico-chemical properties of hydrolysates. Because of the bitter taste that possesses a large number of bioactive peptides derived from whey, their application without essential changes in the taste of the product is possible mainly for products that taste the taste of yogurt, chocolate and coffee as the basic taste. These three tastes greatly mask the bitterness of bioactive peptides without disturbing their structure and activity. In addition to food products, bioactive peptides have been found to be used in pharmacy, treatment.

2.8.1. Application in food products

A modern lifestyle which involves a lot of stress, a little free time and even less time to prepare a meal, with the consumption of fast foods rich in carbohydrates and fats, as well as various preservatives and stabilizers, requires significant correction in the diet and the quality of the foods we consume. Functional food is a brand new product category that has emerged over the last decade, based on the increased interest of consumers in improving the quality of life and health. By the term "functional food" we mean all foods with biologically active activity, which helps to preserve health and influence certain physical functions.

Bioactive peptides are increasingly used in food for athletes. They are used as food supplements to increase muscle mass and repair muscle damage. It is best to consume them with water because if they are taken with milk, casein from milk will slow down their absorption.

Whey protein hydrolysates recently recognized as a food ingredient with important nutritional and functional properties, gaining acceptance as a valuable functional food ingredient. Hydrolysis of whey proteins is known to release bioactive peptides that can exhibit a number of physiological properties and enable them to exhibit antioxidative, antihypertensive, antimicrobial activity, opioid activity and antithrombotic activity[63]. However, a number of researches related to the production of functional chocolate and its placement in commercial flows is quite a little, which could not be explained by the inappropriateness of chocolate matrix[64]. A possible answer to this lack may be attributed to the influence of incorporated functional ingredients on the rheology of chocolate as a parameter that is equally important both for the production process and for the sensory quality of the final product. According to the literature reports[65,66], incorporation of additional ingredients usually increases the rheological parameters of chocolate and negatively influences its flow properties. In addition, there could be a significant correlation between interactions of polyphenols and proteins, but the interference of proteins with polyphenols remains inconclusive, regarding the possibility that proteins could reduce the activity of polyphenols[67,68].

However, due to the grinding stage, friction, and high temperatures that can affect the activity of bioactive peptides, it cannot be added during composing the milk chocolate - at the beginning of the industrial process[69].

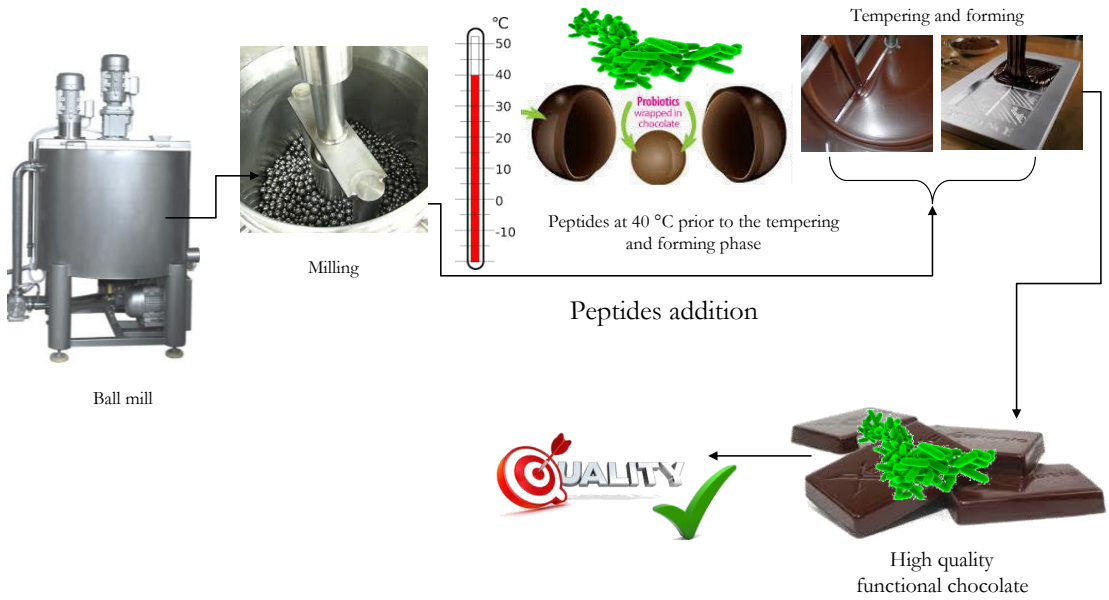

Figure 10. Formulation of functional chocolate enriched with bioactive peptides

The achievement of satisfactory rheology without peptides damage, demands its incorporation at the process stage that allows full homogenization of the chocolate mass. Therefore, the addition of functional ingredients in industrial conditions should be done in the mixing tank (at lower temperatures) before the phase of chocolate shaping.

2.8.2. Application in non-food products

Bioactive peptides in pharmacy - Peptide bioregulators have the power to regain the function of organs and systems in the body, as well as prolong active and healthy life. They contain amino acids identical to the cell structures of the target organ. One of the most famous peptide bioregulators used in pharmacy is collagen. Collagen peptides represent a mixture of important amino acids: glycine, proline and hydroxyproline that play a significant role in the construction of fibrous tissues. Insufficient supply of these acids results in joint pain, nail and hair damage.

Bioactive peptides in cosmetics - In the production of cosmetic preparations, proteins derived from various natural sources are used. The molecular weight of the proteins themselves and their derivatives is of great importance because of its functional

properties (hydration, increasing elasticity, creating a feeling of softness and giving glossy hair). High molecular weight proteins on the skin or hair create a protective film. Peptides maintain a high level of cellular activity. First of all, they serve to regenerate tired and old skin in the smooth, elastic and shiny skin. They also provide protection and cells are more stable due to lack of oxygen and the effects of toxic substances.

Figure 11. Application of bioactive peptides in cosmetic products

Many protein hydrolysates have been used in cosmetic formulations for decades because of their ability to stimulate skin firmness, tone, and elasticity as well as counteract skin aging. Until now, these peptides were poorly studied and their efficacy was poorly demonstrated. During aging, the epidermis and dermis become thin; therefore, an efficient anti-aging product should be able to stimulate the metabolism of senescent fibroblasts and keratinocytes in order to increase the quantity of extracellular matrix components such as collagen and glycosaminoglycans. A parallel study performed on an in vitro skin-equivalent model and with human volunteers demonstrated the efficacy of a specific soy biopeptide against aging. This biopeptide induced a significant increase in glycosaminoglycan synthesis both in vitro and in vivo after a one-month treatment. This new cosmetic ingredient was also able to stimulate collagen synthesis in vitro and in vivo[70]. Enzyme-inhibitory peptides directly or indirectly inhibit the activity of enzymes. Protein or peptides naturally extracted from soybean seeds can inhibit the formation of proteinases. Proteins are frequently used as an anti-aging, skin moisturizing, anti-solar, cleansing, and hair-promoting agent. In a randomized, double-blind, placebo-controlled study[71], soy extract and placebo creams were applied to the volar part of the forearm of 21 healthy women; the papillary index increased to a significantly greater extent with the soy extract cream than with the

placebo cream (4.56 vs. 3.76 arbitrary units, $p<0.05$). Another study with a pseudorandomized design in 10 Caucasian women indicated the superiority of 2% soy biopeptide emulsion to a placebo in terms of collagen synthesis and stimulation of glycosaminoglycan contents[70].

Commercial non-food applications of casein proteins include textile fibers, adhesives, packaging films, biomaterials, plastics and additives in paints, concrete, cement, cosmetics and rubber. Compared to caseins, whey proteins are not so exploited in non-food industries, surfactants in cosmetology and pharmacology, and manufacture of protective films or coatings are some examples. Regarding the milk fat fraction, it is used in the manufacture of emollients and fatty acids. In the current desired green framework, the most important aspect of non-food applications is the valorization of dairy co-products, hence reducing waste streams to compliance with safety and environmental regulations. Cheese whey, a by-product of cheese production, is rich in whey proteins and lactose and some of its value-added applications are manufacture of fermented products such as methane, ethanol, butanol, organic acid, polysaccharides and yeast biomass non-food application[72].

3. EXPERIMENTAL

3.1. Modification of whey proteins by microbial fermentation

3.1.1. Materials

3.1.1.1. Media

Sweet cow's whey powder - CW (Lenic Laboratories, Belgrade, Serbia), with the following composition: proteins 12.11% (w/w), lipids 1.00% (w/w), and carbohydrates 69.62% (w/w), was reconstituted to contain 8% (w/v) dry matter. Sweet goat's whey powder - GW (Stanojević Pharm, Novi Sad, Serbia), with the following composition: proteins 21.99% (w/w), lipids 2.79% (w/w), and carbohydrates 64.39% (w/w), was reconstituted to contain 8% (w/v) dry matter. Whey protein concentrate - WPC (DMV International, Nederland) with the following composition: proteins 80.0% (w/w), lipids 6.30% (w/w), and carbohydrates 6.50% (w/w), was reconstituted to contain 5% (w/v) dry matter. Milk protein concentrate - MPC (Megglosat HP ME) with the following composition: proteins 86.0% (w/w), lipids 1.9% (w/w), and carbohydrates 3.2% (w/w), was reconstituted to contain 5% (w/v) dry matter. The reconstituted substrates were pasteurized at 60 ºC for 60 min and stored at 4 ºC (no longer than one day) prior to its use as a fermentation medium. The chemicals used in this research were of analytical grade purchased from Sigma-Aldrich Chemie GmbH.

3.1.1.2. Microorganisms

The strains *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus reuteri* ATCC 23272, used in this study, were supplied by American Type Culture Collection (ATCC, Rockville, USA). Stock cultures were stored at -18 °C in 3 mL vials containing De Man Rogosa Sharpe (MRS) broth (Fluka, USA) and 50% (v/v) glycerol as a cryoprotective agent. To prepare the laboratory cultures, a drop of desired stock culture was transferred to 3 mL of MRS broth and incubated for 18 h under anaerobic conditions at the optimal growth temperature (37 ºC). The working cultures were pre-cultured twice in MRS broth prior to experimental use.

3.1.2. Methods

3.1.2.1. Fermentation

Experiments were conducted in 200 mL Erlenmeyer flasks containing 100 mL of reconstituted substrates. Samples were inoculated by adding 2% (v/v) of activated cultures and incubated at 37 °C for 24h. During the incubation, samples were taken every 6 h for determination of antioxidant activity.

3.1.2.2. Antioxidant activity

3.1.2.2.1. DPPH scavenging activity

Antioxidant activity of fermented substrates was determined by its ability to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, which was measured according to the modified method[73]. A stock solution of 0.1 mM DPPH (Sigma-Aldrich, Australia) was prepared by dissolving in methanol. After the fermentation samples were macerated with methanol and centrifuged at 8000 rpm for 20 min at 4 ˚C. Methanol (1.5 mL) and DPPH (1.0 mL) were added to the supernatant (0.5 mL). The control sample was prepared by mixing methanol (1.5 mL) and DPPH (1.5 mL), while methanol was used as a blank sample. Mixtures were allowed to stand 30 min in dark, at room temperature. The antioxidant activity was analyzed by reading the absorbance at 517 nm. DPPH scavenging activity was calculated using the following equation Eq. 1:

$$\text{DPPH scavanging activity}\,(\%) = \frac{\text{Ac - As}}{\text{Ac}} \times 100 \qquad (1)$$

Where Aa and Ac represent absorbance of sample and control, respectively.

3.1.2.2.2. FRAP method

The operating FRAP method was conducted following the procedure: 4.5 mL of FRAP solution and 150 μL of extract concentration of 1 mg/mL was poured into the test tube. FRAP solution was prepared just before use. It consists of 25 mL of acetate buffer (300 mmol/L, pH 3.6), 2.5 mL of TPTZ (2,4,6-tripyridyl-s-triazine) and 2.5 mL of 20 mmol/L solution of FeCl3 × 6H2O. The contents of the test tubes were strongly mixed and after 5 min absorbance was measured at 593 nm. Blank was FRAP solution. The results were calculated from the standard curve of $FeSO_4 \times 7H_2O$ (200–1000 μmol/L) and expressed as mmol Fe^{2+}/L[74].

3.1.2.3. Functional properties of WPC hydrolysates

3.1.2.3.1 Emulsifying properties

Emulsifying activity

The emulsifying activity of hydrolysates was estimated according to the method of Pearce and Kinsella (1978)[75] with slight modifications. Briefly, emulsions were prepared by mixing 3 mL of sample and 1 mL of sunflower oil (Vital, Serbia). Mixtures were homogenized for 30 s at room temperature. A 20 μL aliquot of emulsions was immediately pipetted from the emulsions and diluted 250-fold into the 0.1% (w/v) SDS in 0.1 M NaCl, pH 7.0. Diluted emulsions were vortexed for 10 s, transferred into the glass cuvettes (1-cm path length), and the absorbance at 500 nm was recorded against the 0.1% (w/v) SDS in 0.1 M NaCl (pH 7.0) as a blank. Emulsifying activity, expressed as an emulsifying activity index - EAI (m^2/g), was calculated according to equation Eq. 2:

$$\mathrm{EAI}\,(\mathrm{m}^2/\mathrm{g}) = \frac{2.303 \times 2 \times \mathrm{A}}{\Phi \times \mathrm{C} \times \mathrm{L}} \qquad (2)$$

where: A is the absorbance of the emulsion; Φ is the oil phase volume fraction (0.25); C is the concentration of protein before the emulsion is formed (%); L is the light path length (m).

Emulsion stability

The emulsion stability was estimated according to Chobert *et al.* (1988)[76]. The emulsions prepared above were held at 20 °C for 24h and then were heated at 80 °C for 30 min and cooled at room temperature. After cooling, emulsifying activity expressed as emulsifying activity index - EAI (80 °C) was calculated according to equation Eq. 2. The emulsion stability was calculated according to equation Eq. 3:

$$\Delta\,\mathrm{EAI}\,(\%) = \frac{\mathrm{EAI}\,(20°\mathrm{C}) - \mathrm{EAI}\,(80°\mathrm{C})}{\mathrm{EAI}\,(20°\mathrm{C})} \qquad (3)$$

where: EAI (20 °C) is the emulsifying activity index before storing of emulsion (m^2/g); EAI (80 °C)) is the emulsifying activity index after storing of emulsion (m^2/g); The smaller the value of Δ EAI (%) indicates better emulsion stability.

Creaming index

The creaming index was measured according to the method of Firebaugh and Daubert (2005)[77] with slight modifications. Briefly, 5 mL of each emulsion was placed in a glass test tube (internal diameter 16 mm and height 100 mm), then stored at an ambient temperature for 5 days. After storage, three layers, namely a creamed layer at the top, a transparent serum layer at the bottom, and an emulsion layer in the middle were formed in all of the emulsions. The creaming index was calculated according to equation Eq. 4:

$$\text{Creaming index (\%)} = \frac{\text{Hs}}{\text{He}} \times 100 \quad (4)$$

where: He is the total height of the emulsion (mm); Hs is the height of the serum (mm).

3.1.2.3.2. Foaming properties

Foam activity

The foam activity and foam stability were estimated according to Sathe and Salunkhe (1981)[78] method with slight modification. Briefly, the foam was formed by whipping the samples in a homogenizer at 10000 rpm for 5 min at room temperature. The foam activity, expressed as foam expansion (%), was calculated according to equation Eq. 5:

$$\text{Foam expansion (\%)} = \frac{\text{Volume of foam - Volume of protein solution}}{\text{Volume of protein solution}} \times 100 \quad (5)$$

Foam stability

Foam stability was estimated by monitoring drainage after the expanded foam was held for 30 min at 25 °C. Foam stability was calculated according to equation Eq. 6:

$$\text{Foam stability} = \frac{\text{Volume of foam - Volume of drainage}}{\text{Volume of foam}} \quad (6)$$

3.1.2.4. Digestibility and Bioaccessibility of WPC hydrolysates

In vitro protein digestion was carried out according to the method described by Laparra *et al.* (2003)[79] with slight modifications. Briefly, 10 mL of sample was adjusted at pH 2.0 by 6 M HCl, then 10 mg of pepsin was added and the digestion was conducted at 37 °C for 2 h under continuous stirring, then the pH of the samples was adjusted to 8.0 with 1 M NaOH to inactivate the enzyme. In addition, 10 mg of trypsin was added and

samples were incubated at 37 °C for 2 h. Digestion was stopped in accordance with the following measurements.

Digestibility

For measuring digestibility, digestion was stopped by adding 5 mL of 50% trichloroacetic acid (TCA). Samples were allowed to stand for 30 min at 4 °C and then centrifuged at 10,000 × g for 25 min (SIGMA 2-16, rotor 12141, Germany). The resultant precipitate was dissolved in 5 mL of 0.2 M NaOH, and protein concentration was measured using the Bradford (1976)[80] method. Digestibility was calculated according to equation Eq. 7:

$$\text{Digestibility (\%)} = \frac{A - B}{A} \times 100 \quad (7)$$

where: A is the protein content in the sample (mg); B is the protein content (mg) in the TCA precipitate (mg).

Bioaccessibility

For measuring bioaccessibility, digestion was stopped by heating at 95 °C for 10 min. Samples were allowed to stand for 30 min at 4 °C and DPPH scavenging activity was measured as previously described. Bioaccessibility considered as the bioactivity (DPPH scavenging activity) released from the food matrix by *in vitro* gastrointestinal digestion, which is available for absorption, was calculated using Eq. 8[81]:

$$\text{Bioaccessibility (\%)} = \frac{A_{\text{digested}}}{A_{\text{non-digested}}} \times 100 \quad (8)$$

where: A is the DPPH scavenging activities of digested and non-digested samples.

3.1.2.5. Statistical analysis

All experiments were performed in triplicate. All values are expressed as a mean ± standard deviation. Mean values were analyzed using One-way ANOVA. The Tukey post hoc test was performed for means comparison using OriginPro 8 (Origin Lab Co., Northampton, USA). Differences between samples were considered as significant at $p<0.05$.

3.1.3 Results and Discussion

3.1.3.1. Selection of appropriate combination of *Lactobacillus* strain and substrate suitable for the production of bioactive whey protein hydrolysates with high antioxidative potential

Dairy substrates possess antioxidant activity resulting from the presence of such components as bioactive peptides derived from casein, whey proteins, lactoferrin, urate, ascorbate, α-tocopherol, ß-carotene, coenzyme Q10, and various enzymatic systems (superoxide dismutase, catalase, and glutathione peroxidase)[82,83]. All of these compounds possess metal-chelating capabilities and radical scavenging properties[84]. Antioxidant activity of these substrates could be enhanced by fermentation that tends to enhance targeted property by releasing different by-products[85] or by the presence of starter microorganisms that also possess some antioxidative potential[82,83]. During fermentation, dairy proteins could be hydrolyzed by the extracellular proteinase of the lactic acid bacteria, resulting in a formation of peptides that can contribute to the antioxidant activity of fermented substrates. The amount of peptides formed during fermentation depends on the used strain, specifically on it proteolytic activity, as well as the used substrate[86]. The optimal strain-substrate combination is a crucial point in the production of high potent antioxidative formulation that can be easily used in different food products.

The development of antioxidant activity during 24h fermentation was studied with DPPH an FRAP assays. Influences of different microorganisms on changes in antioxidant activities of different whey substrates during 24h of fermentation are shown in Fig. 12, Fig. 13 and Fig. 14.

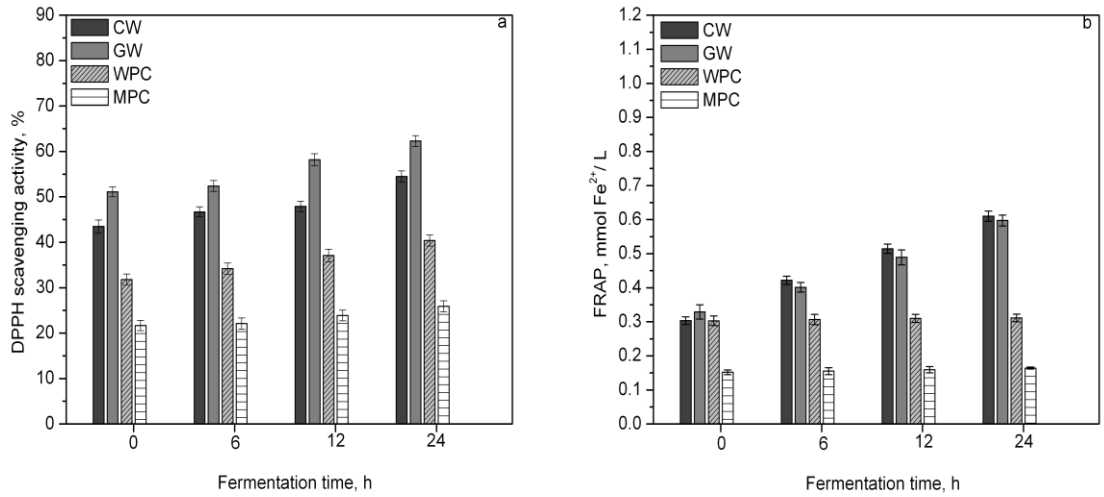

Figure 12. Changes of a) DPPH scavenging activity and b) FRAP antioxidant activity of different substrates fermented with *Lb. acidophilus* ATCC 4356 strain at 37 °C during 24h. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 12, strain *Lb. acidophilus* increases antioxidant activity of all tested substrates during 24h of fermentation. The maximal DPPH scavenging activity of 62.3% (Fig. 12a) was reached after 24h fermentation of goat's whey (GW). The same trend of antioxidant activity increase was observed in FRAP assay, where the maximal metal-chelating ability, i.e. FRAP value of 0.6100 mmol Fe^{2+}/L (Fig. 12b) was registered after 24h fermentation in sample CW (cow's whey). Obtained results, suggests that strain *Lb. acidophilus* is capable to produce a high amount of antioxidant compounds in whey substrates, probably due to its higher proteolytic activity on protein sources that are part of the balanced substrates such as whey. Obtained results are much better than those reported in the literature[86] where the *Lb. acidophilus* is assigned as the strain that produces high antioxidant activity of 42.0% in fermented milk, measured by ABTS assay. Based on the fact that in the present study the minimal DPPH and FRAP values (25.9%, 0.1640 mmol Fe^{2+}/L , respectively) are reached after 24h in milk protein sample (MPC), it could be said that enzymatic system of *Lb. acidophilus* much efficiently hydrolyzes whey protein sources leading to the production of significantly higher antioxidant activity.

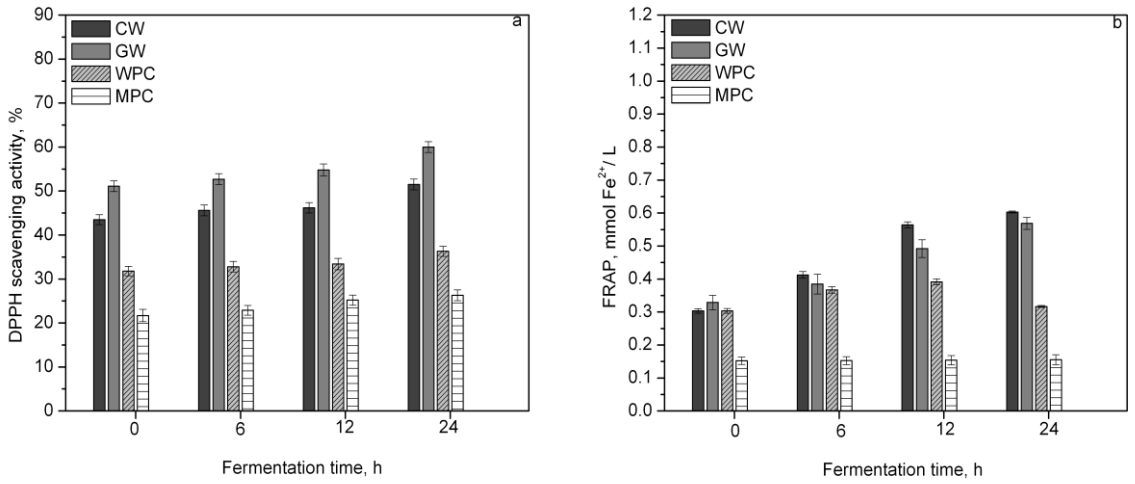

Figure 13. Changes of a) DPPH scavenging activity and b) FRAP antioxidant activity of different substrates fermented with *Lb. reuteri* ATCC 23272 strain at 37 °C during 24h. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 13, strain *Lb. reuteri* significantly ($p<0.05$) increases the antioxidant activity of all tested substrates during 24h of fermentation. The maximal DPPH scavenging activity of 60.0% (Figure 13.a) was reached after 24h fermentation of goat's whey (GW). In addition, the maximal value of metal-chelating ability, i.e. the FRAP value of 0.6031 mmol Fe^{2+}/L (Figure 13b) was reached after 24h fermentation of CW sample. Obtained results are lower than those registered for *Lb. acidophilus* suggesting that *Lb. reuteri* less efficiently hydrolyzes whey proteins than *Lb. acidophilus* strain. Obtained results are in agreement with those reported in the literature[86], where the *Lb. reuteri* produces lower antioxidant activity (30.0%) in milk substrate than *Lb. acidophilus* strain (42.0%), measured by ABTS assay. Based on the literature findings, it was expectable that *Lb. reuteri* reaches lower DPPH and FRAP values than *Lb. acidophilus*, which suggesting that enzymatic system of *Lb. acidophilus* possesses the advanced capability to hydrolyze whey proteins compared to the *Lb. reuteri* strain.

