ZAKLJUČNA KONFERENCA PROJEKTA
LIFE for Acid Whey

CLOSURE CONFERENCE OF THE PROJECT
LIFE for Acid Whey

Napredni načini izrabe potenciala sirotke: prehod iz okoljskega problema v dragocen naravni vir

Advanced ways to exploit the potential of whey: the transition from an environmental problem to a valuable natural resource










Zbornik konference
15. februar 2022
Four Points by Sheraton Ljubljana Mons
Ljubljana, Slovenija

Proceedings of the Conference
February 15, 2022
Four Points by Sheraton Ljubljana Mons
Ljubljana, Slovenia

LIFE FOR ACID WHEY
LIFE16 ENV/SI/000335 LIFE for Acid Whey

INŠTITUT ZA MLEKARSTVO IN PROBIOTIKE
INSTITUTE OF DAIRY SCIENCE & PROBIOTICS

ARHEL
projektiranje in inženiring d.o.o.

Zaključna Konferenca projekta LIFE for Acid Whey
Napredni načini izrabe potenciala sirotke: prehod iz okoljskega problema v dragocen naravni vir
Zbornik konfrence
15. februar 2022, Arhel d.o.o., Pustovrhova 15, 1210 Ljubljana-Šentvid

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Proceedings of the Conference
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PROGRAM KONFERENCE

CONFERENCE PROGRAMME

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15. februar 2022, Four Pionts by Sheraton Ljubljana Mons, Ljubljana
V organizaciji podjetja Arhel d.o.o. in Inštituta za mlekarstvo in probiotike

PROGRAM PREDAVANJ

	Predavatelj	Ustanova	Naslov predavanja
8:00-9:00			Registracija in jutranja kava
9:00-9:10	Nataša Poklar Ulrih	Univerza v Ljubljani, Biotehniška fakulteta	**Otvoritev, pozdravni nagovor**
UVOD, STANJE V SLOVENIJI, TEHNOLOŠKE REŠITVE			
9:10-9:25	Maja Zupančič Justin	Arhel d.o.o.	**Sirotka: okoljski problem ali dragocen naravni vir – izhodišča projekta LIFE for Acid Whey in LAKTIKA**
9:30-9:50	Barbara Rupnik	Gospodarska zbornica Slovenije	**Sirotka v slovenski mlekarski industriji**
9:55-10:10	Svetlana Vidovič, Robert Berce	Bering d.o.o.	**Procesne tehnologije za predelavo sirotke: izzivi in rešitve za mlekarsko industrijo, izdelovalce surovin in biotehnološka podjetja**
10:15-10:30	Irena Petrinič	Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo	**Preizkušanje različnih gonilnih raztopin s procesom osmoze za koncentriranje kisle sirotke**
10:35-10:50	Sara Drmota Prebil	BIA *Separations d.o.o.*	**Uporaba kromatografskih postopkov na monolitnih kolonah za izolacijo proteinov in drugih bioaktivnih molekul v živilski industriji**
10:55-11:10			Odmor za kavo
SIROTKINI PROTEINI, INDUSTRIJSKA IZOLACIJA IN PRIDOBIVANJE			
11:10-11:30	Jernej Oberčkal	Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike	**Hranilne, funkcionalne in bioaktivne lastnosti sirotkinih proteinov ter z njimi povezane rabe**
11:35-11:55	Marko Kete	Arhel d.o.o.	**Razvoj metode in optimiziranega industrijskega postopka izolacije laktoferina**
12:00-12:15	Nika Osel	Univerza v Ljubljani, Fakulteta za farmacijo	**Razvoj analiznih metod za vrednotenje laktoferina in izbranih sirotkinih proteinov**

	Predavatelj	Ustanova	Naslov predavanja
12:20-12:35	Blaž Grilc	Univerza v Ljubljani, Fakulteta za farmacijo	**Razvoj farmacevtske formulacije z laktoferinom za ohranjanje zdravega ravnovesja črevesne mikrobiote**
12:40-12:55	Špela Gruden	Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo	**Protimikrobna aktivnost peptidov, pridobljenih z encimsko hidrolizo laktoferina**
13:00-14:30			Malica, ogled posterjev
BIOTEHNOLOŠKA IZRABA SIROTKE			
14:30-14:50	Bojana Bogovič Matijašić	Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike	**Biotehnološka uporaba sirotke**
14:55-15:10	Diana Paveljšek	Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike	**Izkoriščanje kisle sirotke za gojenje mlečnokislinskih bakterij in pridobivanje koristnih metabolitov**
15:15-15:30	Romana Marinšek Logar	Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za mikrobiologijo	**Pridobivanje bioplina iz sirotke**
15:35-15:50	Irena Barukčić	Univerza v Zagrebu, Fakulteta za živilsko tehnologijo in biotehnologijo	**Sirotka kot dragocen vir funkcionalnih napitkov**
15:55-16:10	Maja Bjelošević	Univerza v Ljubljani, Fakulteta za farmacijo	**Uporaba sirotke v kozmetologiji: primeri kozmetičnih izdelkov**
16:10 – 17:00			Druženje, ogled posterjev, zaključek

ZAKLJUČNA KONFERENCA PROJEKTA LIFE for Acid Whey

Napredni načini izrabe potenciala sirotke: prehod iz okoljskega problema v dragocen naravni vir

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V organizaciji podjetja Arhel d.o.o. in Inštituta za mlekarstvo in probiotike

SEZNAM PREDSTAVITEV S POSTERJI

	Naslov posterja	Avtorji	Ustanova
1	**Analizni pristop za vrednotenje stabilnosti laktoferina**	Nika Osel, Timeja Planinšek Parfant, Jurij Trontelj, Albin Kristl, Robert Roškar	Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija
2	**Ovrednotenje stabilnosti laktoferina v vodnih raztopinah**	Nika Osel, Timeja Planinšek Parfant, Albin Kristl, Robert Roškar	Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija
3	**Analizna metoda za določanje vitamina B12 v vzorcih fermentirane kisle sirotke**	Timeja Planinšek Parfant[1], Nika Osel[1], Albin Kristl[1], Diana Paveljšek[2], Bojana Bogovič Matijašić[2], Robert Roškar[1]	[1]Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija [2]Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija
4	**Stabilnostna študija gastrorezistentnih pelet z laktoferinom**	Nika Osel, Blaž Grilc, Timeja Planinšek Parfant, Albin Kristl, Robert Roškar	Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija
5	**Čiščenje imunoglobulina G iz sirotke in njegova stabilizacija**	Jernej Oberčkal, Bojana Bogovič Matijašić	Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija
6	**Pridobivanje nizina in vitamina B12 z biokonverzijo kisle sirotke**	Diana Paveljšek[1], Jernej Oberčkal[1], Timeja Planinšek Parfant[2], Nika Osel[2], Robert Roškar[2], Bojana Bogovič Matijašić[1]	[1]Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija [2]Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija
7	**Razvoj in optimizacija industrijskega postopka izolacije LPO iz sladke sirotke z uporabo CIM kromatografske kolone**	Marko Kete[1], Jernej Oberčkal[2], Mateja Frančeškin Krapež[1], Alja