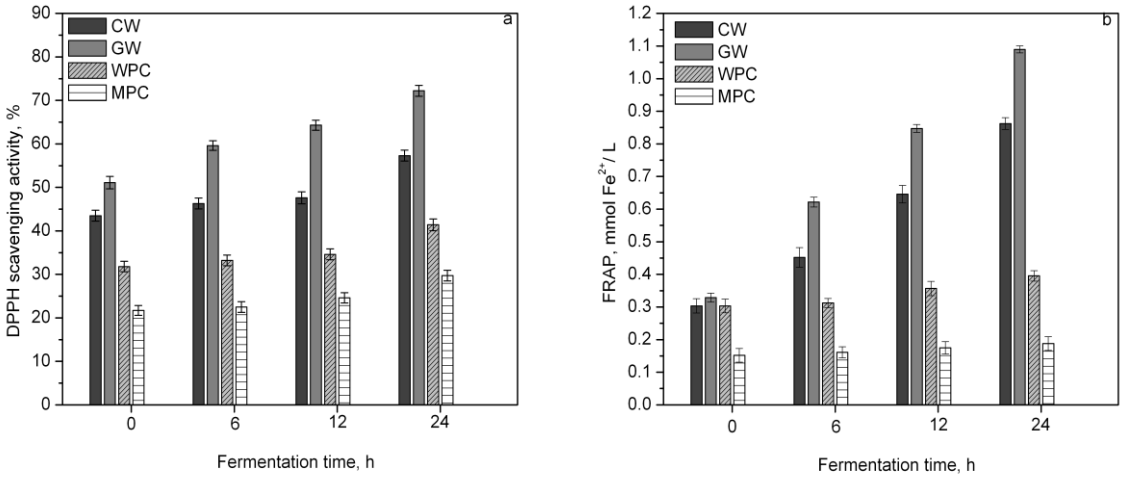

Figure 14. Changes of a) DPPH scavenging activity and b) FRAP antioxidant activity of different substrates fermented with *Lb. rhamnosus* ATCC 7469 strain at 37 °C during 24h. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 14, strain *Lb. rhamnosus* increases antioxidant activity of all tested substrates during 24h of fermentation. The maximal DPPH scavenging activity of 72.2% (Fig. 14a) was reached after 24h fermentation of goat's whey substrate (GW). The same trend of antioxidant activity increase was observed in FRAP assay, where the maximal metal-chelating ability, i.e. FRAP value of 1.0897 mmol Fe^{2+}/L (Fig. 14b) was reached after 24h fermentation of GW sample. Obtained results suggest that strain *Lb. rhamnosus* produces significantly higher antioxidant activity during the hydrolysis of goat's whey compared to the other substrates and microorganisms. It is capable to produce a high amount of antioxidant compounds probably due to high proteolytic activity[87]. Obtained results are in contrast to those reported in the literature[86], where the *Lb. rhamnosus* produces lower antioxidant activity of fermented milk than *Lb. acidophilus* and *Lb. reuteri* strains, measured by ABTS assay. In addition, it could be said that *Lb. rhamnosus* more efficiently hydrolyzes the goat's whey protein substrate than *Lb. acidophilus* and *Lb. reuteri* strains, and leads to the production of significantly higher antioxidant activity in goat's whey compared to the other tested substrate. Obtained DPPH and FRAP values are in agreement with those reported in the literature[88,89,90] related to the production of antioxidant activity in studied substrates. It is interesting to note that the lowest DPPH and FRAP values are obtained in the fermentation of milk protein concentrate (MPC), regardless of the applied microorganism. This observation is in contrast to the literature[86] that reports the high

antioxidant activity measured by ABTS assay obtained during the fermentation of whole milk by studied microorganisms. It is probably due to the fact that casein is slowly degradable protein[91] which digestion does not allow the production of antioxidant peptides by used microorganisms during 24h.

Based on the presented results it is evident that the fermentation of goat's whey by *Lb. rhamnosus* allows the production of the substrate with the high antioxidant activity that could be used as a supplement in a wide range of food products.

3.1.3.2. Functional properties of bioactive whey protein hydrolysates produced by microbial fermentation

Whey proteins by themselves are excellent foaming and emulsifying agents that are often used to improve emulsification in infant formula, beverages, soups, gravies, and coffee whiteners[92]. However, due to the constant development of food products and their production processes, there is a need for its improvement. Two main methods of improving whey proteins functionality are a modification of proteins through chemical and biological treatments. Due to the fact that chemical modifications are not often safe for human health[93], microbial modifications seem to be the most comfortable method for the food industry. The functionality of whey proteins largely depends on its interfacial properties that include emulsification and foaming characteristics[94].

Influence of specific strain-substrate combination on emulsifying and foaming properties as well as digestibility and bioaccessibility of hydrolysates produced after 24h of fermentation using *Lb. rhamnosus* strain is shown in Fig. 15, Fig. 16 and Fig. 17.

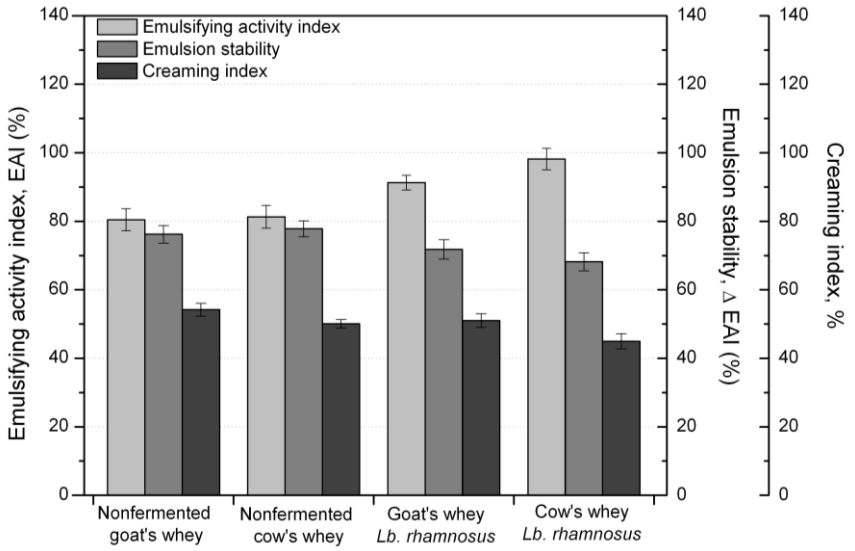

Figure 15. Emulsifying properties of goat's and cow's whey hydrolysates obtained after 24h of fermentation using *Lb. rhamnosus*. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 15, after 24h of fermentation, emulsifying activity for both of produced hydrolysates was increased, compared to the nonfermented substrates. Goat's whey hydrolysate expresses significantly lower EAI (91.3%) than cow's whey hydrolysate (98.2%) obtained using *Lb. rhamnosus*. Observed results could be related to the smaller amount of proteins in cow's whey that allows more efficient degradation of proteins by *Lb. rhamnosus* and higher production of smaller peptides (surfactants) which typically have better emulsifying properties[95]. In addition, after the fermentation stability of formed emulsions was increased regardless of the substrate compared to the nonfermented substrates. Emulsion stability of cow's whey hydrolysates (68.2%) was higher (the lower the value of ΔEAI, %, the greater the stability) than for goat's whey hydrolysates (71.8%), suggesting that efficient protein degradation produces peptides with lower molecular weight that contribute to the emulsion stability rate[96]. As an additional indicator of emulsion stability creaming index was evaluated for measurement whether the fermentation improves the stability of emulsions during 5 days of storage. High creaming index indicates a fast move of droplets and thus a high degree of droplet aggregation[97]. As shown in Fig. 15, creaming index of both substrates fermented by *Lb. rhamnosus* was significantly ($p<0.05$) lower (51.0% for goat's whey and 45.1% for cow's whey hydrolysate) than creaming index of nonhydrolyzed substrates (54.2% for goat's whey and 50.1% for cow's whey). In addition, creaming

index of cow's whey hydrolysate was significantly ($p<0.05$) lower than for goat's whey hydrolysate, suggesting that creaming index is highly dependent on the used substrate. Efficient protein degradation caused by the high proteolytic activity of *Lb. rhamnosus*, implies the higher amount of free amino acids released into the substrate, leads to the reducing of the solution surface hydrophobicity and oiling of (lower creaming index) and influences the stability of the hydrolysate emulsion[98].

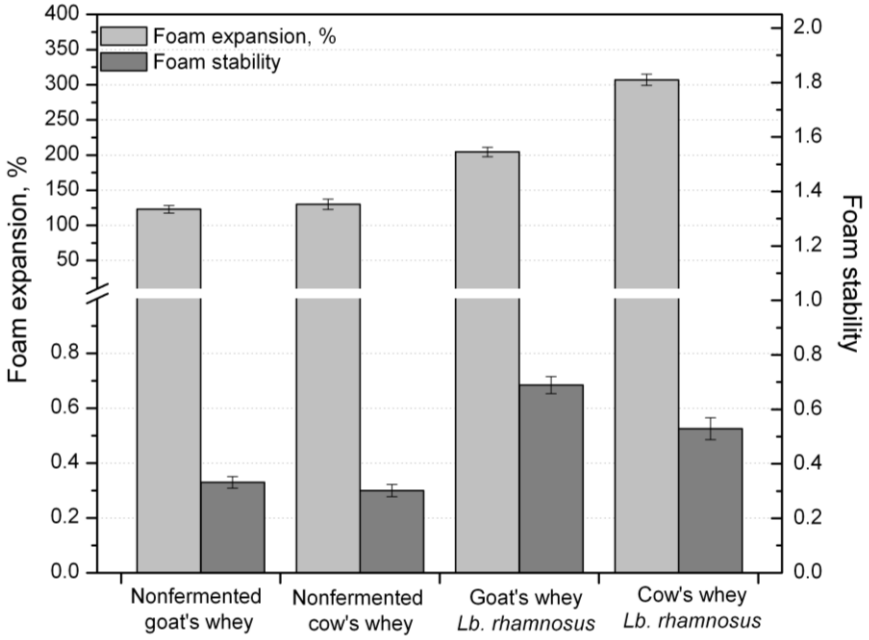

Figure 16. Foaming properties of goat's and cow's whey hydrolysates obtained after 24h of fermentation using *Lb. rhamnosus*. Vertical bars represent the standard deviation (n = 3) for each data point.

Based on the results presented in Fig. 16, foam activity of produced hydrolysates was significantly ($p<0.05$) increased regardless of the substrate, compared to the nonfermented substrates. The obtained results are in agreement with literature data[96] that reports the positive influence of protein degradation on the foam forming ability of hydrolyzed suspension. It is interesting to note that foam expansion of cow's whey hydrolysate (307.3%) was significantly ($p<0.05$) higher than goat's whey hydrolysate (214.6%). Observed results are in agreement with literature data[96], which suggests that smaller peptides produced through more efficient proteolysis, express higher positive influence on the foam forming ability due to more rapid absorption at the interface. In addition, cow's whey hydrolysate expressed significantly ($p<0.05$) lower foam stability

(0.525) compared to the goat's whey hydrolysate (0.684), probably as a consequence of the production of very short peptides that probably contribute to the poor foaming stability.

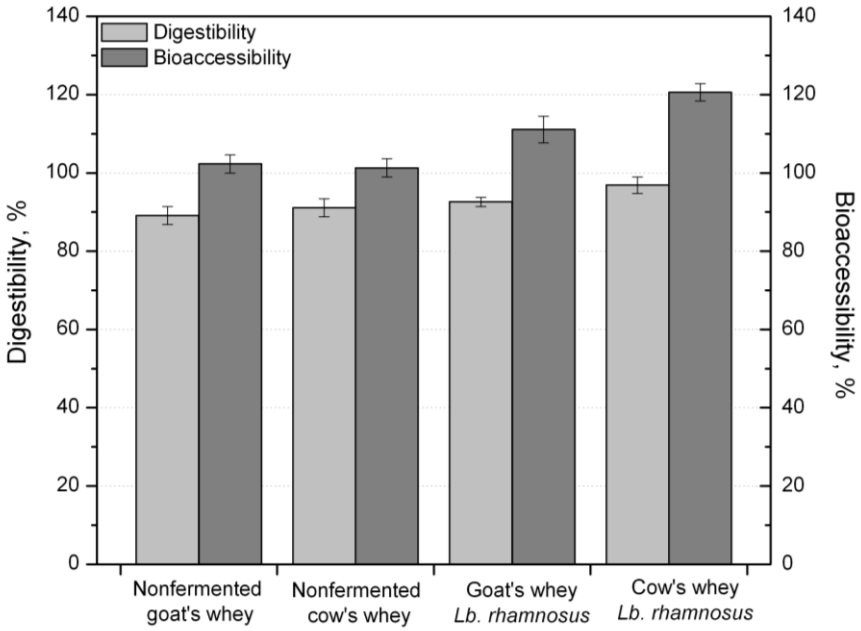

Figure 17. Digestibility and bioaccessibility of goat's and cow's whey hydrolysates obtained after 24h of fermentation using *Lb. rhamnosus*. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 17, the digestibility of both hydrolysates were significantly ($p<0.05$) higher than corresponding nonfermented substrates. Digestibility of cow's whey hydrolysate (96.9%) was significantly ($p<0.05$) higher than goat's whey hydrolysate (92.9%). Respecting the results for DPPH activity registered for cow's whey hydrolysate, it seems that the efficient proteolysis reduces the molecular weight of proteins enhancing its solubility and leads to the production of smaller bioactive peptides that are much more accessible to the gastrointestinal enzymes. In addition, bioaccessibility of both generated hydrolysates was significantly ($p<0.05$) higher than corresponding nonfermented substrates. Based on the results, cow's whey hydrolysate expresses significantly ($p<0.05$) higher bioaccessibility (120.6%) compared to goat's whey hydrolysate (111.1%). Obtained results suggest that generated bioactive peptides are either resistant to the gastric digestion or might be further degraded by digestive enzymes to the smaller peptides with higher antioxidant activity.

3.1.4. Conclusion

Based on the results, increased antioxidant activity was reached in all tested strain-substrate combinations. High levels of antioxidant activities, in the range 29.7-72.2%, were reached in all substrates fermented with *Lb. rhamnosus* strain. Among all combinations, goat's whey fermented with *Lb. rhamnosus* exhibited the highest increase of DPPH scavenging activity (for 21.1%) as well as FRAP antioxidant activity (for 0.761 mmol Fe^{2+}/L) compared to the unfermented substrate. Thus, the study introduces the *Lb. rhamnosus* as highly effective in the production of antioxidants during the fermentation of goat whey. Furthermore, goat whey fermented with *Lb. rhamnosus*, used in liquid or lyophilized form, could be an excellent carrier for delivery of antioxidants into the different dairy and confectionery products. On the other hand, cow's whey fermented with *Lb. rhamnosus* exhibited DPPH scavenging activity of 57.3% as well as FRAP antioxidant activity of 0.862 mmol Fe^{2+}/L. Analyzing simultaneously the results related to the functional properties of hydrolysates it can be noticed that cow's whey hydrolysate satisfies this important application criterion. Namely, compared to the goat's whey, cow's whey hydrolysate exhibited significantly better emulsion activity (98.2%), emulsion stability (68.2%), foam activity (307.1%), digestibility (96.9%) and bioaccessibility (120.6%) as crucial parameters for its application. In addition, compared to the goat's whey, cow's whey is much more accessible and voluminous waste product of the dairy industry and its utilization is much more significant. Therefore, by examining all of these facts, it could be suggested that the process of microbial fermentation of cow's whey using the strain *Lb. rhamnosus* ATCC 74696 represents the appropriate process for the production of bioactive components. The suggested strain-substrate combination allows the production of bioactive peptides with high antioxidant activity as well as the utilization of cow's whey as a waste product that occurs in a very large amount.

3.2. Modification of whey proteins by enzymatic hydrolysis

3.2.1. Materials

3.2.1.1. Media, Enzymes and Reagents

The whey protein concentrate (WPC) with 80.0% of protein, used in this study was purchased from DMV International (5462 GE Veghel, Netherlands). A 10 g of WPC was dissolved in 200 mL of deionized water to produce 5.0% (w/v) of WPC solution that contains 4.0% (w/v) of whey protein equivalent. The obtained suspension was allowed to hydrate for 1h at room temperature with gentle stirring. Pepsin (porcine gastric mucosa, EC 3.4.23.1) and trypsin (porcine pancreas, EC 3.4.21.4), were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), respectively. Bovine serum albumin (BSA) and all other chemicals used in this research were purchased from Sigma-Aldrich Chemie GmbH and were of analytical grade.

3.2.2. Methods

3.2.2.1 Enzymatic hydrolysis

Enzymatic hydrolyses were carried out in a 300-mL mechanically stirred batch reactor with temperature and pH control by using two enzymes separately, namely pepsin and trypsin. The substrate for enzymatic hydrolysis was 200 mL of 5.0% (w/v) aqueous solution of WPC with 44.0 ± 1.363 mg/mL protein content that was adjusted to optimum pH for the particular enzyme. Before hydrolyses, the WPC solutions were adjusted to pH 2.0 by the addition of 1 M HCl, and to pH 8.0 by the addition of 1 M NaOH, for peptic and tryptic hydrolysis, respectively. Solutions were stirred and allowed to equilibrate to the working temperatures for 15 min. Prepared WPC solutions were incubated at 37 and 50 °C for 30, 60, 90, 120 and 240 min with pepsin or trypsin at enzyme/substrate ratios 0.5%, 1.0% and 2.0% on a protein-equivalent basis. During the course of the hydrolyses, the pH of the WPC solutions was kept at pH 2.0 for peptic and pH 8.0 for tryptic hydrolysis, by continuous addition of 0.1 M HCl and 0.1 M NaOH, respectively, using the pH-stat method with an automatic dosage of the base. After hydrolyses, the enzymes were inactivated by heating at 90°C for 15 min, centrifuged at 3,500×g for 30 min, and samples were stored at -20 °C for further analyses.

3.2.2.2. Degree of hydrolysis and average peptide chain length

The degree of hydrolysis (DH) for different incubation times was determined according to Adler-Nissen (1986)[99]. The degree of hydrolysis (DH) is defined as the percent of peptide bonds cleaved during the enzymatic reaction. There is proportionality between the number of peptide bonds cleaved and the acid or base consumption, which can be calculated according to equation Eq. 9:

$$DH(\%) = \frac{h}{h_{tot}} \times 100 = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{M_P} \times \frac{1}{h_{tot}} \times 100 \quad (9)$$

where: h is the number of hydrolyzed peptide bonds; h_{tot} is the sum of the millimoles of individual amino acids per gram of protein associated with the source of protein used in the experiment (for whey protein concentrate is 8.8 meq/g, or simply mmol/g protein); B (mL) is the volume of base or acid consumed for maintaining the pH at 8 or 2; N_B is the normality of the base or acid; α is average degree of dissociation of the α-amino groups related with the pK of the amino groups at particular pH and temperature ($1/\alpha$ = 1.13 at 50 °C and pH 8.0, $1/\alpha$ = 1.26 at 37 °C and pH 8.0, $1/\alpha$ = 1.0 at 50 °C and pH 2.0, $1/\alpha$ = 1.0 at 37 °C and pH 2.0) and M_p (g) is the amount of the protein in the reaction mixture.

Based on the degree of hydrolysis DH (Eq. 1), the average chain length of the peptides was calculated according to equation Eq. 10[99]:

$$APCL = \frac{100}{DH(\%)} \quad (10)$$

3.2.2.3. Total protein and free amino acid quantification

The protein concentration was determined according to Bradford's method[80], using bovine serum albumin (BSA) as a standard. Free amino acid concentration was quantified spectrophotometrically using Ninhydrin method[100] at 570 nm.

3.2.2.4. Antioxidant activity of WPC hydrolysates

In order to estimate the antioxidant activity of the complex biological system that contains a wide range of antioxidants with different chemical structure, DPPH scavenging activity of the samples was estimated according to the combination of the

protocols described by El-Shourbagy and El-Zahar (2014)[101] and McCue and Shetty (2005)[102] with minor modifications. Briefly, 250 μL of each sample was added to 4 mL of 0.1 mM DPPH radical dissolved in acetone (polar aprotic), methanol (polar protic) or hexane (nonpolar) as solvents[101]. Mixtures were vortexed for 1 min, placed on an orbital shaker, and incubated in the dark for 60 min at room temperature. After incubation, samples were centrifuged for 15 min at 6000 rpm at room temperature. Obtained supernatants were filtered using Whatman N^{o} 40 filter paper[156]. At the same time, the control sample that contains the corresponding DPPH radical solution and deionized water instead of the sample was prepared. The absorbance of each supernatant and control was measured at 517 nm using UV-Vis spectrophotometer (Ultrospec 3300 pro, Amerscham Bioscience) against the corresponding solvent as a blank. DPPH radical scavenging activity (%), expressed as the percentage of inhibition, was calculated according to equation Eq. 11:

$$\text{DPPH scavanging activity}\,(\%) = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100 \qquad (11)$$

where: Ac is the absorbance of the control without hydrolysate, and As is the absorbance of the sample with hydrolysate.

Peptic and tryptic hydrolysates with highest antioxidant activities were further examined in terms of its functional properties.

3.2.2.5. Functional properties of WPC hydrolysates

3.2.2.5.1 Emulsifying properties

Emulsifying activity

The emulsifying activity of hydrolysates was estimated according to the method of Pearce and Kinsella (1978)[75] with slight modifications. Briefly, emulsions were prepared by mixing 3 mL of sample and 1 mL of sunflower oil (Vital, Serbia). Mixtures were homogenized for 30 s at room temperature. A 20 μL aliquot of emulsions was immediately pipetted from the emulsions and diluted 250-fold into the 0.1% (w/v) SDS in 0.1 M NaCl, pH 7.0. Diluted emulsions were vortexed for 10 s, transferred into the glass cuvettes (1-cm path length), and the absorbance at 500 nm was recorded against the 0.1% (w/v) SDS in 0.1 M NaCl (pH 7.0) as a blank. Emulsifying activity, expressed

as an emulsifying activity index - EAI (m^2/g), was calculated according to equation Eq. 12:

$$\mathrm{EAI}\,(m^2/g) = \frac{2.303 \times 2 \times A}{\Phi \times C \times L} \quad (12)$$

where: A is the absorbance of the emulsion; Φ is the oil phase volume fraction (0.25); C is the concentration of protein before the emulsion is formed (%); L is the light path length (m).

Emulsion stability

The emulsion stability was estimated according to Chobert *et al.* (1988)[76]. The emulsions prepared above were held at 20 °C for 24h and then were heated at 80 °C for 30 min and cooled at room temperature. After cooling, emulsifying activity expressed as emulsifying activity index - EAI (80 °C) was calculated according to equation Eq. 12. The emulsion stability was calculated according to equation Eq. 13:

$$\Delta\,\mathrm{EAI}\,(\%) = \frac{\mathrm{EAI}\,(20°C) - \mathrm{EAI}\,(80°C)}{\mathrm{EAI}\,(20°C)} \quad (13)$$

where: EAI (20 °C) is the emulsifying activity index before storing of emulsion (m^2/g); EAI (80 °C)) is the emulsifying activity index after storing of emulsion (m^2/g); The smaller the value of Δ EAI (%) indicates better emulsion stability.

Creaming index

The creaming index was measured according to the method of Firebaugh and Daubert (2005)[77] with slight modifications. Briefly, 5 mL of each emulsion was placed in a glass test tube (internal diameter 16 mm and height 100 mm), then stored at an ambient temperature for 5 days. After storage, three layers, namely a creamed layer at the top, a transparent serum layer at the bottom, and an emulsion layer in the middle were formed in all of the emulsions. The creaming index was calculated according to equation Eq. 14:

$$\text{Creaming index (\%)} = \frac{Hs}{He} \times 100 \quad (14)$$

where: He is the total height of the emulsion (mm); Hs is the height of the serum (mm).

3.2.2.5.2. Foaming properties

Foam activity

The foam activity and foam stability were estimated according to Sathe and Salunkhe (1981)[78] method with slight modification. Briefly, the foam was formed by whipping the samples in a homogenizer at 10000 rpm for 5 min at room temperature. The foam activity, expressed as foam expansion (%), was calculated according to equation Eq. 15:

$$\text{Foam expansion (\%)} = \frac{\text{Volume of foam - Volume of protein solution}}{\text{Volume of protein solution}} \times 100 \qquad (15)$$

Foam stability

The foam stability was estimated by monitoring drainage after the expanded foam was held for 30 min at 25 °C. Foam stability was calculated according to equation Eq. 16:

$$\text{Foam stability} = \frac{\text{Volume of foam - Volume of drainage}}{\text{Volume of foam}} \qquad (16)$$

3.2.2.6. Digestibility and Bioaccessibility of WPC hydrolysates

In vitro protein digestion was carried out according to the method described by Laparra *et al.* (2003)[79] with slight modifications. Briefly, 10 mL of sample was adjusted at pH 2.0 by 6 M HCl, then 10 mg of pepsin was added and the digestion was conducted at 37 °C for 2 h under continuous stirring, then the pH of the samples was adjusted to 8.0 with 1 M NaOH to inactivate the enzyme. In addition, 10 mg of trypsin was added and samples were incubated at 37 °C for 2 h. Digestion was stopped in accordance with the following measurements.

Digestibility

For measuring digestibility, digestion was stopped by adding 5 mL of 50% trichloroacetic acid (TCA). Samples were allowed to stand for 30 min at 4 °C and then centrifuged at 10,000 × g for 25 min (SIGMA 2-16, rotor 12141, Germany). The resultant precipitate was dissolved in 5 mL of 0.2 M NaOH, and protein concentration was measured using the Bradford method. Digestibility was calculated according to equation Eq. 17:

$$\text{Digestibility (\%)} = \frac{A - B}{A} \times 100 \quad (17)$$

where: A is the protein content in the sample (mg); B is the protein content (mg) in the TCA precipitate (mg).

Bioaccessibility

For measuring bioaccessibility, digestion was stopped by heating at 95 °C for 10 min. Samples were allowed to stand for 30 min at 4 °C and DPPH scavenging activity was measured as previously described. Bioaccessibility considered as the bioactivity (DPPH scavenging activity) released from the food matrix by *in vitro* gastrointestinal digestion, which is available for absorption, was calculated using Eq. 18 (Rodríguez-Roque *et al.* 2013[81]):

$$\text{Bioaccessibility (\%)} = \frac{A_{\text{digested}}}{A_{\text{non-digested}}} \times 100 \quad (18)$$

where: A is the DPPH scavenging activities of digested and non-digested samples.

3.2.2.7. Statistical analysis

All experiments were performed in triplicate. All values are expressed as a mean ± standard deviation. Mean values were analyzed using One-way ANOVA. The Tukey post hoc test was performed for means comparison using OriginPro 8 (Origin Lab Co., Northampton, USA). Differences between samples were considered as significant at $p<0.05$.

3.2.3 Results and Discussion

3.2.3.1. Selection of enzymes for the production of bioactive whey protein hydrolysates with high antioxidative potential

3.2.3.1.1. Degree of hydrolysis and average peptide chain length

Results related to the effect of different temperatures (37 and 50 °C) and enzyme to substrate (E/S) ratios on the degree of hydrolysis, average peptide chain length, protein and free amino acid concentration of whey protein hydrolysates, obtained during 4h of peptic and tryptic hydrolysis are shown in Fig. 18 and Fig. 19.

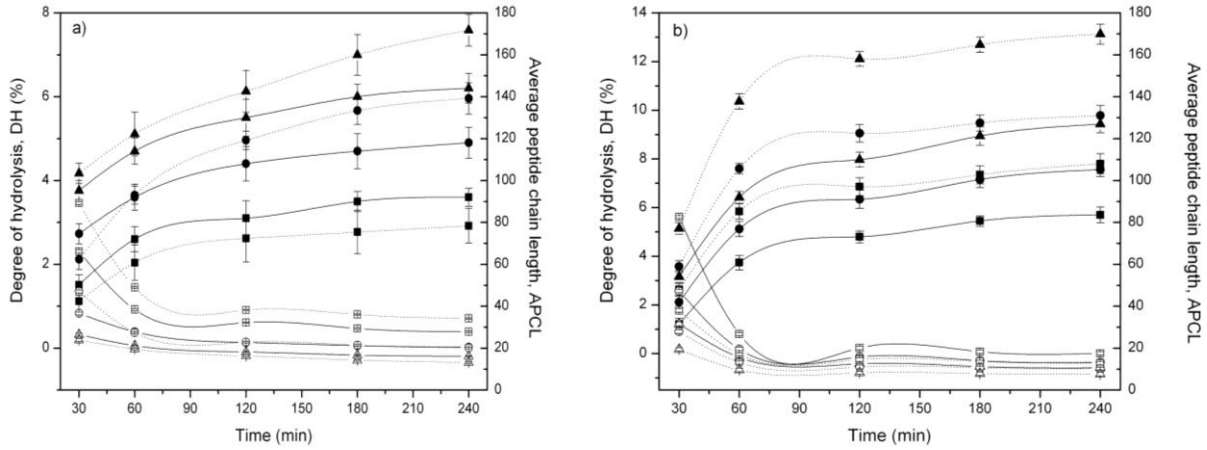

Figure 18. Influence of temperature and enzyme/substrate ratio on the degree of hydrolysis (DH) and average peptide chain length (APCL) of whey protein hydrolysates during 4h of a) peptic and b) tryptic hydrolysis. Legend: closed symbols - degree of hydrolysis; open symbols - average peptide chain length; solid line - 37 °C; dot line - 50 °C; enzyme/substrate ratio (■), (□) - 0.5%; (●), (○) - 1.0%; (▲), (Δ) - 2.0%; Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 18, a rapid degradation of WPC was occurred during 60 min of incubation with both used enzymes. Thereafter, the course of the reaction slowly progresses and leads to the relatively uniform progress of DH until the end of hydrolysis. In addition, the degree of hydrolysis of WPC increases with increasing levels of pepsin and trypsin and with increasing incubation time (Fig. 18a and Fig. 18b). Based on the results, there is statistically significant ($p<0.05$) influence of the E/S ratio on the DH obtained during both tryptic and peptic hydrolysis. Maximal DHs of 13.13 ± 0.412 for tryptic and 7.59 ± 0.378 for peptic hydrolysis were reached at E/S ratio 2.0%. The obtained results are slightly higher compared to DHs reported by Kim *et al.*

(2007)[103] obtained for 2.0% WPC suspension (approximately 12.0% for trypsin and 5.0% for pepsin), due to the higher concentration of the initial WPC suspension (5.0% WPC suspension) used in the present study. Furthermore, the previous study conducted by Mohan *et al*. (2015)[104] has reported lower DH (3%) reached during 5h of peptic hydrolysis of whey protein isolate (WPI) compared to the hydrolysis performed by alcalase and papain. Mohan *et al*. (2015)[104] suggest and confirm that low DH is attributed to the resistance of β-LG to the pepsin digestion. In addition, the difference between DH (4.9%) reported in the present study and the DH of 3.0% reported by Mohan *et al*. (2015)[104], both reached at E/S of 1.0%, is probably due to the differences in substrate composition and measurement method. In addition, statistically significant influence ($p<0.05$) of temperature on DH was observed in samples hydrolysed with trypsin during the whole course of hydrolysis, while in the pepsin hydrolysed sample temperature significantly ($p<0.05$) affects DH only after a 180 min. Maximal DH was reached at 50 °C for both enzymes, which is in agreement with literature findings according to which higher temperatures induce a temperature dependent thermo-denaturation and conformational changes, resulting in exposure of the hydrophobic areas of proteins, alter the number of accessible peptide bonds and alter the rate of proteolysis[105]. As shown in Fig. 18a and Fig. 18b, DH associated with tryptic hydrolysis was significantly higher than DH reached during peptic hydrolysis, especially at a higher temperature. Maximal DH obtained at 50 °C and E/S ratio 2.0%, after 240 min of hydrolysis, was 13.13% for tryptic and 7.59% for peptic hydrolysis. Obtained results suggest that WPC probably has more trypsin specific site than other enzymes' site[106], especially at higher temperatures. On higher temperatures conformational changes and exposure of hydrophobic groups to the solvent, promote the access of the enzyme to at least a few strategic peptide bonds and allow cleavage at many points that induce further structural changes[107], thus, alter the rate of tryptic proteolysis compared with peptic hydrolysis.

Average peptide chain length (APCL) is related to the average molecular weight of peptides in the hydrolysate. As shown in Fig. 18a and Fig. 18b, hydrolysates obtained during peptic hydrolysis are composed of large peptides (more than 7 amino acid residues), while hydrolysate obtained during tryptic hydrolysis represents a mixture of

medium (4-7 amino acid residues) and large peptides. The obtained results are in agreement with literature data according to which trypsin cleavages proteins with high specificity and generates 7–20 amino acids long peptides with a strong C-terminal charge[108], while pepsin, as nonspecific proteases, generates complex peptide pools[109]. In addition to the DH, presented peptide distribution is additional evidence that trypsin causes strong structural changes of proteins, which result in its higher subsequent susceptibility to enzymatic hydrolysis[110].

3.2.3.1.2. Protein and free amino acid concentration

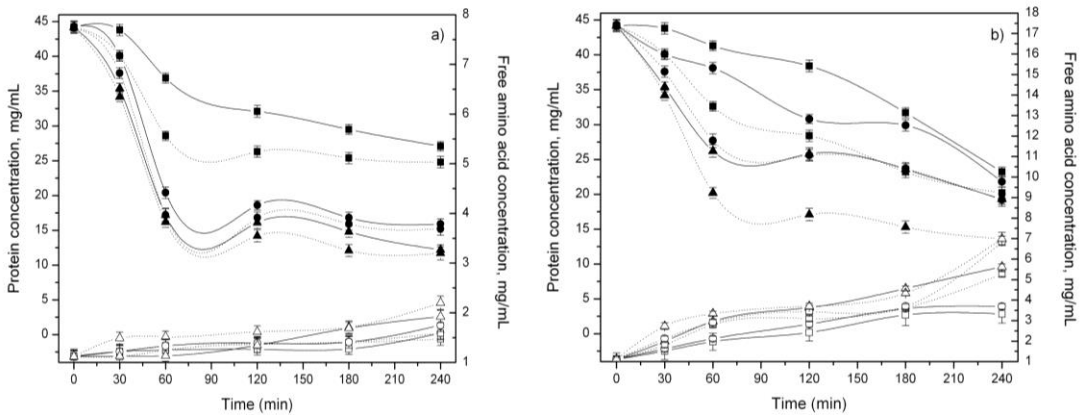

Figure 19. Influence of temperature and enzyme/substrate ratio on protein and free amino acid concentration of whey protein hydrolysates during 4h of a) peptic and b) tryptic hydrolysis. Legend: closed symbols -protein concentration; open symbols - amino acid concentration; solid line - 37 °C; dot line - 50 °C; enzyme/substrate ratio (■), (□) - 0.5%; (●), (o) - 1.0%; (▲), (Δ) - 2.0%; Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 19, changes in protein and free amino acid concentrations of hydrolysates followed the hydrolytic pattern. The concentration of proteins in WPC hydrolysates rapidly decreases, while the concentration of free amino acids steadily increases until the end of hydrolysis (Fig. 19a and Fig. 19b). The highest free amino acid concentration of 6.99 mg/mL registered after 240 min of incubation with trypsin at 50 °C is in accordance with the fact that tryptic hydrolysis reaches significantly ($p<0.05$) higher DH (13.13%) than peptic hydrolysis (7.59%). In addition, obtained results suggest the existence of a positive correlation between the DH and free amino acid content as well as a negative correlation between the DH and protein concentration also reported by Morais *et al.* (2013)[111]. Based on the literature findings, according to

which greater amount of free amino acid implies the presence of a smaller amount of large peptides[112], it could be assumed that trypsin hydrolysis leads to the achievement of suitable peptide profile rich in small peptides.

3.2.3.2. DPPH scavenging activity of WPC hydrolysates

A major step of the study was to contribute to the previous studies dealing with the antioxidant activity of WPC hydrolysates, commonly expressed as total DPPH scavenging activity, by determining the nature of antioxidant species that most notably affect the antioxidant properties of WPC hydrolysates. For this reason, the antioxidant activity of WPC hydrolysates was measured by DPPH method using methanol, acetone and hexane as polar protic, polar aprotic and nonpolar solvents, respectively, and the results are shown in Fig. 20.

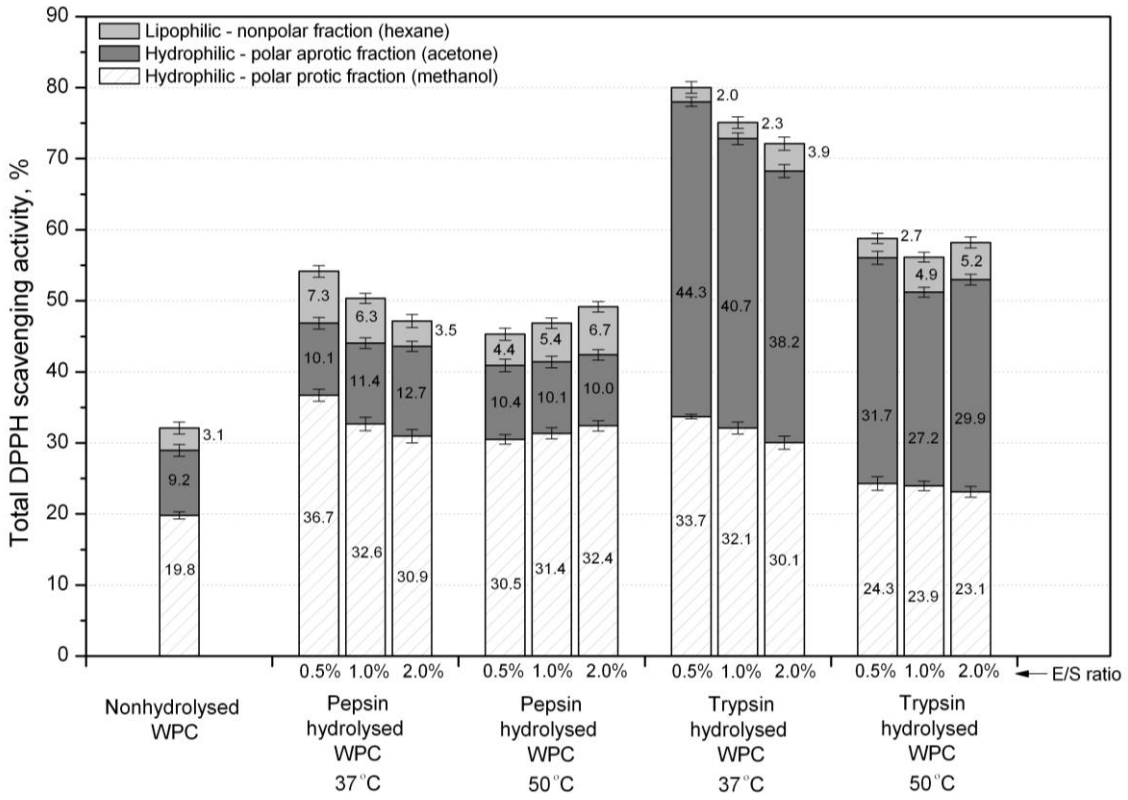

Figure 20. Influence of temperature and enzyme to substrate ratio on total antioxidant activity after 4h of peptic and tryptic hydrolyses conducted at 37 and 50 °C, and E/S ratios 0.5, 1.0 and 2.0%. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 20, nonhydrolyzed WPC showed rather low total DPPH radical scavenging activity of 32.1%, corresponding to 3.2% for a lipophilic fraction, 19.8%,

and 9.2% for hydrophilic protic and aprotic fractions, respectively. The obtained results related to the antioxidant activity of whey proteins are in agreement with previous studies[113,114], especially with the findings of Colbert *et al.* (1991)[115] which reported that whey is mainly composed of polar antioxidant compounds. Based on the results hydrolysis significantly ($p<0.05$) contributes to the total DPPH scavenging activity of WPC hydrolysates, regardless of the used enzyme, temperature and E/S ratio. In addition, total DPPH scavenging activity of pepsin and trypsin hydrolysates was negatively influenced by temperature, as well as E/S ratio at the lower temperature (37 °C) for both used enzymes.

After 4h of peptic digestion, total DPPH scavenging activity increased to the maximal 54.1% obtained at temperature of 37 °C and E/S ratio 0.5%. Mohan *et al*. (2015)[104] shows that even at low DH (3%) pepsin hydrolysate possesses the satisfactory antioxidant activity expressed as reducing capacity. The contribution of hydrophilic (polar) antioxidant fraction of 46.8% was significantly ($p<0.05$) higher than the lipophilic fraction that participates with 7.3% in total DPPH scavenging activity. Similarly, Rodríguez-Roque *et al.* (2013)[81] reported that pepsin digestion highly influences the total antioxidant activity of soya milk, with antioxidant distribution in favor to the hydrophilic (polar) fraction of antioxidant species. In addition, the hydrophilic protic fraction with DPPH scavenging activity of 36.7% significantly ($p<0.05$) contributes to the total antioxidant activity compared to the hydrophilic aprotic fraction (10.1%).

On the other hand, after 4h of tryptic digestion, total DPPH scavenging activity increased to the maximal 80.0% obtained at temperature of 37 °C and E/S ratio 0.5%. The contribution of hydrophilic (polar) antioxidant fraction of 78.0% was higher than the lipophilic fraction that participates with 2.0% in total DPPH scavenging activity. The hydrophilic aprotic fraction with DPPH scavenging activity of 44.3% significantly ($p<0.05$) contributes to the total antioxidant activity compared to the hydrophilic protic fraction (33.7%).

The obtained results are in agreement with findings observed by Baublis *et al.* (2000)[116] who reported that the gastric digestion (pepsin and trypsin) of wheat-based cereals

increases concentration and activity of low-molecular-weight hydrophilic antioxidants and leads to the higher antioxidant potential of the tested food matrix.

It is interesting to note the significant difference in antioxidant activities between polar fractions of peptic and tryptic hydrolysates. The results could be related to the fact that pepsin preferentially cleaves peptide bonds at Phe, Tyr, Trp and Leu[117], producing peptides with polar uncharged (Tyr) amino acid residues soluble in polar protic solvents such as methanol and nonpolar (Phe, Trp, Leu) amino acid residues[118] soluble in nonpolar solvents such as hexane. On the other hand, trypsin preferentially cleaves peptide bonds at Arg and Lys[119], producing peptides with polar positively charged amino acid residues[118] soluble in polar aprotic solvents such as acetone. Therefore, results suggest that whey protein hydrolysate obtained after peptic hydrolysis composed mainly of peptides with uncharged amino acid residues such as Tyr, while hydrolysate obtained after tryptic hydrolysis composed mainly of peptides with positively charged amino acid residues such as Arg and Lys.

Based on the results presented in Fig. 20, tryptic hydrolysis produces peptides with higher antioxidant activity compared with peptic hydrolysis, thus significantly improves the total antioxidant activity of WPC hydrolysates. Results are in agreement with the earlier study[120] that reported the production of antioxidant peptides is much more pronounced in tryptic than in peptic hydrolysis. In addition, the study conducted by Hernandez-Ledesma *et al.* (2005)[121] reported the higher antioxidant activity of β-LG hydrolysate obtained after trypsin digestion compared to the pepsin digestion, which is probably attributed to the resistance of β-LG to the pepsin digestion confirmed by Mohan *et al.* (2015)[104]. Whey protein hydrolysate with the maximal total antioxidant activity of 80.0% produced after 4h of tryptic hydrolysis at 37 °C and E/S ratio 0.5% mainly composed of hydrophilic (polar aprotic) antioxidant species. In addition, presented results suggest that tryptic hydrolysis significantly improves the release of antioxidant peptides coded in parent whey proteins as a complex food matrix. Due to the fact that the antioxidant activity of tryptic hydrolysate was mainly composed of hydrophilic antioxidant peptides, it could be assumed that obtained hydrolysate is most suitable for preventing oxidation processes in polar charged food ingredients. Both peptic and tryptic hydrolysates with highest total antioxidant activities were obtained at

37 °C and E/S ratio 0.5% and were further examined in terms of its functional properties.

3.2.3.3. Functional properties of bioactive whey protein hydrolysate produced by enzymatic hydrolysis

3.2.3.3.1. Emulsifying properties

Influence of peptic and tryptic hydrolysis on emulsifying and foaming properties as well as digestibility and bioaccessibility of produced hydrolysates are shown in Fig. 21 and Fig. 22.

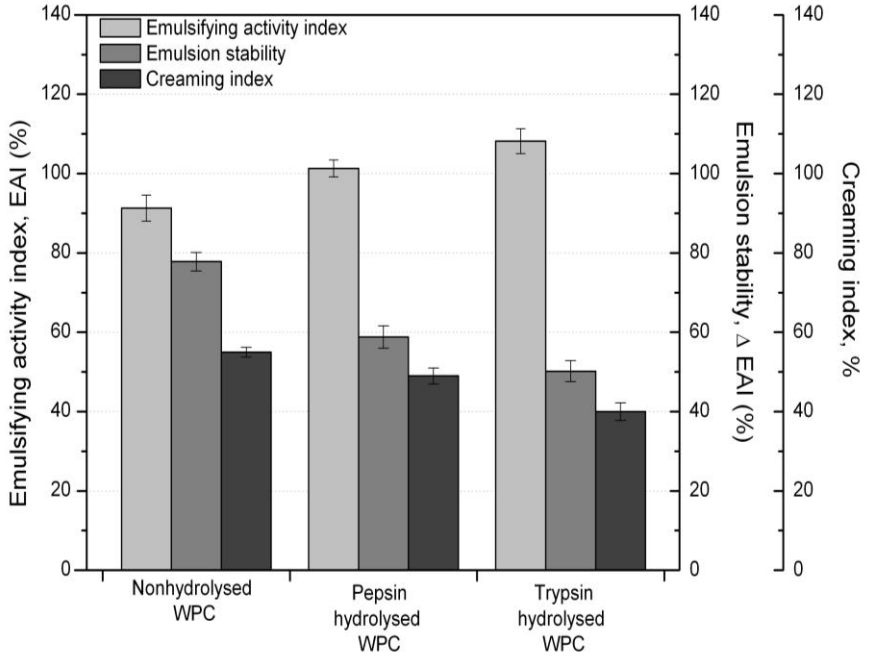

Figure 21. Emulsifying properties of peptic and tryptic hydrolysates obtained after 4h of hydrolysis at 37 °C and E/S ratio 0.5%. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 21, after 4h of hydrolysis emulsifying activity was increased regardless of the used enzyme. Pepsin hydrolysate expresses significantly lower EAI than trypsin hydrolysate. Observed results could be related to the higher DH of trypsin hydrolysate that allows the production of smaller peptides (surfactants) which typically have better emulsifying properties[122]. In addition, compared to the nonhydrolyzed WPC, the stability of formed emulsions was increased regardless of the used enzyme. The emulsion stability of trypsin hydrolysate was higher than for pepsin hydrolysate,

suggesting that higher DH produces peptides with a lower molecular weight that contributes to the emulsion stability[123].

As an additional indicator of the emulsion stability, creaming index was evaluated for measurement whether the hydrolysis improves the stability of emulsion during 5 days of storage. The high creaming index indicates a fast move of droplets and thus a high degree of droplet aggregation[124]. As shown in Fig. 21, creaming index of both hydrolysates was significantly ($p<0.05$) lower (49.0 ± 2.02 for pepsin and 40.1 ± 2.17 for trypsin hydrolysate) than creaming index of nonhydrolyzed WPC (55.2 ± 1.24). After 4h of hydrolysis, creaming index of trypsin hydrolysate was significantly ($p<0.05$) lower than for pepsin hydrolysate. It could be assumed that higher DH obtained after 4h of tryptic hydrolysis implies the higher amount of free amino acids, leads to the reducing of the solution surface hydrophobicity and oiling of (lower creaming index), and influences the stability of the hydrolysate emulsion[125]. The observed results are in agreement with literature reports which suggest that creaming index is highly dependent on DH of hydrolysates[125]. It is interesting to note that the reported results are in agreement with the results related to the higher stability of trypsin hydrolysate emulsion. It could be explained by the better ability of trypsin hydrolysate to form relatively thick interfacial films on the surface of oil droplets and prevent droplet aggregation and oiling of, which led to the enhanced stability of formed emulsion[125].

3.2.3.3.2. Foaming properties

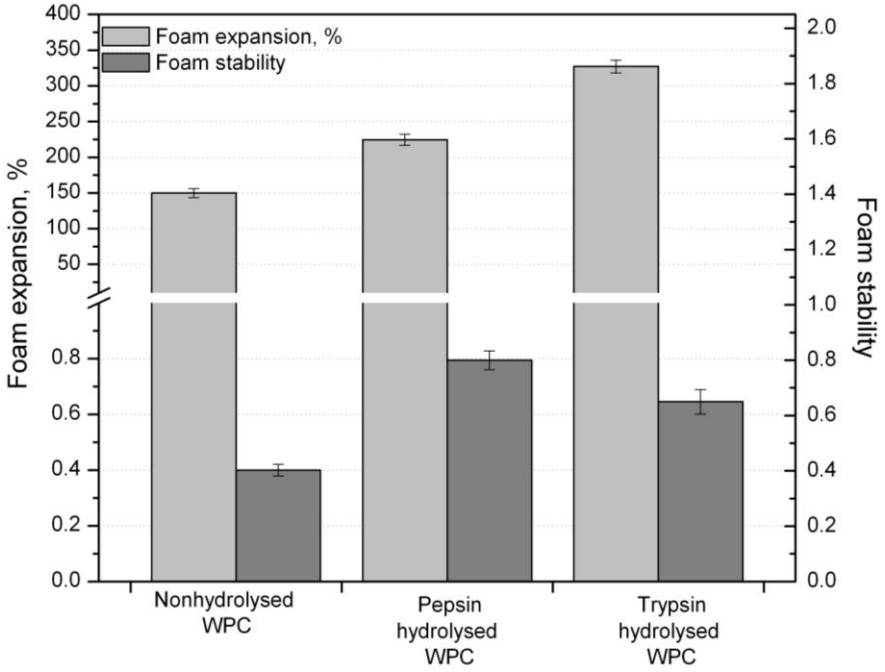

Figure 22. Foaming properties of peptic and tryptic hydrolysates obtained after 4h of hydrolysis at 37 °C and E/S ratio 0.5%. Vertical bars represent the standard deviation (n = 3) for each data point.

Based on the results presented in Fig. 22, foam activity of produced hydrolysates was significantly ($p<0.05$) increased regardless of the used enzyme, compared to the unhydrolyzed WPC. The obtained results are in agreement with literature data[123] that reports the positive influence of pepsin and trypsin hydrolysis on the foam forming ability of WPC hydrolysates. It is interesting to note that the foam expansion of trypsin hydrolysate (327.1%) was significantly ($p<0.05$) higher than pepsin hydrolysate (224.4%). Observed results are in agreement with literature data[123], which suggests that smaller peptides produced through limited enzymatic hydrolysis, express higher positive influence on the foam forming ability of hydrolysates due to more rapid absorption at the interface. On the other hand, trypsin hydrolysate expressed significantly ($p<0.05$) lower foam stability compared to the pepsin hydrolysate. It could be explained by higher DH, which implies the production of very short peptides that probably contribute to poor foaming stability. Similarly, comparing the 44 hydrolysates of whey proteins, Van der Ven (2002)[123] reported that fractions with the molecular weight larger than 7 kDa express higher foam stability. However, limited enzymatic hydrolysis was found to

be successful in improving interfacial properties (both foaming and emulsification properties) of whey protein hydrolysates[126].

3.2.3.4. Digestibility and bioaccessaibility of WPC hydrolysates

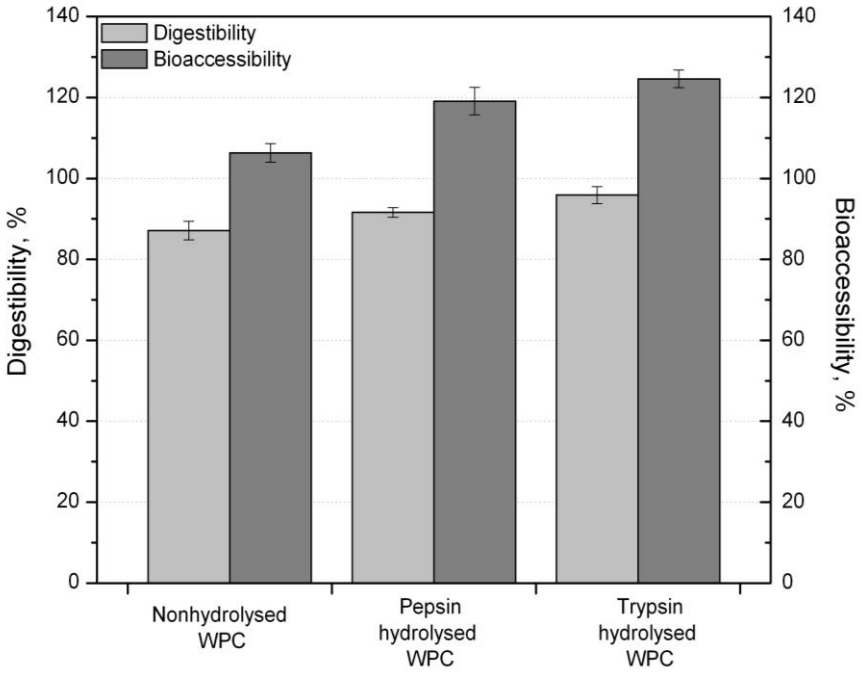

Figure 23. Digestibility and bioaccessibility of peptic and tryptic hydrolysates obtained after 4h of hydrolysis at 37 °C and E/S ratio 0.5%. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 23, the digestibility of both generated peptic and tryptic WPC hydrolysates were significantly ($p<0.05$) higher than nonhydrolyzed WPC. Digestibility of trypsin hydrolysate (95.9%) was significantly ($p<0.05$) higher than pepsin hydrolysate (91.6%). Respecting the results for DH, APCL (Fig. 18b) and DPPH (Fig. 20) registered for trypsin hydrolysate, it seems that the reduced molecular weight of proteins enhances its solubility and allows better accessibility of smaller hydrophilic peptides to the enzymes included in the gastrointestinal digestion in the human body. The obtained results are in agreement with the literature results[127] that suggest a better digestibility of WPC 80 hydrolysates obtained after enzymatic hydrolysis. Presented results suggest that enzymatic modification of whey proteins significantly contributes to their digestibility, which is often the limiting factor for their use as food supplements.

On the other hand, the biological activity does not necessarily correlate with *in vivo* bioactivity features. Therefore, bioaccessibility is an additional factor that gives a rough

picture of the stability of produced bioactivity in simulated gastrointestinal digestion conditions[128].

As shown in Fig. 23, bioaccessibility of both generated peptic and tryptic WPC hydrolysates were significantly ($p<0.05$) higher than nonhydrolyzed WPC. Based on the results, trypsin hydrolysate expresses significantly ($p<0.05$) higher bioaccessibility (124.6%) compared to pepsin hydrolysate (119.1%). Obtained results suggest that generated bioactive peptides are either resistant to the gastric digestion or might be further degraded by digestive enzymes to the smaller peptides with higher antioxidant activity. The results are supported by DH results presented in this study, according to which 4h was not the endpoint of hydrolysis. However, time of hydrolysis used in this study represents an optimal time to obtain hydrolysates with excellent antioxidant activity and improved functional properties.

3.2.4. Conclusion

In conclusion, this study shows that tryptic hydrolysis of whey proteins can enable the release of peptides with high antioxidant activity, originating from parent whey proteins as a complex food matrix. A trypsin hydrolysate, with a maximal total antioxidant activity of 80.0%, can be produced after 4h by hydrolysis at 37 °C and an E/S ratio of 0.5%. The trypsin hydrolysate exhibited 47.9% higher antioxidant activity compared to the nonhydrolysed whey proteins, as well as 25.9% higher antioxidant activity compared to the pepsin hydrolysate produced under the same conditions. The trypsin hydrolysate, which is mainly composed of hydrophilic (polar aprotic) antioxidant species, exerted an improved digestibility of 95.9% and bioaccessibility of 124.6%, compared to the native whey proteins and peptic hydrolysate. In addition to its good antioxidant properties, the trypsin hydrolysate had excellent functional properties, such as emulsifying and foam-forming abilities. These properties indicate it to be a desirable raw material for the food industry that could replace whey proteins as a traditional additive for product fortification. Based on the improved properties, trypsin hydrolysate can be considered as an excellent carrier for the delivery of antioxidants that contribute to both the health and functional properties of dairy or confectionery products. The main contribution of the present study was the determination of the antioxidant activity of peptides with different polarities, contributing to a more precise

determination of the total antioxidant activity of the resulting hydrolysates. This detailed analysis of antioxidant activity allows the precise selection of optimal conditions for the production of hydrolysates with maximal antioxidant activity. The hydrophilic nature of its antioxidant activity shows that tryptic hydrolysate is a natural ingredient most suitable for preventing oxidation processes in polar charged food ingredients. The high bioaccessibility suggests there would be the preservation of peptide bioactivity during the process of gastrointestinal digestion, which is a particular challenge in terms of the application of such additives.

3.3. Production of bioactive whey protein hydrolysate powders

3.3.1. Materials

3.3.1.1. Media, Culture, Enzyme and Reagents

The sweet cow's whey powder (12.11% (*w/w*) of proteins, Lenic Laboratories, Belgrade, Serbia), and whey protein concentrate powder (with 80.0% (*w/w*) of proteins DMV International, 5462 GE Veghel, Netherlands), were used as sources of whey proteins. The sweet cow's whey powder (W) was reconstituted to contain 8% (*w/v*) of dry matter, while whey protein concentrate powder (WPC) was reconstituted to contain 5% (*w/v*) of dry matter. Both suspensions were allowed to hydrate for 1h at room temperature with gentle stirring and subjected to the following processes. The strain *Lactobacillus rhamnosus* ATCC 7469 was supplied by American Type Culture Collection (ATCC, Rockville, USA). Stock culture was stored at -18 °C in 3 mL vials containing De Man Rogosa Sharpe (MRS) broth (Fluka, USA) and 50% (*v/v*) glycerol as a cryoprotective agent. To prepare the laboratory culture, a drop of stock culture was transferred to 3 mL of MRS broth and incubated for 18 h under anaerobic conditions at 37 ºC. The working culture was pre-cultured twice in MRS broth prior to experimental use. The trypsin (porcine pancreas, EC 3.4.21.4), were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The chemicals used in this research were of analytical grade purchased from Sigma-Aldrich Chemie GmbH.

3.3.2. Methods

3.3.2.1. Enzymatic Hydrolysis

The enzymatic hydrolysis was carried out in a 500-ml mechanically stirred batch reactor with temperature and pH control, by commercial enzyme trypsin. The substrate for enzymatic hydrolysis was 400 mL of 5% (*w/v*) aqueous solution of WPC with 44.0 ± 1.363 mg mL^{-1} of protein content (*Section 3.2.2.1*). Before hydrolysis, the WPC solution was adjusted to pH 8 by addition of 1 M NaOH, then stirred and allowed to equilibrate to the working temperature for 15 min. Prepared WPC solution was then incubated at 37 °C for 240 min with 0.5% of trypsin on a protein-equivalent basis. During the course of the hydrolysis, the pH of the WPC solution was kept at pH 8 by continuous addition of

0.1 M NaOH, using the pH-stat method with a contentious dosage of the base. After the hydrolysis, the enzyme was inactivated by heating at 90°C for 15 min, whey protein hydrolysate (WPH) was cooled at room temperature, centrifuged at 6000 rpm for 15 min and subjected to the further spray drying process.

3.3.2.2. Fermentation

The fermentation was conducted in a 500-ml Erlenmeyer flask containing 400 mL of reconstituted whey (W). Sample were inoculated with 2% (*v/v*) *Lb. rhamnosus* ATCC 7469 strain and incubated at 37 °C for 24 h. After the fermentation, fermented whey hydrolysate (FWH) was cooled at room temperature, centrifuged at 6000 rpm for 15 min and subjected to the further spray drying process.

3.3.2.3. Spray drying

The produced hydrolysate solutions (WPH, FWH) as well as corresponding liquid controls (WPC, W), were spray dried in order to produce powdered hydrolysate samples (WPH-P, FWH-P) and corresponding powdered controls (WPC-P, W-P). The spray drying process was performed using a laboratory scale Mini spray dryer B-290 (BUCHI, Labortechnik AG, Switzerland), in conjunction with a peristaltic pump, with the following conditions: inlet temperature at 120 °C, corresponding to a reading of outlet temperature 68-70 °C. The batches of powdered samples were stored in a desiccator (at room temperature) before its use in the following assays.

3.3.2.4. Antioxidant activity

DPPH radical-scavenging test: In order to estimate the antioxidant activity of the complex biological system that contains a wide range of antioxidants with different chemical structure, DPPH scavenging activity of the samples was estimated according to the protocol described by McCue and Shetty (2005)[102] with minor modifications. Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (50, 100, 150, 200 and 250 mg mL^{-1}). An aliquot of each sample solution (250 µL) was added to 4 mL of 0.1 mM DPPH radical dissolved in *ethanol* as solvent. Mixtures were vortexed for 1 min, placed on an orbital shaker, and incubated in the dark for 60 min at room temperature. After incubation, the samples were centrifuged for 15 min at 6000 rpm at

room temperature. Obtained supernatants were filtered using Whatman N^{o} 40 filter paper. The control sample contained DPPH radical solution and deionized water. The absorbance of each supernatant and control was measured at 517 nm against the *ethanol* as a blank. The DPPH radical scavenging activity (%) was calculated according to equation Eq. 19:

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100 \qquad (19)$$

where: Acontrol is the absorbance of the control without hydrolysate, and Asample is the absorbance of the sample with hydrolysate. The antioxidant activity was expressed as the inhibitory concentration ($IC_{50,}$ mg mL^{-1}) of the hydrolysate needed to scavenge 50% of the DPPH free radicals.

3.3.2.5. Lipid peroxidation inhibitory activity

Ferric thiocyanate assay: The oxidation of oleic acid, a monounsaturated fatty acid that is the major constituent of triglycerides, as the model compound, and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), as the free radical initiator, was monitored using the ferric thiocyanate method (FTC) for up to at least 5 h, as described by Azuma *et al.* (1999)[129] with slight modifications. Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (100, 250, 500, 750 and 1000 mg mL^{-1}). An aliquot of the each sample solution (0.30 mL) was placed in a screw-cap vial and mixed with 1.3% (*w/v*) oleic acid in methanol (1.40 mL), 0.2 M phosphate buffer (pH 7.0, 1.40 mL), and water (0.70 mL). The vial was incubated at 50.0 ± 0.1 ºC in the dark and sampling was carried out every hour for up to at least 5 h until the absorbance of the control reaches the value of 0.500 ± 0.030 at 500 nm. The degree of oxidation was measured in triplicate according to the ferric thiocyanate method [33]. The reaction mixture (0.10 mL) was diluted with 75% aqueous (*v/v*) methanol (9.70 mL) and mixed with 20 mM $FeCl_2$ solution in 3.5% (*w/w*) HCl (0.10 mL) and 10% (*w/w*) aqueous NH_4SCN solution (0.10 mL). After precisely 3 min the absorbance was measured at 500 nm, versus 75% methanol. The results are expressed as the percentage of lipid peroxidation inhibitory activity and calculated according to equation Eq. 20:

$$\text{Lipid peroxidation inhibitory activity}\,(\%) = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100 \qquad (20)$$

where: Acontrol and Asample were the absorbance values of control and sample, at the time that control sample reached its maximum absorbance, respectively. The lipid peroxidation inhibitory activity was expressed as the inhibitory concentration (IC_{50}, mg mL^{-1}) of the hydrolysate needed to suppress 50% of lipid peroxidation.

3.3.2.6. Membrane stabilizing activity

Membrane stabilizing activity assay[130]: Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (50, 250, 500, 1000 and 2000 µg mL^{-1}). An aliquot of each sample solution (2 mL) was added in 1 mL of 10% human red blood cells (HRBCs) suspension. A control sample was prepared in the same manner using saline instead of the test sample solution. Diclofenac sodium (250 µg mL^{-1}) was used as reference standard drug. Centrifuge tubes were incubated at 56 ºC for 30 minutes and cooled down to room temperature. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicate. The percentage of membrane stabilizing activity was calculated according to Eq. 21:

$$\text{Membrane stabilizing activity (\%)} = \frac{\text{Acontrol - Asample}}{\text{Acontrol}} \times 100 \qquad (21)$$

where: Acontrol is the absorbance of control and Asample is the absorbance of test sample. The membrane stabilizing activity was expressed as the inhibitory concentration (IC_{50}, µg mL^{-1}) of the hydrolysate needed to suppress 50% of HRBC lyses.

3.3.2.7. Protein denaturation inhibition activity

Inhibition of albumin denaturation assay [131]: Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (50, 250, 500, 1000 and 2000 µg mL^{-1}). An aliquot of each sample solution (2 mL) was added in 3 mL of 1% aqueous solution of bovine albumin fraction, and pH (6.4) of the reaction mixture was adjusted using a small amount of 1N HCl. The samples were incubated at 37 ºC for 20 min and then heated to 51 °C for 20 min, cool down and turbidity was measured at 660nm. Diclofenac sodium (250 µg mL^{-1}) was used as reference standard drug. A control sample was prepared in the same manner using distilled water instead of the test sample

solution. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated according to Eq. 22:

$$\text{Protein denaturation inhibition activity } (\%) = \frac{\text{Acontrol - Asample}}{\text{Acontrol}} \times 100 \quad (22)$$

where: Acontrol is the absorbance of control and Asample is the absorbance of test sample. The protein denaturation inhibition activity was expressed as the inhibitory concentration (IC_{50}, μg mL^{-1}) of the hydrolysate needed to suppress 50% of protein denaturation.

3.3.2.8. Antibacterial activity

Liquid challenge method: Quantitative tests of the antimicrobial activity of the spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) against three Gram-positive (G^+) bacteria *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes,* and Gram-negative (G^-) bacteria *E. coli* (ATCC 25922), were performed according to the liquid challenge method. Overnight cultures (*S. aureus* and *E. coli*) were diluted (10^{-1}) in normal saline solution (NaCl, 0.85% w/v). 2% (*v/v*) of prepared cultures was used to inoculate test tubes that contained 2.5 mL of sterile nutrient broth (NB) and 2.5 mL of spray dried hydrolysate solution at concentration 100 mg mL^{-1}. The initial number of bacteria in each suspension was between 10^5–10^6 colony forming units per milliliter (CFU mL^{-1}) and concentration of spray dried hydrolysate was approximately 50 mg mL^{-1}. Blank was prepared in the same manner, with bacteria in sterile nutritious broth (NB) and sterile distilled water instead of spray dried hydrolysate solution, and used as a control. Thus, the prepared samples were incubated for 24 h at 37 ºC. The number of bacteria was assessed at the start and after 24 h of incubation. Serial dilutions were prepared and 100 μL of appropriate suspensions were plated on nutrient agar. After 24 h incubation at 37 ºC, the plates containing 25–250 colonies were enumerated using a colony counter and the viable cell count was expressed as $\log_{10}$ (CFU mL^{-1}).

3.3.2.9. Stability of whey protein hydrolysate powders during storage

Stability of whey protein hydrolysate powders during storage was monitored trough determination of antioxidant activity. Antioxidant activity tests were performed

immediately after the production (0 day) and during180 days of storage at 20 ± 2 °C and 4 ± 2 °C, by the method described in *Section 3.3.2.4.*

3.3.2.10. Statistical analysis

All absorbance measurements were performed using UV-Vis spectrophotometer (Ultrospec 3300 pro, Amerscham Bioscience). The IC_{50} values were calculated by the formula Y = 100*A1/(X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration (linear regression analysis, $p < 0.0001$ was considered significant) using OriginPro 8 (Origin Lab Co., Northampton, USA) software. The experiments were performed in triplicate and values are expressed as a mean ± standard deviation. Mean values were analyzed using One-way ANOVA. The Tukey post hoc test was performed for means comparison using OriginPro 8 (Origin Lab Co., Northampton, USA). Differences between samples were considered significant at $p<0.05$.

3.3.3. Results and Discussion

3.3.3.1. Comparative analysis of whey protein hydrolysate powders obtained by enzymatic and microbial hydrolysis

3.3.3.1.1. Antioxidant activity of whey protein hydrolysate powders

Free radical-mediated reactions have a significant role in many biological phenomena such as cellular damage and aging by stimulating oxidation of lipids and formation of secondary lipid peroxidation products. The antioxidant activity of produced hydrolysate powders was measured by DPPH method and the results are shown in Fig. 24.

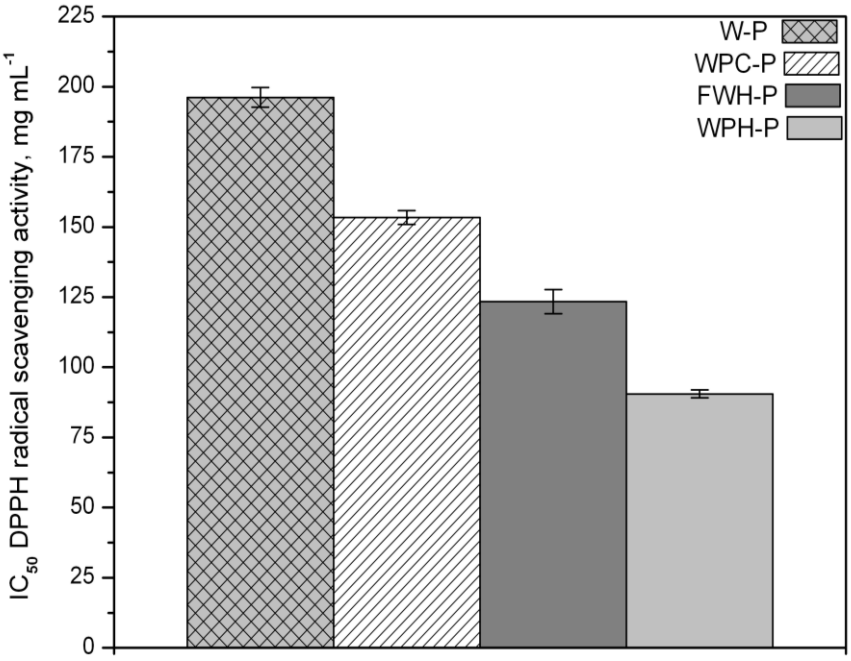

Figure 24. DPPH radical scavenging activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as inhibitory concentration (IC_{50}, mg mL^{-1}) needed to scavenge 50% of the DPPH free radicals.

As shown in Fig. 24 both hydrolysate powders (FWH-P and WPH-P) expressed significantly higher DPPH radical scavenging activities compared to the corresponding controls (W-P and WPC-P). The WPH-P showed significantly ($p<0.05$) higher DPPH radical scavenging activity, corresponding to the IC_{50} value of 90.5 ± 1.4 mg mL^{-1}, compared to the FWH-P that expresses DPPH radical scavenging activity corresponding to the IC_{50} value of 123.4 ± 4.2 mg mL^{-1}. Obtained results are slightly lower than those reported in the literature[132] according to which the trypsin hydrolysed WPC expresses

67.01% of DPPH radical scavenging activity at a concentration of 10 mg mL^{-1}. The observed difference could be explained by the significantly higher degree of hydrolysis (20.17%) induced by higher enzyme concentration (2%), compared to the degree of hydrolysis of 5.7% (*Section 3.2.3.1.1.*) induced by enzyme concentration of 0.5% used for the production of tested WPH-P. However, during the hydrolysis and fermentation of food proteins, commercial enzymes and enzymes produced by starter cultures cleave the proteins and produce peptides of different sizes. Thus, regardless of the proteolytic activity of lactic acid bacteria, it could be assumed that enzymatic hydrolysis probably releases much smaller peptides with a higher antioxidant activity that significantly improves antioxidant activity of produced hydrolysate. This observation is supported by fact that different proteolytic enzymes cleave proteins based on their specificity, and produce peptides with different sizes, sequences, and characteristics responsible for different functional properties[133]. In addition, the use of enzymatic hydrolysis to produce bioactive peptides is preferred than microbial fermentation due to the short reaction time, easier scalability and predictability.

3.3.3.1.2. Inhibition of lipid peroxidation of whey protein hydrolysate powders

In the last years, special attention has been dedicated to searching whey-derived peptides with lipid peroxidation inhibitory activities. The lipid peroxidation inhibitory activity of produced hydrolysate powders was measured by lipid peroxidation method and the results are shown in Fig. 25.

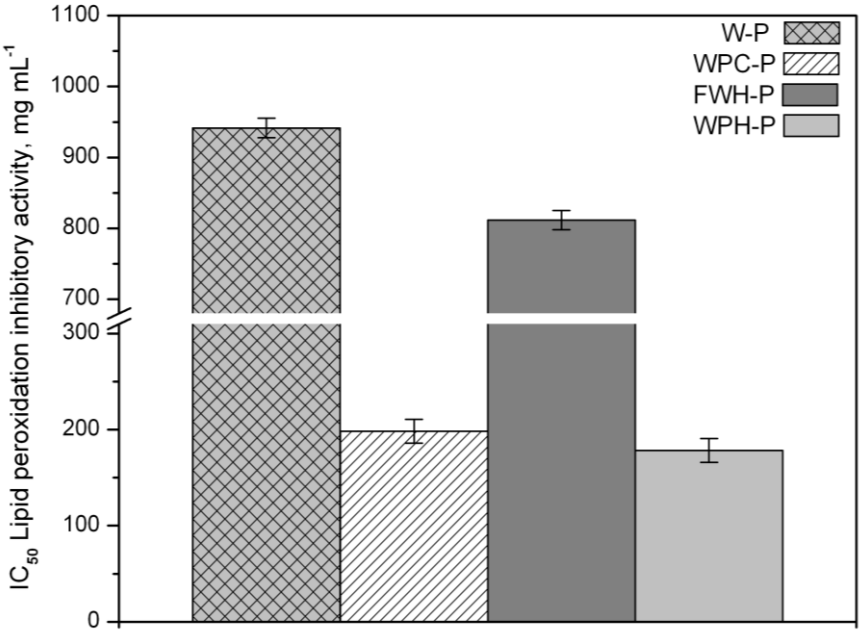

Figure 25. Lipid peroxidation inhibitory activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as inhibitory concentration (IC_{50}, mg mL^{-1}) needed to suppress 50% of lipid peroxidation.

Comparing the activities of hydrolysate powders (FWH-P and WPH-P) and corresponding controls (W-P and WPC-P) it can be noticed that fermentation significantly contributes to the lipid peroxidation inhibitory activity compared to the process of whey protein hydrolysis. In addition, the WPH-P showed significantly ($p<0.05$) higher lipid peroxidation inhibitory activity corresponding to the IC_{50} value of 178.4 ± 12.4 mg mL^{-1}, compared to the FWH-P that expresses lipid peroxidation inhibitory activity corresponding to the IC_{50} value of 811.5 ± 13.7 mg mL^{-1}. Comparing the results of lipid peroxidation inhibitory activity with results of DPPH radical scavenging activity it could be assumed that the concentration of lipid peroxidation inhibitors was substantially lower than the concentration of hydrogen donating compounds, for both hydrolysate powders. Obtained results are in agreement with the literature findings[132,134] that reports similar relation of lipid peroxidation inhibitors and hydrogen donating compounds at a higher degree of hydrolysis (DH > 5%) as well as the longer time of fermentation (> 20h). However, oxidation of food constituents is a key event in food spoilage. It is well known that lipid peroxidation of food products can cause deterioration in food quality, shorten the shelf life and decrease the acceptability

of processed foods. Lipid oxidation can generate free radicals that can lead to fatty acid decomposition, which may reduce the nutritional value and safety of food by producing undesirable flavors and toxic substances[135]. Therefore, it is important to delay the lipid oxidation and the formation of free radical in food containing lipids and/or fatty acids[136]. Therefore, it could be assumed that WPH-P could be promising from a food technology perspective since it can help to delay the peroxidation and subsequently prevent food rancidity. On the other hand, many food allergens are stable and resistant to digestion by gastrointestinal enzymes or are processed into high molecular weight peptides which retain the IgE binding and T-cell-stimulating properties as is the case of bovine whey protein β-lactoglobulin[137,138]. Therefore, although whey protein hydrolysates represent a promising source of bioactive peptides that can be used for the production of improved functional food products, it needs to be more explored in terms of their antigenicity. Due to these facts, hydrolysate powders produced by procedures described in the present study cannot be used in food products intended for cow milk allergic persons.

3.3.3.1.3 Membrane stabilizing activity of whey protein hydrolysate powders

Inflammation is the normal body response triggered by the release of chemical mediators from injured tissue and migrating cells. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bacterial enzymes and proteases which cause further tissue inflammation and damage[139]. *In-vitro* membrane stabilizing activity of hydrolysate powders was screened using a membrane stabilizing model against HRBC (human red blood cells) and the obtained results are presented in Fig. 26.

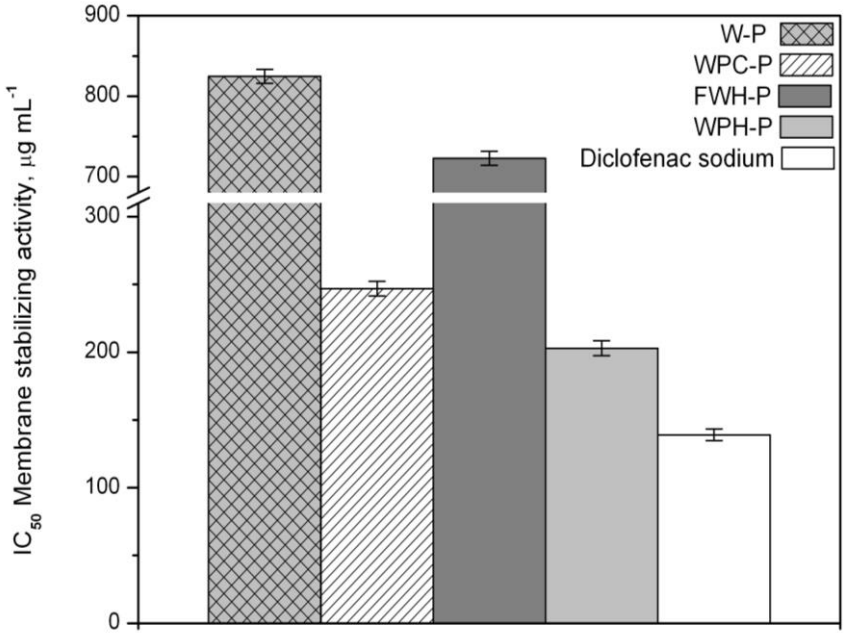

Figure 26. Membrane stabilizing activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as inhibitory concentration (IC_{50}, μg mL^{-1}) needed to suppress 50% of HRBC lyses.

As shown in Fig. 26, both hydrolysate powders (FWH-P and WPH-P) were effective in inhibiting the heat-induced hemolysis of erythrocyte membrane. In addition, hydrolysate powders expressed significantly higher membrane stabilizing activity compared to the corresponding controls (W-P and WPC-P). The membrane stabilizing activities of both hydrolysate powders were concentration dependent and comparable to the standard diclofenac sodium. The WPH-P showed significantly ($p<0.05$) higher membrane stabilizing activity corresponding to the IC_{50} value of 202.9 ± 5.4 μg mL^{-1}, compared to the FWH-P that expresses membrane stabilizing activity corresponding to the IC_{50} value of 722.8 ± 8.6 μg mL^{-1}. The erythrocyte membrane is analogous to the lysosomal membrane [140] and its stabilization implies that the produced hydrolysates may also well stabilize lysosomal membrane. Compared to the diclofenac sodium, a standard anti-inflammation drug that showed 50% membrane stabilizing activity at the concentration of 139.0 ± 4.2 μg mL^{-1}, it could be assumed that WPH-P exhibits moderate membrane stabilizing activity. Obtained results are in agreement with previous studies[141,142] according to which whey protein hydrolysates can inhibit the inflammatory responses in respiratory and intestinal epithelial cells.

3.3.3.1.4. Protein denaturation inhibition of whey protein hydrolysate powders

The results of *in-vitro* protein denaturation inhibition activity of produced hydrolysate powders were presented in Fig. 27.

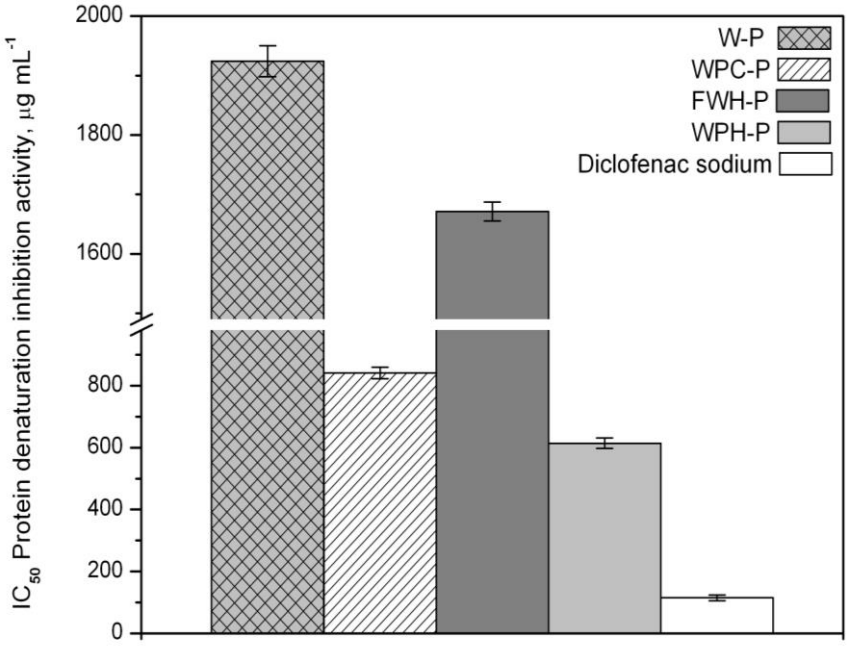

Figure 27. Protein denaturation inhibition activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as inhibitory concentration (IC_{50}, μg mL^{-1}) needed to suppress 50% of protein denaturation.

As shown in Fig. 27, both hydrolysate powders (FWH-P and WPH-P) expressed significantly higher protein denaturation inhibition activity compared to the corresponding controls (W-P and WPC-P). The produced hydrolysate powders were effective in inhibiting the protein denaturation at significantly ($p<0.05$) different concentrations. The inhibition activity of at least 50% was registered at 614.2 ± 16.6 μg mL^{-1} for WPH-P powder, as well as at 1671.2 ± 15.9 μg mL^{-1} for FWH-P. The WPH-P at the concentrations greater than 614.2 ± 16.6 μg mL^{-1} provided significant protection (≥50%) against denaturation of proteins. Compared to the diclofenac sodium, a standard anti-inflammation drug that showed 50% protein denaturation inhibition activity at the concentration of 114.7 ± 9.2 μg mL^{-1}, it could be assumed that WPH-P exhibits low protein denaturation inhibition activity. Inhibitory activity of WPH-P was six times weaker than of diclofenac sodium but triple stronger than protein denaturation inhibitory activity of FWH-P powder. From the results of the present study, it can be suggested

that both hydrolysate powders (WPH-P and FWH-P) are capable to inhibit denaturation of the protein. Based on the literature, a whey protein hydrolysates can attenuate dermatitis in NC/Nga mice[143], while casein hydrolysates have shown promise in treating adjuvant arthritis in rats and chemically induced colitis in mice through modulation of both chronic and acute inflammatory responses[144,145].

3.3.3.1.5. Antibacterial activity of whey protein hydrolysate powders

The growth inhibition observed at the end of 24 h of incubation in the presence of WPH-P at a concentration of 50 mg mL^{-1} is shown in Table 5.

Table 5. Antibacterial activity of whey protein hydrolysate powder (WPH-P), tested at concentration of 50 mg mL^{-1} against pathogenic bacteria

Sample after 24h of incubation	Viable cell count, log_{10} (CFU mL^{-1})			
	S. aureus	***B. cereus***	***E. coli***	***L. monocytogenes***
Control	8.301	6.301	8.342	8.342
WPH-P	7.361	5.301	8.361	7.342

The WPH-P has shown growth inhibition of 1.000 log_{10} (CFU mL^{-1}) for *B. cereus* and *L. monocytogenes* and 0.940 log_{10} (CFU mL^{-1}) for *S. aureus*. The WPH-P hasn't shown any growth inhibition for Gram-negative *E. coli*. This is in line with previous research where WPH-P hasn't shown antibacterial activity against *E. coli*[146]. Also, Pellegrini *et al.* (2001)[147] have shown that the tryptic digestion of the whey protein yielded four antibacterial peptides which were inhibitory only to Gram-positive bacteria.

The growth inhibition observed at the end of 24 h of incubation in the presence of FWH-P at a concentration of 50 mg mL^{-1} is shown in Table 6.

Table 6. Antibacterial activity of fermented whey hydrolysate powder (FWH-P), tested at concentration of 50 mg mL^{-1} against pathogenic bacteria

Sample after 24h of incubation	Viable cell count, log_{10} (CFU mL^{-1})			
	S. aureus	***B. cereus***	***E. coli***	***L. monocytogenes***
Control	8.204	7.204	8.653	7.255
FWH-P	5.477	3.477	4.301	6.204

As shown in Table 6, the antibacterial activity of FWH-P was higher than that of WPH-P for all bacterial strains. The growth inhibition was 2.727 log10 (CFU mL-1) for *S.*

aureus, 3.727 log10 (CFU mL-1) for *B. cereus*, 4.342 log10 (CFU mL-1) for *E. coli*, and 1.051 log10 (CFU mL-1) for *L. monocytogenes*. In this study, FWH-P has shown significantly ($p<0.05$) higher growth inhibition for *S. aureus*, *B. cereus*, *E. coli* compared to the WPH-P. Several studies indicate the lactic acid bacteria possess high antimicrobial activity as a result of their highly developed proteolytic system[148,149]. In addition, the inhibitory effects might be originated of proteins such as lactoperoxidase and lactoferrin as minor but very important whey constituents[150]. This could also be due to the low pH of the fermented whey that resulted from lactic acid produced particularly by lactic acid bacteria. Based on the literature findings[151,152] the *Lactobacillus* species could inhibit growth of various Gram positive and Gram negative bacteria through the production of hydrogen peroxide, bacteriocins and organic acids such as lactic and acetic acids. These substances inhibit the growth of pathogenic bacteria and also improve the commensal balance between some gut flora and humans[153]. Also, Sagong *et al.* (2011)[154] revealed the effectiveness of lactic acid for reduction of *E. coli* and *L. monocytogenes.* In this study, FWH-P has shown significantly ($p<0.05$) higher growth inhibition for *S. aureus*, *B. cereus*, *E. coli* compared to the WPH. Due to the low pH value of FWH-P, it can be adequately used as a supplement in the products such as fermented beverages that are characterized by a low pH value.

3.3.3.2. Stability of whey protein hydrolysate powders

Stabilities of whey protein hydrolysate powders, measured as changes of the antioxidant activity of whey protein hydrolysate powders during the 6 months (180 days) of storage at 4 ± 2 °C and 20 ± 2 °C, are presented in Fig. 28 and Fig. 29. The antioxidant activity of powders was expressed as the inhibitory concentration ($IC_{50,}$ mg mL^{-1}) of the hydrolysate needed to scavenge 50% of the DPPH free radicals.

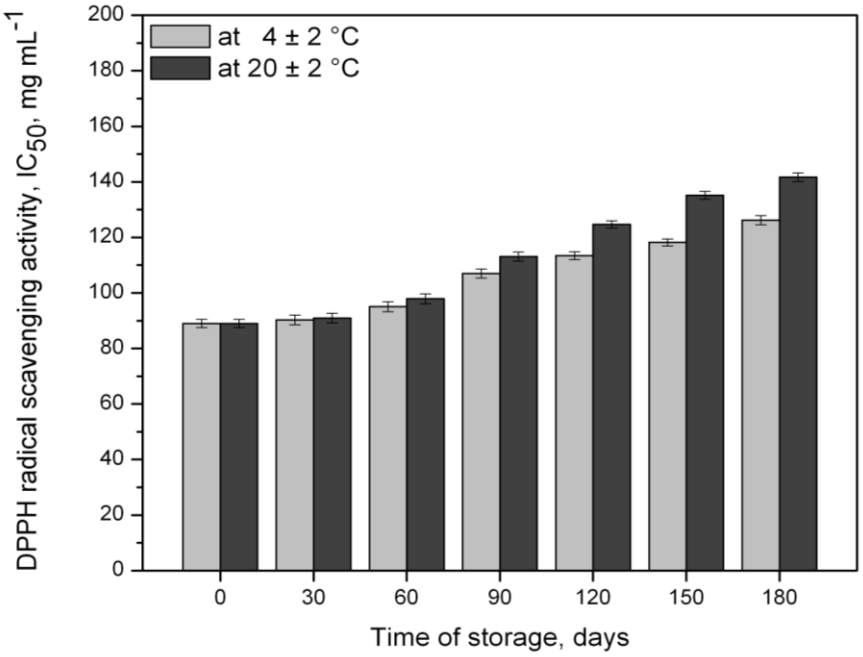

Figure 28. Stability of DPPH scavenging activity of whey protein hydrolysate (WPH-P) powder during 180 days of storage at 4 ± 2 °C and 20 ± 2 °C. Vertical bars represent the standard deviation (n = 3) for each data point.

As indicated in Fig. 28, the antioxidant activity of whey protein hydrolysate (WPH-P) powder decrease during the whole storage period, reaching the minimal level of 141.7 mg mL^{-1} after 180 days of storage at 20 ± 2 °C. During the 60 days, there is no significant difference between samples stored at 20 ± 2 °C and 4 ± 2 °C. In addition, during the 60 days, both samples (stored at 20 ± 2 °C and 4 ± 2 °C) retain the antioxidative potential at a level that corresponds to the level observed immediately after the production (approximately 90.0 mg mL^{-1}). After that period, sample stored at 4 ± 2 °C retains much of antioxidant potential compared to the sample stored at 20 ± 2 °C, during the whole storage period. However, in the period 60-180 days, the antioxidant activity of samples is significantly ($p<0.05$) lower than the activity registered after the production. Based on this finding it could be suggested that the antioxidant activity of produced whey protein hydrolysate (WPH-P) retain at a satisfactory level during the 60 days of storage.

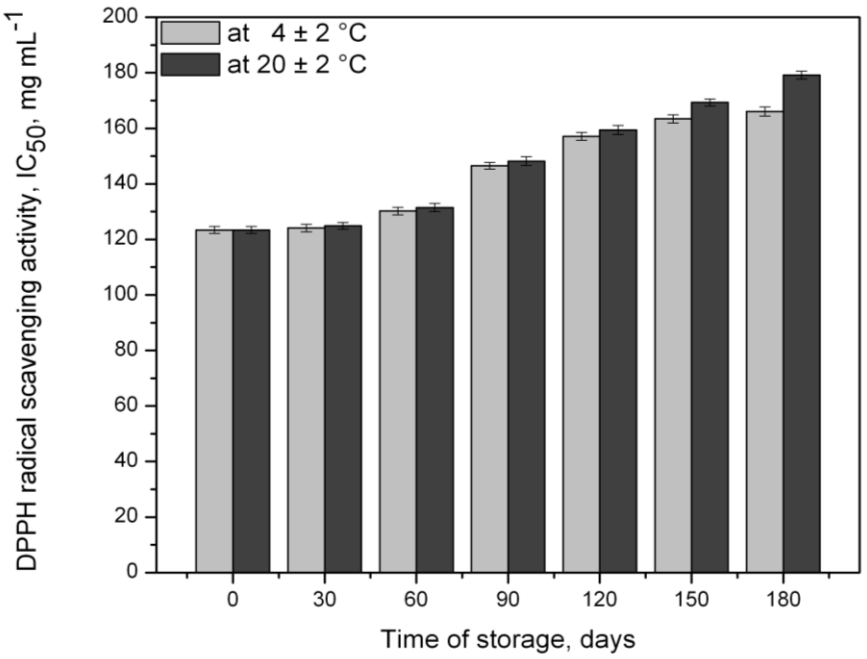

Figure 29. Stability of DPPH scavenging activity of fermented whey hydrolysate (FWH-P) powder during 180 days of storage at 4 ± 2 °C and 20 ± 2 °C. Vertical bars represent the standard deviation (n = 3) for each data point.

As indicated in Fig. 29, the antioxidant activity of fermented whey hydrolysate (FWH-P) powder decrease during the whole storage period, reaching the minimal level of 179.2 mg mL^{-1} after 180 days of storage at 20 ± 2 °C. During the 120 days, there is no significant difference between samples stored at 20 ± 2 °C and 4 ± 2 °C. In addition, during the 30 days, both samples (stored at 20 ± 2 °C and 4 ± 2 °C) retain antioxidative potential at a level that corresponds to the level observed immediately after the production. After 30 days, the sample stored at 4 ± 2 °C retains much of the antioxidant potential compared to the sample stored at 20 ± 2 °C. After 30 days of storage, the antioxidant activity of samples is significantly ($p<0.05$) lower than the activity registered after the production. Based on this finding it could be suggested that the antioxidant activity of produced fermented whey hydrolysate (FWH-P) powder remains at satisfactory level during the 30 days of storage.

3.3.4. Conclusion

Based on the results presented for different bioactivities of whey protein hydrolysate powder produced by tryptic digestion, it can be highlighted that addition of at least 178.4 mg mL^{-1} of whey protein hydrolysate could inhibit processes of lipid peroxidation for 50% as well as microbial contamination caused by *S. aureus* ATCC25923, *B. cereus* ATCC 11778 and *L. monocytogenes*. In addition, the proposed concentration will surely provide antioxidant, protein denaturation inhibition and membrane stabilizing activity greater than 50%. On the other hand, hydrolysate powder obtained by whey fermentation should be added in a concentration of at least 811.5 mg mL^{-1} to achieve all of the tested bioactivities, with emphasis on significantly more pronounced antimicrobial activity against all tested strains. Therefore, it could be concluded that enzymatic hydrolysis represents the optimal process for the production of hydrolysate powder with pronounced bioactive properties that could be considered as a very promising natural food supplement.

3.4. Application of bioactive whey protein hydrolysate powder

3.4.1. Materials

3.4.1.1. Whey protein hydrolysate powders

Whey protein hydrolysate powders (WPH-P and FWH-P) were produced as described in *Section 3.3.2.*

3.4.1.2. Confectionery fat filling

The confectionery fat filling was produced at the Soko-Štark plant (Belgrade, Serbia) in Bühler ball mill. All components (except lecithin and aroma) are automatically dosed and homogenized in the Buhler mixer, and then transferred continuously to the receiving vessel, and then in the ball mill, by the pump of a specific flow, to grind. The grinding is based on the friction/passing of all the greasy particles through the Buhler mill equipped with the mixer. According to the demand of the desired viscosity and composition of the raw materials, the required flow (grinding speed) and maximal temperature that must not be exceeded during grinding is defined. The number of pellets does not change. Higher speed, shorter passage through the mill - low friction, provided the requirement of particle size is below 20μm. Lecithin is added in two stages, half at the start of grinding in a ball mill, and half 15 minutes before the end together with the aromas. The confectionery fat filling then pumped into the larger mixer to cool down.

3.4.2. Methods

3.4.2.1. Formulation of confectionery fat filling supplemented by whey protein hydrolysate

The produced whey protein hydrolysate powders (WPH-P and FWH-P) were added in confectionery fat filling at concentrations of 1.0, 2.5 and 5.0%. Following the addition of whey protein hydrolysate powders (WPH-P and FWH-P), the samples were homogenized (15000 rpm) to obtain a uniform raw material base. The final products were packed in aluminium foil and marked in blank paper/cardboard, and then stored at 20 ± 2 °C and 4 ± 2 °C.

3.4.2.2. Chemical composition of confectionery fat filling

The chemical composition of the final confectionery fat filling tablets was determined using standard AOACC (2000) methods[155]. The basic chemical composition of confectionery fat filling is determined using standard AOACC methods: moisture - thermogravimetric, total fat - Soxlet method, proteins - Kjeldal method, carbohydrates and sucrose - polarimetric, lactose - iodometric titration, and is shown in Table 7. The energy value was calculated based on the raw material composition of the confectionery fat filling tablets.

3.4.2.3. DPPH scavenging activity of confectionery fat filling

DPPH scavenging activity of the samples was estimated according to the protocol described by McCue and Shetty (2005)[156] with minor modifications. Briefly, five dilutions of confectionery fat filling (50, 100, 150, 200 and 250 mg mL^{-1}) were prepared. 250 μL of each dilution was added to 4 mL of 0.1 mM DPPH radical dissolved in *ethanol* as solvent. Mixtures were vortexed for 1 min, placed on an orbital shaker, and incubated in the dark for 60 min at room temperature. After incubation, samples were centrifuged for 15 min at 6000 rpm at room temperature. The obtained supernatants were filtered using Whatman N^o 40 filter paper. At the same time, the control sample that contains the DPPH radical solution and deionized water instead of the sample was prepared. The absorbance of each supernatant and control was measured at 517 nm against the *ethanol* as a blank. The DPPH radical scavenging activity (%) was calculated according to equation Eq. 23:

$$\text{DPPH scavanging activity}\,(\%) = \frac{\text{Ac - As}}{\text{Ac}} \times 100 \qquad (23)$$

where: Ac is the absorbance of the control without hydrolysate, and As is the absorbance of the sample with hydrolysate. The antioxidant activity was expressed as the inhibitory concentration ($IC_{50,}$ mg mL^{-1}) of the fat filling needed to scavenge 50% of the DPPH free radicals.

3.4.2.4. Determination of confectionery fat filling texture

In order to determine the texture of confectionery fat filling, a cone penetration method was applied at a temperature of 20 ± 2 °C, on the texture meter TA.XT Plus (Stable

Micro System, UK), according to the Chocolate Spread method - SPRD2_SR_PRJ. The accessories used are a conical piston, cups, which are attached to the metal platform (Fig. 30).

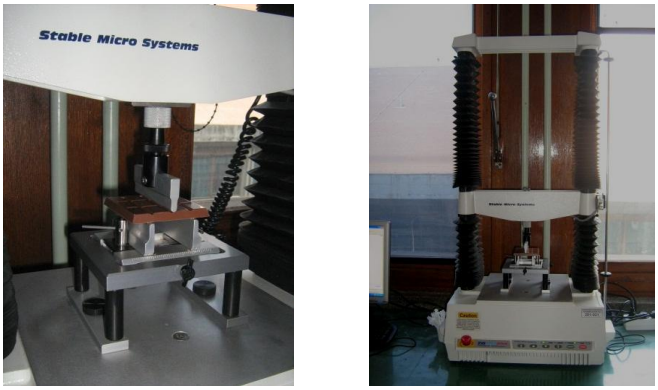

Figure 30. Texture meter TE32 and accessories used for textural analysis of confectionery fat filling

Each sample was placed into the cone sample holder and pressed down in order to eliminate air pockets. Any excess of the sample was scraped off with a knife. The filled cone sample holder was then put in the base holder and 45° cone probe with the diameter of 38 mm was used to penetrate the samples at 3 mm/s. During the analysis, the force is measured to reach the maximum. This force is defined as the strength at the specific penetration depth. The size below the surface of the obtained force dependence curve (F) from the time (t) represents the shear operation in the applied force.

3.4.2.5. Rheological properties of confectionery fat filling

Rheological properties were determined in the rotation viscometer RheoStress (600 HP, Haake, Germany), according to the IOCCC method[157], at the temperature of 40 ± 0.1 °C using a concentric cylinder system (sensor Z20 DIN, Fig. 31).

Figure 31. RheoStress 600, Haake and accessories used in the rheological analysis of confectionery fat filling

Flow curves were determined using the method of the hysteresis loop within the shear rate interval of 1-60 1/s. The shear rate was increased from 1 to 60 s^{-1} in the period of 240 s, then maintained at the maximum speed of 60 s^{-1} for 60 s, and the decreasing of shear rate from 60 to 1 s^{-1} also lasted for 240 s.

3.4.2.6. The distribution of particle size distribution in confectionery fat filling

Influence of milling time on particle size distribution in confectionery fat filling samples was determined by Mastersizer 2000 (Malvern Instruments, England) laser diffraction particle size analyzer equipped with a Hydro 2000 μP dispersion unit (Malvern Instruments, England).

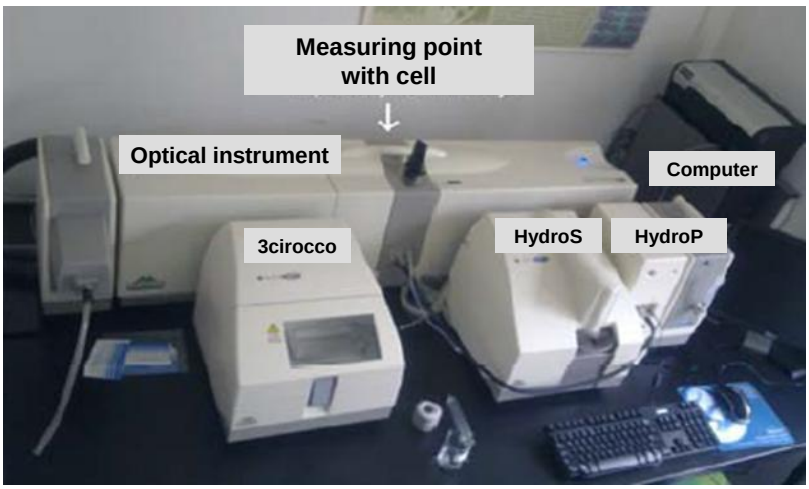

Figure 32. Mastersizer 2000, Malvern and accessories used in the texture analysis of confectionery fat filling

Confectionery fat filling samples were dispersed in sunflower oil at ambient temperature (20 ± 2 °C) and added until adequate obscuration was obtained (10-20%). The results were quantified as volume-based particle size distribution, using Mastersizer 2000 Software

3.4.2.7. Sensory analysis in confectionery fat filling

Quantitative descriptive analysis (QDA)

Detailed sensory analysis of confectionery fat filling samples was done by conducting quantitative descriptive analysis (QDA) with a trained sensory panel according to the method described by De Pelsmaeker et al.[158], with slight modifications. The panel

consisted of 15 assessors (8 being women and 7 men, age between 25 and 55) selected from a pool of the 60 possible candidates which were included in acceptance testing. Panellists were selected based on their abilities to identify and describe differences in confectionery fat filling samples and their recognizing the presence of different ingredients. They participated in a 3 month training period when the sensory descriptors including texture quality parameters (hardness, brittleness, dryness, stickiness and toughness), as well as melting parameters (melting point, melt rate, cooling, meltability) were chosen, defined and measured. The panellists were trained over a period of 15 h to perform the quantitative descriptive analysis.

During the QDA test, each panellist received the seven confectionery fat filling samples (20 g) at a time, in individual packs coded with 3-digit numbers, in random order, a pencil, a questionnaire and a glass of cold water to rinse their mouths between samples. The samples were presented monadically at 20 ± 2 ˚C. Each questionnaire consists of questions: name, age, sex as well as hardness, brittleness, dryness, stickiness, toughness, melting point, melt rate, cooling, meltability for seven consumed samples. The panellists have been asked to mark a value which best represents the tested quality parameter for each of seven samples, on the 5-point scale ranging from 1 = low to 5 = high. The QDA analysis has consisted of 105 questionnaires distributed into 7 sessions (7 samples). Prior to serving in both analyses (acceptance testing and QDA) all samples were subjected to counts of yeasts, molds and coliforms to evaluate the hygienic and sanitary conditions of the products.

3.4.2.7. Stability of confectionery fat filling during storage

Stability of confectionery fat filling during storage was monitored trough determination of antioxidant activity. Antioxidant activity tests were performed immediately after the production (0 day) and during 180 days of storage at 20 ± 2 °C, by the method described in *Section 3.4.2.3.*

3.4.3. Results and Discussion

3.4.3.1. Chemical composition of confectionery fat filling

The basic chemical composition of confectionery fat filling was determined using standard AOACC methods and obtained results are shown in Table 7.

Table 7. Chemical composition of confectionery fat filling

Chemical composition of confectionery fat filling, per 100g		
Ingredient	**Without peptides**	**Supplemented with 5%WPH-P**
Energy, kcal	566.24 kcal	558.04 kcal
Total carbohydrates, g	43.99	42.12
Sucrose, g	24.19	22.98
Lactose, g	19.80	19.14
Total fat, g	37.18	35.64
Milk fat, g	3.60	3.74
Proteins, g	13.91	17.21

3.4.3.2. Influence of bioactive whey protein hydrolysate powder on antioxidative activity of confectionery fat filling

Bioactive peptides with an antioxidative potential obtained by enzymatic hydrolysis and microbial fermentation of whey proteins are a new way of providing positive effects on human health and at the same time representing a good alternative to synthetic antioxidants. The antioxidant activity of confectionery fat filling expressed as IC_{50} (mg mL^{-1}) is the minimal amount of fat filling that is required to inhibit 50% of DPPH radical activity. Influence of different concentrations of bioactive whey protein hydrolysate powders on the antioxidative potential of confectionery fat filling is shown in Fig. 33.

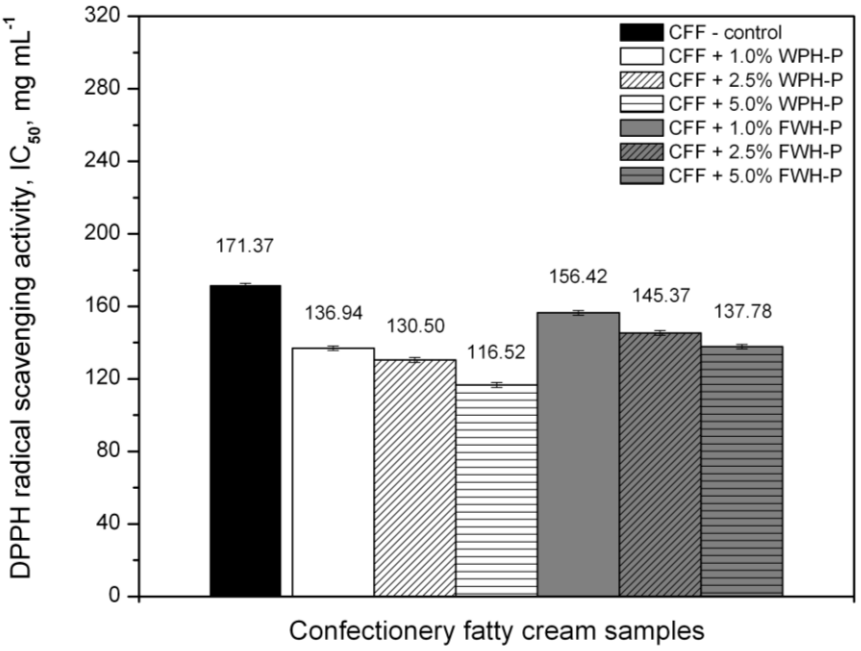

Figure 33. Influence of different concentrations of bioactive whey protein hydrolysate powders on DPPH scavenging activity of confectionery fat filling. Vertical bars represent the standard deviation (n = 3) for each data point.

As shown in Fig. 33, all samples of the fat filling enriched with peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) exhibit significantly ($p<0.05$) stronger antioxidant activity compared with the control confectionery fat filling sample (CFF). Their activities in relation to the control sample (CFF) are 20-32% higher depending on the peptide concentration. The strongest DPPH scavenging activity that corresponds to IC_{50} value of 116.52 mg mL^{-1} was registered in the sample with 5% of WPH-P. Fat filling sample with 5% of WPH-P expresses for 32% stronger antioxidative power than control sample (171.37 mg mL^{-1}). Samples of the fat filling enriched with peptides produced by fermentation of whey (FWH-P) exhibit 9-20% stronger antioxidant activity compared with the control sample (CFF) depending on the peptide concentration. The strongest DPPH scavenging activity that corresponds to IC_{50} value of 137.78 mg mL^{-1} was registered in the fat filling sample with 5% of FWH-P. Fat filling sample with 5% of FWH-P expresses for 20% stronger antioxidative power than control sample (171.37 mg mL^{-1}).

In addition, based on the presented results it is evident that all samples of confectionery fat filling enriched with peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) exhibit significantly ($p<0.05$) stronger antioxidant activity compared to the fat

filling samples enriched with peptides produced by microbial fermentation of whey (FWH-P). Presented results revealed that minimal concentration of fat filling (116.52 mg mL^{-1}) that is capable to inhibits the DPPH radical activity for 50% and reduce the harmful effect of these radicals, corresponds to the fat filling sample supplemented with 5% of WPC hydrolysate.

3.4.3.3. Influence of bioactive whey protein hydrolysate powder on hardness of confectionery fat filling

Influence of different concentrations of bioactive whey protein hydrolysate powders on the hardness of confectionery fat filling is shown in Fig. 34.

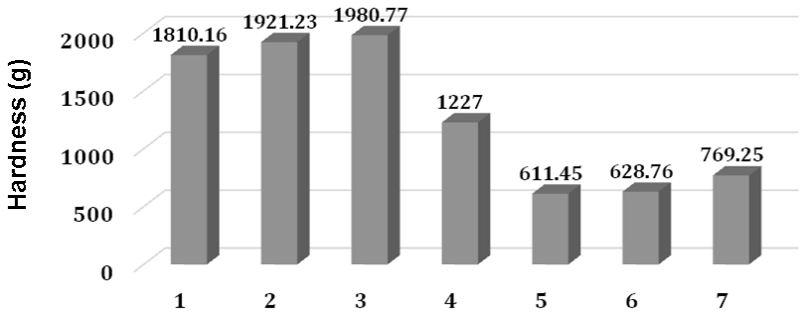

Figure 34. Hardness of confectionery fat filling supplemented with different concentrations of bioactive whey protein hydrolysate powders. Legend: 1- CFF+1%WPH-P; 2- CFF+2.5%WPH-P; 3- CFF+5%WPH-P; 4- CFF+1%FWH-P; 5- CFF+2.5%FWH-P; 6- CFF+5%FWH-P; 7- CFF (confectionery fat filling)

As shown in Fig. 34, the addition of peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) significantly influences the hardness of the confectionery fat filling. Compared to the control sample (CFF), the hardness of fat filling samples was increased from 2.3 to 2.6 times for WPH-P concentrations of 1-5%, respectively. On the other hand, the addition of peptides produced by fermentation of whey proteins (FWH-P) at a concentration of 1% leads to the increase of fat filling hardness by 1.6 times, while higher concentrations of 2.5 and 5% results in the opposite effect and decrease the hardness of fat filling compared to the control sample (CFF).

Generally, the optimal supplementation of confectionery fat filling cannot be determined because it depends on the potential application of fat filling in stuffed confectionery products. If the fat filling is used for stuffed chocolate it should be a fat filling with a hardness of about 1000 g[159] or a filling supplemented with peptides

produced by fermentation of whey proteins (FWH-P) at a concentration of 1%. If the fat filling should be used for the filling of biscuits, this should be substantially harder filling that corresponds to the sample supplemented with peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) at a concentration of 5%.

However, the general observation related to the influence of bioactive whey protein hydrolysate powders on hardness of confectionery fat filling revealed that addition of peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) increases the hardness of the fat filling, while the addition of peptides produced by fermentation of whey proteins (FWH-P) reduces the hardness of confectionery fat filling compared to the control sample (CFF).

3.4.3.4. Influence of bioactive whey protein hydrolysate powder on rheological properties of confectionery fat filling

The confectionery fat filling, as well as chocolate, is a rheological system with the solid phase (particles of sucrose, cocoa powder and powdered milk) dispersed in the fat phase[160]. It is characterized by a nonuniform distribution of solid particles and shows thixotropic properties characterized by a yield stress and plastic flow[159]. Influences of different concentrations of bioactive whey protein hydrolysate powders on thixotropic loops and rheological properties of confectionery fat filling are shown in Fig. 35.

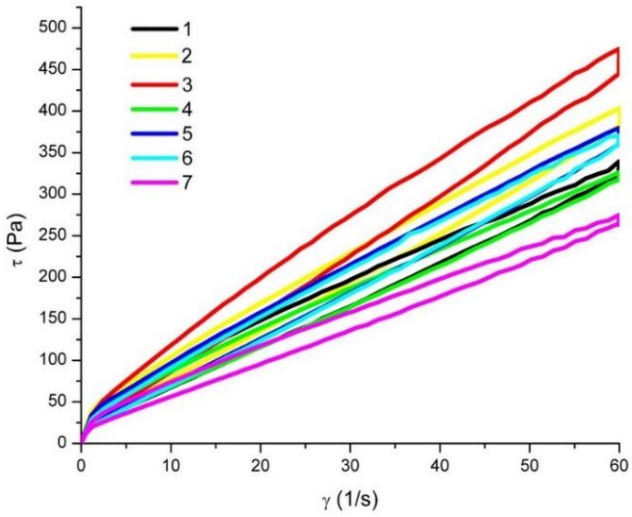

Figure 35. Flow curves of confectionery fat filling supplemented with different concentrations of bioactive whey protein hydrolysate powders. Legend: 1- CFF+1%WPH-P; 2- CFF+2.5%WPH-P; 3- CFF+5%WPH-P; 4- CFF+1%FWH-P; 5- CFF+2.5%FWH-P; 6- CFF+5%FWH-P; 7- CFF (confectionery fat filling)

Influence of bioactive whey protein hydrolysate powders on the rheological properties of all fat filling samples is shown in Table 8.

Table 8. Particle size distribution (PSD) parameters of confectionery fat filling supplemented with different concentrations of bioactive whey protein hydrolysate powders.

Sample	Casson yield stress, Pa	Casson viscosity, Pa·s	Thixotropic curve area, $Pa \cdot s^{-1}$
CFF+1% WPH-P	6.521	4.019	1630
CFF+2.5% WPH-P	5.825	5.210	2123
CFF+5% WPH-P	5.461	6.066	2455
CFF+1% FWH-P	5.770	3.983	1177
CFF+2.5% FWH-P	4.790	4.781	1786
CFF+5% FWH-P	3.441	4.952	1628
CFF - confectionery fat filling	5.010	3.307	1210

As shown in Fig. 35, thixotropic loops of all samples have the same properties as a control sample (7 - CFF) without incorporated hydrolysates. The control sample (7 - CFF) of confectionery fat filling exhibits thixotropic flow and has good homogeneity, indicated also by the surface value of the thixotropic loop of 1210 $Pa \cdot s^{-1}$, and the Casson viscosity value of 3.307 Pa·s (Table 8).

The difference in rheological properties of samples is solely due to the presence of various concentrations of protein supplements. The addition of peptides produced by enzymatic hydrolysis of whey protein concentrate (WPH-P, loops 1-3) and peptides produced by fermentation of whey proteins (FWH-P, loops 4-6) influences the packing of particles. It is important to note that the addition of peptides affects more Casson viscosity rather than Casson yield, and the increase in viscosity can be avoided by a better selection of the emulsifier.

As the particles are smaller the specific surface area of the particles increases, and therefore the amount of fat required for the coating of the particles. Accordingly, the fat filling with the largest mean particle diameter, a sample enriched with 5% of peptides produced by fermentation of whey proteins (FWH-P), has the lowest Casson yield (3.441 Pa), i.e. requires the smallest force that must be applied to make the fat filling flow.

Conversely, the fat filling with the highest viscosity values according to Casson (6,066 Pa·s), and the surface of the thixotropic loop (2,455 $Pa \cdot s^{-1}$) has the highest hardness

(1980.7 g, Fig. 34), which is a sample with 5% of peptides produced by enzymatic hydrolysis of whey protein concentrate (WPH-P). The most homogeneous structure, similar to the peptide-free control sample (CFF), has sample enriched with 1% of peptides produced by the fermentation of whey proteins (FWH-P), but the most optimal sample from the rheology point of view is the fat filling enriched with 5% of peptides produced by the fermentation of whey proteins (FWH-P).

3.4.3.5. Influence of bioactive whey protein hydrolysate powder on particle size distribution in confectionery fat filling

Confectionery fat filling contains a large fat content (37%) that represents a continuous fat phase and directly affects the consistency of the final product. Confectionery products containing fat filling can be melted faster or slower depending on the textural and sensory characteristics of fat filling. For this reason, the grinding phase is very important in order to obtain an adequate distribution of the particle size in the fat filling that will affect the quality of the final product[161]. Influence of bioactive whey protein hydrolysate powders on particle size distribution in confectionery fat filling is presented in Table 9.

Table 9. Influence of bioactive whey protein hydrolysate powders on particle size distribution in confectionery fat filling

	CFF samples						
	Without	**+1% WPH-P**	**+2.5% WPH-P**	**+5% WPH-P**	**+1% FWH-P**	**+2.5% FWH-P**	**+5% FWH-P**
d^a(0.1) (µm)	3.91	3.69	3.24	3.92	3.51	2.59	3.46
d^a(0.5) (µm)	16.48	15.32	13.49	15.15	15.37	12.18	14.75
d^a(0.9) (µm)	46.97	46.95	44.14	44.93	47.73	43.72	53.12
D^b (µm)	21.68	21.24	19.37	20.40	21.40	18.50	22.73
Specific surface (m^2g^{-1})	0.67	0.72	0.80	0.68	0.74	0.98	0.75
Distribution width	2.61	2.82	3.03	2.71	2.88	3.37	3.37

[a]d (0.1), d (0.5), d (0.9) respectively represent 10%, 50%, and 90% of all particles with this size.
[b]D - volume weighted mean diameter.

Based on the results presented in Table 9, it can be seen that even 50% of the sample volume of all the examined fat filling samples has particles with size less than 16.48 µm, the highest value is measured in the control sample without added peptides. The

mean volume particle diameter is lower in the fat filling enriched with peptides produced by fermentation of whey proteins (FWH-P), which is consistent with the hardness of the fat filling. The parameter d (0.9) has values of 44.14 μm in a fat filling CFF+2.5%WPH-P to 53.12 μm in fat filling CFF+5%FWH-P.

The appearance of the particle size distribution curve of the fat filling produced in a ball mill in industrial conditions is shown in Fig. 36.

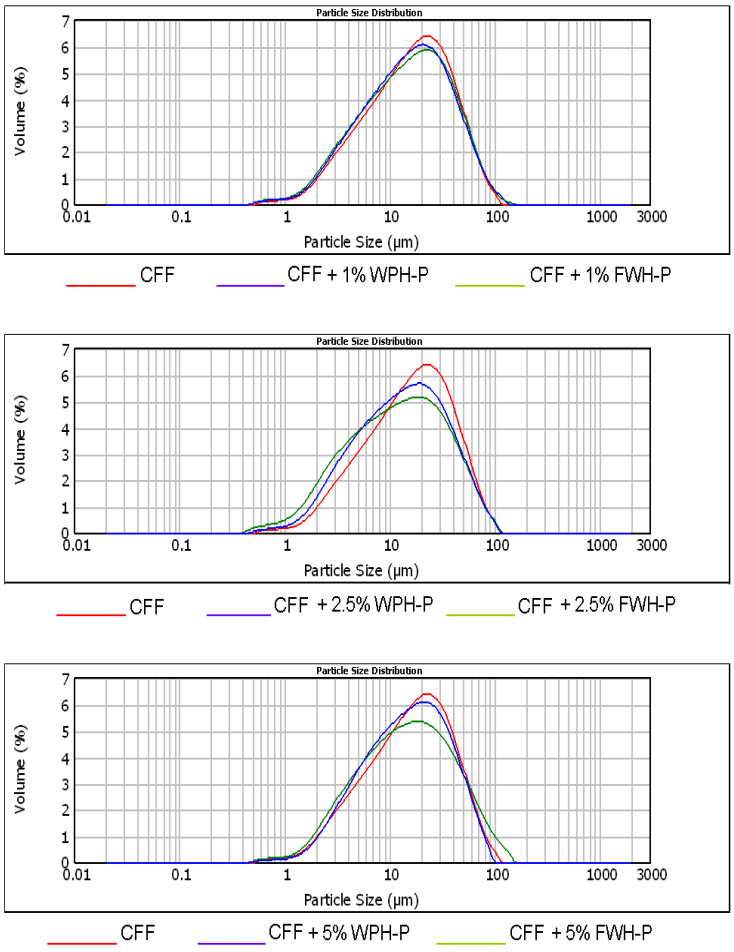

Figure 36. The appearance of the particle size distribution curves of the fat filling supplemented with different concentrations of bioactive whey protein hydrolysate powders

The appearance of the curve is very similar to Gauss's distribution curve (Fig. 36), where 90% of the sample volume has particles of less than 46.97 μm and the mean diameter of the bulk distribution less than 21.68 μm (Table 9). As shown in Figure 36 the narrowest span, and thus the simplest structure, has a control sample (CFF) without added peptides. A slight increase of only 3.8% has a sample with 5% of peptides

produced by enzymatic hydrolysis of whey proteins (WPH-P). The most similar particle size distribution as fat filling with no added peptides, was registered in fat fillings with 1% added peptides regardless of the type of peptide (CFF+1%WPH-P and CFF+1%FWH-P), which is somewhat understandable, since it is not a large amount of peptides to impair the structure of the fat filling. An addition of 2.5% of both types of whey protein hydrolysate led to the complexity of the structure and appearance of wider distribution. The sample with 5% peptides produced by enzymatic hydrolysis of whey protein concentrate (CFF+5%WPH-P) can be considered as optimal, from the point of view of particle size distribution in the fat filling.

3.4.3.6. Influence of bioactive whey protein hydrolysate powder on sensory properties of confectionery fat filling

Influence of different concentrations of bioactive whey protein hydrolysate powders on sensory properties of confectionery fat filling is shown in Fig. 37 and Fig. 38.

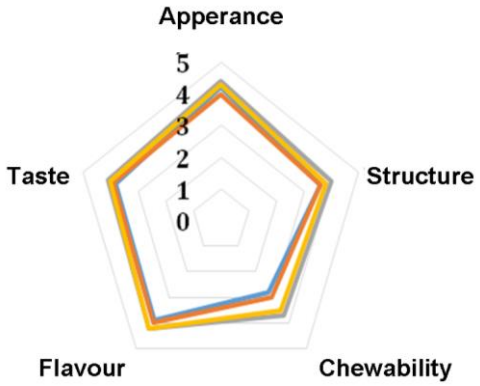

Figure 37. Sensory characteristics of confectionery fat filling supplemented with different concentrations of peptides produced by enzymatic hydrolysis of whey protein concentrate.

The addition of whey protein hydrolysates in confectionery fat filling does not lead to a change in the sensory quality of fat filling. Results presented in Fig. 37 show the fully uniform quality of all samples regardless of the amount of added hydrolysates compared to the control sample without added peptides (CFF), especially in the appearance, flavor and taste. The samples of confectionery fat filling, in which hydrolysates are added in an amount of 5%, have a better structure and chewiness compared to the other tested samples, even to the control sample (CFF). This is in accordance with the results obtained for hardness (Fig. 34). Due to the fact that hydrolysates are finer particles that

cause an increase in the hardness of the film, they additionally influence the improvement of the fat filling structure. This filling could certainly be applied to stuffed chocolate products, as there was a slight increase in the hardness of the fat filling that would not affect other sensory characteristics. The best sensory characteristics have a confectionery fat filling with 5% peptides produced by enzymatic hydrolysis of whey protein concentrate (CFF+5%WPH-P), followed by control sample (CFF) and fat fillings with 2.5% and 1% peptides produced by enzymatic hydrolysis of whey protein concentrate.

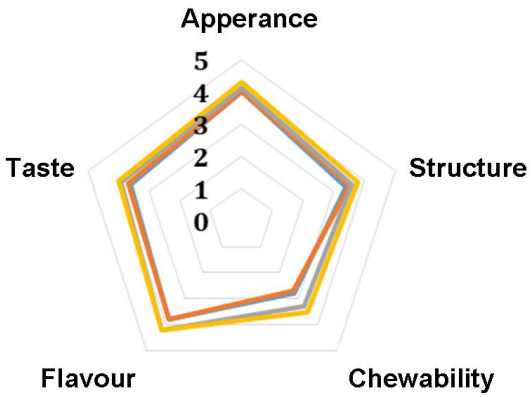

Figure 38. Sensory characteristics of confectionery fat filling supplemented with different concentrations of peptides produced by fermentation of whey proteins.

The addition of hydrolysates obtained by fermentation of whey in fat filling does not lead to significant changes in the sensory quality, especially in the structure of the taste and appearance. As indicated in Fig. 38, there are slight differences in flavor and chewing. The best sensory characteristics have a control sample (CFF), followed by a confectionery fat filling with 5% peptides produced by fermentation of whey proteins (CFF+5%FWH-P) and fat fillings with 2.5% and 1% peptides produced by fermentation of whey proteins that reached a lowest sensory values.

3.4.3.7. Stability of antioxidant activity of confectionery fat filling supplemented with bioactive whey protein hydrolysate powder

Stability during the storage period is the crucial parameter related to achieving and maintaining the functional properties of confectionery fat filling. Changes of the antioxidant activity of fat filling supplemented with 5% WPH-P during the 6 months (180 days) of storage at 20 ± 2 °C, are presented in Fig. 39 The antioxidant activity was

expressed as the inhibitory concentration ($IC_{50,}$ mg mL^{-1}) of the hydrolysate needed to scavenge 50% of the DPPH free radicals.

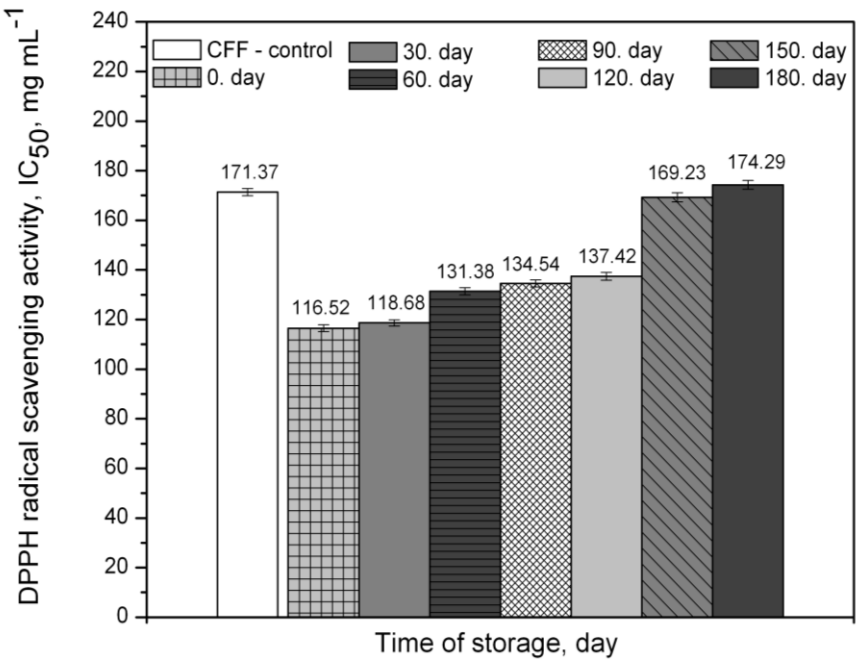

Figure 39. Stability of DPPH scavenging activity of confectionery fat filling supplemented with 5% WPH-P during 180 days of storage at 20 ± 2 °C. Vertical bars represent the standard deviation (n = 3) for each data point.

The obtained results indicate a significant ($p<0.05$) increase in the antioxidant activity of fat filling after the addition of 5%WPH-P (116.52 mg mL^{-1}) compared to the control sample (CFF) with the antioxidant activity of 171.37 mg mL^{-1}. As indicated in Fig. 39, antioxidant activity of fat filling decreases during the whole storage period, reaching the minimal level of 174.29 mg mL^{-1} after 180 days of storage. It is interesting to note that there are three levels of fat filling stability. During the 30 days of storage fat filling supplemented with 5%WPH-P expresses high stable antioxidant activity. In this storage interval, the antioxidant activity of fat filling decreases for only 1.8% compared to the 0 day of storage. In the next three mounts (30-120 days) antioxidant activity of fat filling decreases and reaches a moderate level of at last 137.42 mg mL^{-1} registered at 120. day of storage, which is for 15.2% less than activity registered at 0. day. The observed results suggest that WPH-P still achieves its antioxidant activity and is capable to protects the fat filling from potential free radicals that can be formed during the storage process. After four months, the antioxidant activity of fat filling decreases for

approximately 33.1% and reaches a level of at last 174.29 mg mL^{-1}. Based on the observed results it is evident that after 5-6 months, the antioxidant activity of fat filling drops below the activity registered in the control sample (CFF) without added WPH-P (171.37 mg mL^{-1}). This observation suggests that the antioxidative potential of WPH-P is fully spent, probably on the removal of free radical species formed during the storage of the fat filling. However, it could be suggested that the addition of 5%WPH-P could protect and preserve the fat filling during the four months of storage. In this interval, fat filling expresses high to moderate antioxidant activity. From the presented results, it can be concluded that peptides produced by enzymatic hydrolysis of whey proteins (WPH-P) are a very good option for the supplementation of confectionery fat filling in order to improve its nutritional and functional properties. The antioxidant activity expresses very good stability during the four months of storage, which means that this the optimal way to obtain an enriched product with unchanged nutritional and functional properties during a longer storage period. It is important to emphasize that fat filling represents a very good choice for filling of various confectionery products. Using the enriched fat filling, it is possible to fill products such as napkins, biscuits, chocolate and many others, thus enabling the functional properties of these products to be improved. As this fat filling has proven to be a suitable environment for bioactive peptides, that do not lose their activity during the storage period, it represents a good choice for filling those products that show a decline in bioactivity or stability during storage, or that are not suitable to be enriched with other proteins for any reason.

3.4.4. Conclusion

Based on the results presented in this section, fat filling supplemented with 5% of bioactive whey protein hydrolysate powder (WPH-P) expresses for 32% stronger antioxidative power, which corresponds to the IC_{50} of 116.52 mg mL^{-1}, than control sample CFF (171.37 mg mL^{-1}). This fat filling expresses 2.6 times higher hardness than control sample (CFF), which is very important if the fat filling would be used for the filling of biscuits that demands substantially harder fat filling. Confectionery fat filling supplemented with 5% of WPH-P exhibits the highest Casson viscosity values (6,066 Pa·s), the surface of the thixotropic loop (2,455 $Pa \cdot s^{-1}$) and hardness (1980.7 g), which are certainly not an advantages but can be avoided by a better selection of the

emulsifier. This fat filling can be considered as optimal, from the point of view of particle size distribution and sensory characteristics. In addition, it could be suggested that the addition of 5%WPH-P could protect and preserve the fat filling during the four months of storage. After 120 days of storage antioxidant activity of fat filling corresponds to the IC_{50} of 137.42 mg mL^{-1}, suggesting that WPH-P still achieves its antioxidant activity and is capable to protect the fat filling from potential free radicals that can be formed during the storage process.

4. CONCLUSIONS

This thesis deals with the procedures of microbial fermentation and enzymatic hydrolysis, as two approaches that can be used for the production of whey protein hydrolysates. The main objective of the thesis was to examine the possibility of using different protein substrates, *Lactobacillus* strains and proteolytic enzymes in the production of whey protein hydrolysates that expresses high bioactive potential and are suitable for use in targeted confectionery products. Based on the results presented in this doctoral dissertation the following conclusions can be established:

The results related to the microbial fermentation as a possible approach in the production of bioactive protein hydrolysate revealed that the fermentation of cow's whey using the strain *Lb. rhamnosus* ATCC 7469 represents the appropriate process for the production of bioactive components. Cow's whey fermented with *Lb. rhamnosus* exhibited DPPH scavenging activity of 57.3% as well as FRAP antioxidant activity of 0.862 mmol Fe^{2+}/L. Cow's whey hydrolysate exhibited excellent emulsion activity (98.2%), emulsion stability (68.2%), foam activity (307.2%), digestibility (96.9%) and bioaccessibility (120.6%), as crucial parameters for its application. The suggested strain-substrate combination allows the production of hydrolysate with high antioxidant activity and satisfactory functional properties, as well as the utilization of cow's whey as a waste product that occurs in a very large amount and represents a serious threat to the environment.

The results related to the enzymatic hydrolysis as a possible approach in the production of bioactive protein hydrolysate revealed that tryptic hydrolysis of whey protein concentrate can enable the release of peptides with high antioxidant activity, originating from parent whey proteins as a complex food matrix. A trypsin hydrolysate, with a maximal total antioxidant activity of 80.0%, can be produced after 4h by hydrolysis at 37 °C and an E/S ratio of 0.5%. The trypsin hydrolysate is mainly composed of hydrophilic (polar aprotic) antioxidant species and in addition to its good antioxidant properties, it had an excellent functional properties, such emulsion activity (108.2%), emulsion stability (50.2%), foam activity (327.1%), digestibility (95.9%) and

bioaccessibility (124.6%). These properties indicate it to be a desirable raw material for the food industry that could replace whey proteins as a traditional food supplement for product fortification.

The transformation of the selected hydrolysates into the powders and its comparative analysis related to the bioactive properties revealed that enzymatic hydrolysis represents the optimal process for the production of hydrolysate with pronounced bioactive properties that could be considered as a very promising natural food supplement. The whey protein hydrolysate produced by tryptic digestion, in the amount of at least 178.4 mg mL^{-1} could inhibit processes of lipid peroxidation for 50% as well as microbial contamination caused by *S. aureus* ATCC25923, *B. cereus* ATCC 11778 and *L. monocytogenes*. In addition, the proposed concentration will surely provide antioxidant, anti-arthritic and anti-inflammatory activity greater than 50%. On the other hand, hydrolysate obtained by whey fermentation should be used in a concentration of at least 811.5 mg mL^{-1} to achieve all of the tested bioactivities, with emphasis on significantly more pronounced antimicrobial activity against all tested strains.

In addition to production, the method of administration is also an important segment that contributes to the utilization of all the potentials of the produced bioactive supplement. The detailed analysis related to the application of bioactive whey protein hydrolysate powder in confectionery fat filling revealed that it should be used at the concentration of 5% (w/w). Confectionery fat filling supplemented with 5% of bioactive whey protein hydrolysate (WPH-P) powder expresses for 32% higher antioxidant activity, which corresponds to the IC_{50} of 116.52 mg mL^{-1}, than control sample (CFF) without added hydrolysate (171.37 mg mL^{-1}). This fat filling expresses 2.6 times higher hardness than control sample (CFF) without added hydrolysate, which is very important if the fat filling would be used for the filling of biscuits that demands substantially harder fat filling. Confectionery fat filling supplemented with 5% of WPH-P exhibits high Casson viscosity values (6,066 Pa·s), the surface of the thixotropic loop (2,455 $Pa \cdot s^{-1}$) and hardness (1980.7 g), which are certainly not an advantages, but can be avoided by a better selection of the emulsifier. This combination of fat filling and whey protein hydrolysate powder can be considered as optimal, from the point of view of particle size distribution and sensory characteristics. In addition, it could be suggested that the addition of 5%WPH-P could protect and preserve the fat filling during the four

months (120 days) of storage. During 120 days of storage antioxidant activity of fat filling reaches the minimal level that corresponds to IC_{50} of 137.42 mg mL^{-1}, suggesting that WPH-P still achieves satisfactory antioxidant activity and is capable to protect the fat filling from potential free radicals that can be formed during the storage process.

Based on the review of the previously published experimental data in the literature and the results presented in this doctoral dissertation, a significant contribution has been made in terms of the utilization and processing of whey using the processes of microbial and enzymatic modification of whey proteins. According to the results of detailed characterization, the functionally improved whey protein hydrolysate powder can be produced after 4h by hydrolysis at 37 °C and E/S ratio of 0.5%. The produced hydrolysate exerts remarkable antioxidant activity and functional properties and can be applied in a confectionary fat filling without disturbing its technological properties. The supplemented fat filling can be further used for fortification of a wide range of confectionery products, which greatly contributes to the development of the food industry. The results and conclusions presented in this dissertation are the basis for the further development of functionally improved supplements that can be applied in a wide range of confectionery products.

LIST OF TABLES

LIST OF FIGURES

5. LITERATURE REVIEW

[1] Midmore P., Naspetti S., Sherwood A. M., Vairo D., Wier M., Zanoli R., Consumer attitudes to quality and safety of organic and low input foods: a review. University of Wales, Aberystwyth, UK (2005).

[2] Papas A. M. (1999). Diet and antioxidant status. *Food and Chemical Toxicology* 37, 999-1007.

[3] Young I. S., Woodside J.V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology* 54, 176–86.

[4] Stadtman E. R. (2006). Protein oxidation and aging. *Free radical research* 40, 1250-1258.

[5] Halliwell B. (1997). Antioxidants: the basics – what they are and how to evaluate them. *Advances in Pharmacology* 38, 3-20.

[6] Halliwell B. (1995). How to characterize an antioxidant - An update. *Biochemical Society Symposia* 61, 73–101.

[7] Sies H. (1997). Oxidative stress: oxidants and antioxidants, *Experimental Physiology* 82, 291–5.

[8] Waraho T., McClements D. J., Decker E. A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science and Technology* 22, 3-13.

[9] Barrefors P., Granelli K., Appelqvist L. A., Bjoerck L. (1995). Chemical characterization of raw milk samples with and without oxidative off-flavor. *Journal of Dairy Science* 78, 2691-2699.

[10] Chen J., Lindmark-Månsson H., Gorton L., Åkesson B. (2003). Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods. *International Dairy Journal* 13, 927–935.

[11] Kullisaar T., Zilmer M., Mikelsaar M., Vihalemm T., Annuk H., Kairane C., Kilk A. (2002). Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology* 72, 215–224.

[12] Lopes G. K. B., Schulman H. M., Hermes-Lima M. (1999). Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1472, 142-152.

[13] Yang J. H., Mau J. L., Ko P. T., Huang L. C. (2000). Antioxidant properties of fermented soybean broth. *Food Chemistry* 71, 249–254.

[14] Virtanen T., Pihlanto A., Akkanen S., Korhonen H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of Applied Microbiology* 102, 106–115.

[15] Sugiarto M., Ye A., Singh H. (2009). Characterisation of binding of iron to sodium caseinate and whey protein isolate. *Food Chemistry* 114, 1007–1013.

[16] Hernandez-Ledesma B., Davalos A., Bartolome B., Amigo L. (2005). Preparation of antioxidant enzymatic hydrosylates from a-lactalbumin and b-lactoglobulin. Identification of active peptides by HPLC–MS/MS. *Journal of Agricultural and Food Chemistry* 53. 588–593.

[17] Tong L. M., Sasaki S., McClements D. J., Decker E.A. (2000). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry* 48,1473–1478.

[18] Colbert L. B., Decker E. A. (1991). Antioxidant activity of an ultrafiltration permeate from acid whey. *Journal of Food Science* 56, 1248–1250.

[19] Gauthier S. F., Pouliot Y. (2003). Functional and biological properties of peptides obtained by enzymatic hydrolysis of whey proteins. *Journal of Dairy Science* 86, E78–E87.

[20] Bulatović M., Rakin M., Mojović LJ., Nikolić S., Vukašinović Sekulić M., Đukić Vuković A., (2012). Whey as a raw material for the production of functional beverages. *Chemical Industry*, 66, 567 – 579

[21]Source:https://stats.oecd.org/Index.aspx?DataSetCode=HIGH_AGLINK_2010

[22]Source:https://www.agrarije.com/sites/default/files/Efekti%20liberalizacije%20na%20poljoprivredu%20Srbije.pdf

[23] Tsakali E., Petrotos K., D' Allessandro A., Goulas P. (2010). A review on whey composition and the methods used for its utilization for food and pharmaceutical products Conference: 6th International Conference on Simulation and Modelling in the Food and Bio-Industry FOODSIM 2010, At CIMO Research Centre, Braganca, Portugal.

[24] Carić M., Milanović S., Gavarić D. (1979). Some aspects of industrial whey processing, *Mljekarstvo* 29, 232-236.

[25] Tratnik, Lj. (2003) The role of whey in the production of functional food, *Mljekarstvo* 53, 325-352.

[26] Jeličić I., Božanić R., Tratnik Lj. (2008). Whey-based beverages - a new generation of dairy products, *Mljekarstvo* 58, 257–274.

[27] Kontopidis G., Holt C., Sawyer L. (2004) Invited review: beta-lactoglobulin: binding properties, structure, and function. *Journal of Dairy Science* 87, 785–796.

[28] Ye X., Yoshida S., Ng T. B. (2000). Isolation of lactoperoxidase, lactoferrin, alpha-lactalbumin, beta-lactoglobulin B and beta-lactoglobulin A from bovine rennet whey using ion exchange chromatography. *The International Journal of Biochemistry & Cell Biology* 32, 1143-1150.

[29] Farrell H. M., Jimenez-Flores R., Bleck G. T., Brown E. M., Butler J. E., Creamer L. K., Hicks, C. L., Hollar, C. M. (2004). Nomenclature of the proteins of cows' milk-sixth revision. *Journal of Dairy Science* 87, 1641–1674.

[30] Marshall K. (2004). Therapeutic Applications of Whey Protein, Alternative medicine review, *Journal of Clinical Therapeutic* 9, 136-56.

[31] Morris P. E., Fitzgerald R. J. (2008). Whey Proteins and Peptides in Human Health - Whey Processing, Functionality and Health Benefits, Wiley-Blackwell, IFT Press, 285–343.

[32] Chatterton D. E. W., Smithers G., Roupas P., Brodkorb, A. (2006). Bioactivity of β-lactoglobulin and α-lactalbumin β-Technological implications for processing. *International Dairy Journal* 16, 1229–1240.

[33] Heine W. E., Klein P. D., Reeds P. J. (1991). The importance of alpha-lactalbumin in infant nutrition, *The Journal of Nutrition* 121, 277-283.

[34] Haque E., Chand R. (2006). Milk protein derived bioactive peptides. URL: http://www.dairyscience.info/bio-peptides.htm.

[35] Haque E., Chand R., Kapila, S. (2009). Biofunctional properties of bioactive peptides of milk origin. *Food Reviews International* 25, 28–43.

[36] Sousa G. T., Lira F. S., Rosa J. C., de Oliveira E. P., Oyama L. M., Santos R. V., Pimentel G.D. (2012). Dietary whey protein lessens several risk factors for metabolic diseases: a review. *Lipids in Health and Diseases* 11, 67.

[37] Tavares T. G, Malcata F. X. (2013). Whey Proteins as Source of Bioactive Peptides Against Hypertension, Dr. Blanca Hernández-Ledesma (Ed.), Bioactive Food Peptides in Health and Disease, InTech,182 :https://www.intechopen.com/books/bioactive-food-peptides-in-health-anddisease/whey-proteins-as-source-of-bioactive-peptides-against

[38] Madureira A. R., Pereira C. I., Gomes A. M. P., Pintado M. E., Malcata F.X. (2007). Bovine whey proteins – Overview on their main biological properties, *Food Research International* 40, 1197–1211.

[39] Requena P., Daddaoua A., Guadix E., Zarzuelo A., Suárez M. D., Sánchez de Medina F., Martínez-Augustin O. (2009). Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-kappaB signal transduction pathways. *British Journal of Pharmacol*ogy 157,1232–1240.

[40] Gobbetti M., Minervini F., Rizzello C.G. (2007). Bioactive peptides in dairy products, in: Hui, Y.H., Chandan, R., Clark, S., Cross N.A. (Eds.), Handbook of Food Products

Manufacturing: Health, Meat, Milk, Poultry, Seafood, and Vegetables. John Wiley & Sons, Hoboken, New Jersey, 489–517.

[41] Herceg Z., Režek A. (2006). Nutritional and functional properties of the whey protein concentrate and isolate. *Mljekarstvo* 56, 379-396.

[42] Wouters A. G. B., Rombouts I., Fierens E., Brijs K., Delcou J. A. (2016). Relevance of the Functional Properties of Enzymatic Plant Protein Hydrolysates in Food Systems. *Comprehensive Reviews in Food Science and Food Safety* 15, 786–800.

[43] Rinderknecht H. (1993). Pancreatic secretory enzymes. *The pancreas, biology, pathobiology, and disease* 219–251.

[44] Walsh G. (2005). "Therapeutic insulins and their large-scale manufacture," *Applied Microbiology and Biotechnology* 67, 151–159.

[45] Dunn B. M. (2001). Overview of pepsin-like aspartic peptidases. *Current Protocols in Protein Science*. 21 doi:10.1002/0471140864.ps2103s25.

[46] Papachristodoulou D., Snape A., Elliott W. H., Elliott D. C. (2014). *Biochemistry and molecular biology*. Oxford University Press.

[47] Dunn B. M. (2002). Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chemical Reviews* 102, 4431–4458.

[48] Fisher E. H. (1956) Structure of Enzyme Loci in: Seventeenth Annual Biology Colloquium Friday and Saturday, 36.

[49] Bulatović M. (2015). Production and characteristics of functional fermented whey based beverages. PhD Thesis, Faculty of Technology and Metallurgy, University of Belgrade.

[50] Terzic-Vidojevic A., Mihajlovic S., Uzelac G., Veljovic K., Tolinacki M., Nikolic M., Topisirovic Lj., Kojic M. (2014). Characterization of lactic acid bacteria isolated from artisanal Travnik young cheeses, sweet creams and sweet kajmaks over four seasons. *Food Microbiology* 39, 27–38.

[51] Pescuma M., Hebert E. M., Mozzi F., de Valdez G. F. (2008). Whey fermentation by thermophilic lactic acid bacteria: Evolution of carbohydrates and protein content. *Food Microbiology* 25, 442–451.

[52] Bulatović M., Krunić T.,Vukašinović-Sekulić M., Zarić D., Rakin M. (2014). Quality attributes of a fermented whey-based beverage enriched with milk and a probiotic strain, *RSC Advances* 4, 55503–55510.

[53] Miclo L., Roux E., Genay M., Brusseaux E., Poirson C., Jameh N., Perrin C., Dary A. (2012). Variability of hydrolysis of β-, αs1-, and αs2-caseins by 10 strains of Streptococcus thermophilus and resulting bioactive peptides. *Journal of. Agricultural and. Food Chemistry* 60, 554–565.

[54] Hrnjez D. (2015) Biological activity of fermented dairy products obtained using kombucha and conventional starter cultures, PhD Thesis, Faculty of Technology, Univeristy of Novi Sad.

[55] Paltroineri P. (2012). Whey: Types, Composition and Health Implications, Chapter: Bioactive Peptides with Health Benefit and their Differential Content in: Whey of Different Origin, Publisher: Nova, Hauppauge, NY, Editors: Benitez RM, Ortero GM, pp.153-168.

[56] Korhonen H., Pihlanto A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current pharmaceutical design* 9, 1297-1308.

[57] Christensen, Jeffrey E. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 76, 217-246.

[58] Lukić J. R. (2015). Improvement of functional characteristics of fermented whey based beverage by the addition of bioactive peptides. Master thesis, Faculty of Technology and Metallurgy, University of Belgrade.

[59] Pennings B., Groen B., de Lange A., Gijsen A. P., Zorenc A. H., Senden J. M., van Loon L. J. (2012). Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *American Journal of Physiology. Endocrinology and Metabolism* 15, E992-999.

[60] Urso M. L., Clarkson P. M. (2003). Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* 189, 41-54.

[61] Jisha S., Padmaja G. (2011). Whey protein concentrate fortified baked goods from cassava- based Composite Flours: Nutritional and functional properties. *Food and Bioprocess Technology* 4, 92-101.

[62] Wronkowska M., Jadacka M., Soral-Smietana M., Zander L., Dejnowiec F., Banaszczyk P., Jelinski T., Szmatowicz B. (2015). ACID whey concentrated by ultrafiltration a tool for modeling bread properties. *LWT-Food Science and Technology* 61, 172–176.

[63] Park Y. W., Nam M. S. (2015). Bioactive peptides in milk and dairy products: a review. *Korean Journal for Food Science of Animal Resources* 35, 831.

[64] Konar N., Toker O. S., Oba S., Sagdic O. (2016). Improving functionality of chocolate: A review on probiotic, prebiotic, and/or synbiotic characteristics. *Trends in Food Science and Technology* 49, 35-44.

[65] Laličić-Petronijević J., Popov-Raljić J., Obradović D., Radulović Z., Paunović D., Petrušić M., Pezo L. (2015). Viability of probiotic strains *Lactobacillus acidophilus* NCFM® and *Bifidobacterium lactis* HN019 and their impact on sensory and rheological properties of milk and dark chocolates during storage for 180 days. *Journal of Functional Foods* 15, 541-550.

[66] Żyżelewicz D., Nebesny E., Motyl I., Libudzisz Z. (2010). Effect of milk chocolate supplementation with lyophilised *Lactobacillus* cells on its attributes. *Czech Journal of Food Sciences* 28, 392-406.

[67] Serafini M., Crozier A. (2003). Nutrition: milk and absorption of dietary flavanols. *Nature* 426, 788-788.

[68] Tabernero M., Serrano J., Saura-Calixto F. (2006). The antioxidant capacity of cocoa products: contribution to the Spanish diet. *International Journal of Food Science and Technology* 41, 28-32.

[69] Zarić D., Bulatović M., Rakin M., Krunić T., Lončarević I., Pajin B. (2016). Functional, rheological and sensory properties of probiotic milk chocolate produced in a ball mill, *RSC Advances* 6, 13934–13941.

[70] Andre-Frei V., Perrier E., Augustin C., Damour O., Bordat P., Schumann K., Förster T., Waldmann-Laue M. (1999). A comparison of biological activities of a new soya biopeptide studied in an in vitro skin equivalent model and human volunteers. *International Journal of Cosmetic Science* 21, 299-311.

[71] Sudel K. M., Venzke K., Mielke H., Breitenbach U., Mundt C., Jaspers S., Koop U., Sauermann K., Knussman-Hartig E., Moll I., Gercken G., Young A. R., Stäb F., Wenck H., Gallinat S. (2005). Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract. *Photochemistry and Photobiology* 81, 581–587.

[72] Audic J. L., Chaufer B., Daufin G. (2003). Non-Food Applications of Milk Components and Dairy Co-Products: A Review. *Le Lait* 83, 417-438.

[73] Balakrishnan G., Agrawal R. (2014). Antioxidant activity and fatty acid profile of fermented milk prepared by Pediococcus pentosaceus. *Journal of Food Science and Technology* 51, 4138-4142.

[74] Pavlović M. D., Buntić A. V., Šiler-Marinković S. S., Dimitrijević-Branković S. I. (2013). Ethanol influenced fast microwave-assisted extraction for natural antioxidants obtaining from spent filter coffee. *Separation and Purification Technology* 118, 503–510.

[75] Pearce K. N., Kinsella J. E. (1978). Emulsifying Properties of Proteins: Evaluation of a Turbidimetric Technique. *Journal of Agricultural and Food Chemistry* 26, 716–723.

[76] Chobert J. M., Bertrand-Harb C., Nicolas M. G. (1988). Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin. *Journal of Agricultural and Food Chemistry* 36, 883–892.

[77] Firebaugh J. D., Daubert C. R. (2005). Emulsifying and foaming properties of derivatized whey protein ingredient. *International Journal of Food Properties* 8, 243–253.

[78] Sathe S. E., Salunkhe D. K. (1981). Functional properties of the great Northem bean proteins: emulsion, foaming, viscosity and gelation properties. *Journal of Food Science* 46, 71–75.

[79] Laparra J. M., Velez D., Montoro R., Barbera R., Farre R. (2003). Estimation of arsenic bioaccessibility in edible seaweed by an in vitro digestion method. *Journal of Agricultural and Food Chemistry* 51, 6080–6085.

[80] Bradford M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72**,** 248–254.

[81] Rodríguez-Roque M. J., Rojas-Graü M. A., Elez-Martínez P., Martín-Belloso O. (2013). Soymilk phenolic compounds, isoflavones and antioxidant activity as affected by in vitro gastrointestinal digestion. *Food Chemistry* 136, 206–212.

[82] Chen J., Lindmark-Månsson H., Gorton L., Åkesson B. (2003). Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods. *International Dairy Journal* 13, 927–935.

[83] Kullisaar T., Zilmer M., Mikelsaar M., Vihalemm T., Annuk H., Kairane C., Kilk A. (2002). Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology* 72, 215–224.

[84] Lopes G. K. B., Schulman H. M., Hermes-Lima M. (1999). Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1472, 142-152.

[85] Yang J. H., Mau J. L., Ko P. T., Huang L. C. (2000). Antioxidant properties of fermented soybean broth. *Food Chemistry* 71, 249–254.

[86] Virtanen T., Pihlanto A., Akkanen S., Korhonen H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of Applied Microbiology* 102, 106–115.

[87] Cagnoa R. D., Quintob M., Corsettic A., Minervinia F., Gobbettia. (2006). Assessing the proteolytic and lipolytic activities of single strains of mesophilic lactobacilli as

adjunct cultures using a Caciotta cheese model system. *International Dairy Journal* 16, 119-130.

[88] Najgebauer-Lejko D., Grega T., Tabaszewska M. (2014). Yoghurts with addition of selected vegetables: acidity, antioxidant properties and sensory quality. Acta scientiarum polonorum. *Technologia alimentaria* 13, 35-42.

[89] Yadav D., Vij S., Hati S., Singh B. P., Dhanday M., Dahiya M., Vandna V. (2012). Evaluation of Total Antioxidantxidant Activity of Soy Yoghurt. *Indian Journal of Dairy Science* 65, 220-224.

[90] Najgebauer-Lejko D., Sady M. (2015). Estimation of the antioxidant activity of the commercially available fermented milks. Acta scientiarum polonorum. *Technologia alimentaria* 14, 387–396.

[91] Luhovyy B. L., Akhavan T., Anderson G. H. (2007). Whey Proteins in the Regulation of Food Intake and Satiety. *Journal of the American College of Nutrition* 26, 704S–712S.

[92] Gauthier S. F., Pouliot Y. (2003). Functional and biological properties of peptides obtained by enzymatic hydrolysis of whey proteins. *Journal of Dairy Science* 86, E78–E87.

[93] Mahmoud M. I. (1994). Physicochemical and functional properties of protein hydrosylates in nutritional products. *Food Technology* 48, 89-95.

[94] Krešic G., Lelas V., Herceg Z., Režek A. (2006). Effects of high pressure on functionality of whey protein concentrate and whey protein isolate. *Lait* 86**,** 303-315.

[95] Walstra P. (2002). *Physical chemistry of foods*. New York: Marcel Dekker Inc.

[96] Van der Ven C. (2002). Biochemical and functional characterisation of casein and whey protein hydrolysates: a study on the correlations between biochemical and functional properties using multivariate data analysis. PhD Thesis, Wageningen: Wageningen University.

[97] Varka E. M., Ampatzidis C., Kostoglou M., Karapantsios T., Dutschk V. (2010). On the use of electrical conductance measurements for the stability of oil-in-water

Pickering emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 365, 81–188.

[98] Mujun L., Guoping Y., Yuxiu Y., Peipei G., Wei-wei Q. I., Xing-hang C. (2015). Solubility, Free Amino and Freeze-Thaw Stability of Soy Protein Isolate Prepared by Papain Modification. *International Conference on Materials Chemistry and Environmental Protection*.

[99] Adler-Nissen J. (1986). *Enzymic hydrolysis of food proteins*. London: Elsevier Applied Science.

[100] Lie S. (1973). The EBC-Ninhydrin method for determination of free alpha amino nitrogen. *Journal of the Institute of Brewing* 79**,** 37–41.

[101] El-Shourbagy G A and El-Zahar K M (2014) Oxidative stability of ghee as affected by natural antioxidants extracted from food processing wastes. *Annals of Agricultural Sciences* 59, 213–220.

[102] McCue P. P., Shetty K. (2005). Phenolic antioxidant mobilization during yogurt production from soymilk using kefir cultures. *Process Biochemistry* 40, 1791–1797.

[103] Kim S. B., Ki K. S., Khan M. A., Lee W. S., Lee H. J., Ahn B. S., Kim H. S. (2007). Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *Journal of Dairy Science* 90, 4043–4050.

[104] Mohan A., Udechukwu M. C., Rajendran S. R. C. K. and Udenigwe C. C. (2015). Modification of peptide functionality during enzymatic hydrolysis of whey proteins. *RSC Advances* 5, 97400-97407.

[105] Kella N. K. Kinsella J. E. (1988). Enhanced thermodynamic stability of ß-lactoglobulin at low pH: A possible mechanism. *Biochemical Journal* 255, 113–118.

[106] Monti J. C., Jost R. (1978). Enzymatic solubilization of heat denatured cheese whey protein. *Journal of Dairy Science* 61, 1233–1237.

[107] Mullally M. M., Meisel H., FitzGerald R. J. (1997). Angiotensin-I converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *International Dairy Journal* 7, 299–303.

[108] Tran B. Q., Hernandez C., Waridel P., Potts A., Barblan J., Lisacek F., Quadroni M. (2011). Addressing trypsin bias in large scale (phospho) proteome analysis by size exclusion chromatography and secondary digestion of large post-trypsin peptides. *Journal of Proteome Research* 10, 800–811.

[109] Ahn J., Cao M. J., Yu Y. Q., Engen J. R. (2013). Accessing the Reproducibility and Specificity of Pepsin and other Aspartic Proteases. *Biochimica et biophysica acta* 1834, 1222–1229.

[110] Stefanović A. B., Jovanović J. R., Grbavčić S. Ž., Šekuljica N. Ž., Manojlović V. B., Bugarski B. M., Knežević-Jugović Z. D. (2014). Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production. *European Food Research and Technology* 239, 979–993.

[111] Morais H. A., Silvestre M. P., Silva V. D., Silva M. R., Simões e Silva A. C., Silveira J. N. (2013). Correlation between the degree of hydrolysis and the peptide profile of whey protein concentrate hydrolysates: effect of the enzyme type and reaction time. *American Journal of Food Technology* 8, 1–16.

[112] Silvestre M. P., Silva M. C., Silva V. D., Silva M. R., Amorin L. L. (2011). WPC hydrolysates obtained by the action of pancreatin: preparation, analysis and phenylalanine removal. *Asian Journal of Scientific Research* 4**,** 302–314.

[113] Tong L. M., Sasaki S., McClements D. J., Decker E. A. (2000). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry* 48, 1473–1478.

[114] Sugiarto M., Ye A., Singh H. (2009). Characterisation of binding of iron to sodium caseinate and whey protein isolate. *Food Chemistry* 114, 1007–1013.

[115] Colbert L. B., Decker E. A. (1991). Antioxidant activity of an ultrafiltration permeate from acid whey. *Journal of Food Science* 56, 1248–1250.

[116] Baublis A., Decker E. A., Clydesdale F. M. (2000). Antioxidant effect of aqueous extracts from wheat based ready-to-eat breakfast cereals. *Food Chemistry* 68, 1–6.

[117] Whitaker J. R. (1993). *Principles of enzymology for the food sciences*. New York: Marcel Decker Inc.

[118] Pommié C., Levadoux S., Sabatier R., Lefranc G., Lefranc M. P. (2004). IMGT standardized criteria for statistical analysis of immunoglobulin V-REGION amino acid properties. *Journal of Molecular Recognition* 17**,** 17–32.

[119] Barrett A J, Woessner J F and Rawlings N D (2012) *Handbook of proteolytic enzymes*. London: Elsevier Academic Press.

[120] Suju L., Weina T., He L., Jiankang C., Weibo J. (2012). Improving antioxidant activities of whey protein hydrolysates obtained by thermal preheat treatment of pepsin, trypsin, alcalase and flavourzyme. *International Journal of Food Science and Technology* 47, 2045–2051.

[121] Hernandez-Ledesma B., Davalos A., Bartolome B., Amigo L. (2005). Preparation of antioxidant enzymatic hydrosylates from a-lactalbumin and b-lactoglobulin. Identification of active peptides by HPLC–MS/MS. *Journal of Agricultural and Food Chemistry* 53, 588–593.

[122] Walstra P. (2002). *Physical chemistry of foods*. New York: Marcel Dekker Inc.

[123] Van der Ven C. (2002). Biochemical and functional characterisation of casein and whey protein hydrolysates: a study on the correlations between biochemical and functional properties using multivariate data analysis. PhD Thesis, Wageningen: Wageningen University.

[124] Varka E. M., Ampatzidis C., Kostoglou M., Karapantsios T., Dutschk V. (2010). On the use of electrical conductance measurements for the stability of oil-in-water Pickering emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 365, 181–188.

[125] Liu M. J., Yu G. P., Yao Y. X., Guo P. P., Qi W. W., Cai X. H. (2015). Solubility, Free Amino and Freeze-Thaw Stability of Soy Protein Isolate Prepared by Papain Modification. 1st International Conference on Materials Chemistry and Environmental Protection. Paris: Atlantis Press.

[126] Foegeding E. A., Davis J. P., Doucet D., McGuffey M. K. (2002). Advances in modifying and understanding whey protein functionality. *Trends in Food Science and Technology* 13, 151–159.

[127] Clemente A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology* 11, 254-262.

[128] Hartmann R., Meisel H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology* 18, 163–169.

[129] Azuma K., Nakayama N., Koshioka M., Ippoushi K., Yamaguchi Y., Kohata K., Yamauchi Y., Ito H., Higashio H. (1999). Phenolic antioxidants from the leaves of Corchorus olitorius L. *Journal of Agricultural and Food Chemistry* 47, 3963-3966.

[130] Dam Arawwawalaa L. D. A. M., Arambewelaa L. S. R., Ratnasooriya W. D. (2012). Alpinia calcarata Roscoe: A potent antiinflammatory agent. *Journal of Ethnopharmacology* 139, 889-892.

[131] Govindappa M., Naga Sravya S., Poojashri M. N., Sadananda T. S., Chandrappa C. P. (2011). Antimicrobial, antioxidant and in vitro anti-inflammatory activity of ethanol extract and active phytochemical screening of Wedelia trilobata (L.) Hitchc. *J Pharmacognosy and Phytotherapy* 3, 43-51.

[132] Zhang Q., Wu H., Ling Y., Lu R. (2013). Isolation and identification of antioxidant peptides derived from whey protein enzymatic hydrolysate by consecutive chromatography and Q-TOF MS. *Journal of Dairy Research* 80: 367-373.

[133] Mojica L., de Mejía E. G. (2016). Optimization of enzymatic production of anti-diabetic peptides from black bean (Phaseolus vulgaris L.) proteins, their characterization and biological potential. *Food and Function* 7: 713-727.

[134] Virtanen T., Pihlanto A., Akkanen S., Korhonen H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of Applied Microbiology* 102**,** 106-115.

[135] Niki E., Yoshida Y., Saito Y., Noguchi N. (2005). Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications* 338, 668-676.

[136] Peng X., Kong B., Xia X., Liu Q. (2010). Reducing and radical-scavenging activities of whey protein hydrolysates prepared with Alcalase. *International Dairy Journal* 20, 360-365.

[137] [41] Breiteneder H, Mills ENC. Molecular properties of food allergens. *J Allergy Clin Immunol Pract.* 2005; 115: 14-23.

[138] Tsabouri S, Douros K, Priftis, KN. Cow Milk Allergenicity. *Endocr Metab Immune Disord Drug Targets.* 2014; 14: 16–26.

[139] Vane J.R., Bolting R.M. (1995). New insights into the mode of action of anti-inflammatory drugs. *Inflamatory Research* 44, 1-10.

[140] Chou C. T. (1997). The Antiinflammatory Effect of an Extract of Tripterygium wilfordii Hook F on Adjuvant-induced Paw Oedema in Rats and Inflammatory Mediators Release. *Phytotherapy Research* 11, 152-154.

[141] Iskandar M. M., Dauletbaev N., Kubow S., Mawji N., Lands L. C. (2013). Whey protein hydrolysates decrease IL-8 secretion in lipopolysaccharide (LPS)-stimulated respiratory epithelial cells by affecting LPS binding to Toll-like receptor 4. *British Journal of Nutrition* 110, 58-68.

[142] Piccolomini A. F., Iskandar M. M., Lands L. C., Kubow S. (2012). High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food Nutrition Research* 56: doi: 10.3402/fnr.v56i0.17549.

[143] Shimizu N., Dairiki K., Ogawa S., Kaneko T. (2006). Dietary whey protein hydrolysate suppresses development of atopic dermatitis-like skin lesions induced by mite antigen in NC/Nga mice. *Allergology International* 55, 185-189.

[144] Hatori M., Ohki K., Hirano S., Yang X. P., Kubok H., Abe C. (2008). Effects of a casein hydrolysate prepared from Aspergillus oryzae protease on adjuvant arthritis in rats. *Bioscence,i Biotechnology, and Biochemistry* 72, 1983-1991.

[145] Pescuma M., Espeche Turbay M. B., Mozzi F., de Valdez G. F., de Giori G. S., Hebert E. M. (2013). Diversity in proteinase specificity of thermophilic lactobacilli as revealed by hydrolysis of dairy and vegetable proteins. *Applied Microbiology and Biotechnology* 97, 7831-7844.

[146] Théolier J., Hammami R., Labelle P., Fliss I., Jean J. (2013). Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. *Journal of Functional Foods* 5, 706-714.

[147] Pellegrini A., Dettling C., Thomas U., Hunziker P. (2001). Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochimica et Biophysica Acta* 1526, 131-140.

[148] Cascales E., Buchanan S. K, Duche D., Kleanthous C., Lloubes R., Postle K., Riley M., Slatin S., Cavard D. (2007). Colicin biology. *Microbioogy andl Molecular Biology Review* 71, 158-229.

[149] Abriouel H., Franz C. M., Ben Omar N., Galvez A. (2011). Diversity and applications of Bacillus bacteriocins. *FEMS Microbiology Reviews* 35, 201-232.

[150] Tenovuo J. (2002). Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: efficacy and safety. *Oral diseases* 8, 23-29.

[151] Gilliland S. E., Speck M. L. (1977). Antagonistic action of Lactobacillus acidophilus toward intestinal and foodborne pathogens in associative cultures. *Journal of Food Proection.* 40, 820-823.

[152] Warny M., Fatimi A., Bostwick E. F., Laine D. C., Lebel F., LaMont J. T., Pothoulakis C., Kelly C. P. (1999). Bovine immunoglobulin concentrate-clostridium difficile retains C difficile toxin neutralising activity after passage through the human stomach and small intestine. *Gut* 44, 212-217.

[153] Olorunfemi O. B., Adebolu T. T., Adetuyi F. C. (2006). Antibacterial Activities of Micrococcus lactis strain isolated from Nigerian fermented cheese whey against Diarrhoea causing organisms. *Research Journal of Biological Sciences* 1, 24-27.

[154] Sagong H. G., Lee S. Y., Chang P. S., Heu S., Ryu S., Choi Y. J., Kang D. H. (2011). Comed effect of ultrasound and organic acids to reduce Escherichia coli O157: H7, Salmonella Typhimurium, and Listeria monocytogenes on organic fresh lettuce. *International Journal of Food Microbiology* 145, 287-292.

[155] AOAC, Official Methods of Analysis, 17th ed., Associationof Official Analytical Chemists, Maryland, USA, 2000

[156] McCue P. P., Shetty K. (2005). Phenolic antioxidant mobilization during yogurt production from soymilk using kefir cultures. *Process Biochemistry* 40**,** 1791–1797.

[157] IOCCC (2000). Viscosity of cocoa and chocolate products. Analytical Method 46-2000., International Office of Cocoa, Chocolate and Confectionary, Geneva.

[158] De Pelsmaeker S., Gellynck X., Delbaere C., Declercq N., Dewettinck K. (2015). Consumer-driven product development and improvement combined with sensory analysis: A case-study for European filled chocolates. *Food quality and preference* 41, 20-29.

[159] Pajin B. (2014). Tehnologija čokolade i kakao proizvoda, Tehnološki fakultet Novi Sad, Univerzitet u Novom Sadu.

[160] Bueschelberger H. G. (2004). Lecithins. In: Emulsifiers in Food Technology. Editor: R.J. Whitehurst, pp.18., Northampton, Blackwell.

[161] Lončarević I., Fišteš A., Rakić D., Pajin B., Petrović J., Torbica A., Zarić D. (2017). Optimization of the ball mill processing parameters in the fat filling production, *Chemical Industry and Chemical Engineering Quarterly* 23, 197−206.

BIOGRAPHY

Salem Mohamed Embiriekah was born on May 25, 1972. in Iddabia, Libya, where he finished basic and high school. He graduated Chemistry (Bachelor level) at Faculty of Science, University of Misurata, on 1995. He finished Master degree in Scientific Research, at Chemistry Department of the Faculty of Science, University of Misurata, at year 2009. In the period 2009-2012 he was a lecturer on the Analytical Chemistry subject at the Faculty of Science, University of Misurata. In the period 2011-2013, Salem Embiriekh conducted theoretical and practical lessons from the Biochemistry and Organic Chemistry course at the Faculty of Education in Libya.

University of Belgrade has conducted the process of recognition and evaluation of the study program and performed the nostrification on June 03, 2014, and issued a document in the name of Salem Mohamed Embiriekh for the second level of higher education obtained at the University of Misurata, Libya.

In the 2012/2013 he started his doctoral studies at Faculty of Technology and Metallurgy, University of Belgrade, at Department of Biochemical Engineering and Biotechnology, under the direction of supervisor prof. dr Marica Rakin, full profesor. All exams under the plan and program of the studies, he passed with an average value of 9.58. He speaks and writes English.

BIOGRAFIJA

Salem Mohamed Embiriekah je rođen 25. maja 1972. godine u Libiji gde je završio osnovnu i srednju školu. Diplomirao na studijskom programu Hemija na Fakultetu prirodnih nauka, Univerziteta u Misurati 1995. godine. Master akademske studije je završio na hemijskom odseku Prirodno-matematičkog fakulteta, Univerziteta u Misurati 2009. godine. U periodu 2009-2012 godine bio je predavač na predmetu Analitička hemija na Prirodno-matematičkom fakultetu Univerziteta u Misurati. U periodu od 2011. do 2013. godine, Salem Embiriekh je izvodio teorijsku i praktičnu nastavu na predmetima Biohemija i Organska Hemija na Pedagoškom fakultetu u Libiji.

Univerzitet u Beogradu sproveo je proces priznavanja i evaluacije studijskog programa, izvršio nostrifikaciju 03. juna 2014. godine i izdao dokument na ime Salem Mohameda Embiriekha za drugi nivo visokog obrazovanja stečen na Univerzitetu u Misurati, Libija.

U periodu 2012/2013 započeo je doktorske studije na Tehnološko-metalurškom fakultetu Univerziteta u Beogradu, na odseku za biohemijsko inženjerstvo i biotehnologiju, pod rukovodstvom prof. dr Marica Rakin, redovniog profesora Tehnološko-metalurškog fakulteta, Univerziteta u Beogradu. Sve ispite predviđene planom i programom doktorskih akademskih studija položio je sa prosečnom ocenom 9,58. Govori i piše engleski.

Prilog 1.

ИЗЈАВА О АУТОРСТВУ

Име и презиме аутора: Salem Mohamed Embiriekah

Број индекса: 4051/2013

Изјављујем

да је докторска дисертација под насловом

„Funkcionalna svojstva i primena hidrolizata proteina surutke dobijenih biotehnološkim putem“ („Functional properties and possibility of application of whey protein hydrolysates obtained by biotechnological processing“)

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

Потпис аутора

У Београду, _____

_____

Prilog 2.

ИЗЈАВА О ИСТОВЕТНОСТИ ШТАМПАНЕ И ЕЛЕКТРОНСКЕ ВЕРЗИЈЕ ДОКТОРСКОГ РАДА

Име и презиме аутора: Salem Mohamed Embiriekah

Број индекса: 4051/2013

Студијски програм: Biohemijsko inženjerstvo i biotehnologija

Наслов рада:

„Funkcionalna svojstva i primena hidrolizata proteina surutke dobijenih biotehnološkim putem“ („Functional properties and possibility of application of whey protein hydrolysates obtained by biotechnological processing“)

Ментор: dr Marica Rakin, red. prof

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, _____

_____

Prilog 3.

ИЗЈАВА О КОРИШЋЕЊУ

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

„Funkcionalna svojstva i primena hidrolizata proteina surutke dobijenih biotehnološkim putem“ („Functional properties and possibility of application of whey protein hydrolysates obtained by biotechnological processing“)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство (CC BY)
2. Ауторство – некомерцијално (CC BY-NC)
3. Ауторство – некомерцијално – без прерада (CC BY-NC-ND)
4. Ауторство – некомерцијално – делити под истим условима (CC BY-NC-SA)
5. Ауторство – без прерада (CC BY-ND)
6. Ауторство – делити под истим условима (CC BY-SA)

(Молимо да заокружите само једну од шест понуђених лиценци.

Кратак опис лиценци је саставни део ове изјаве).

Потпис аутора

У Београду, _____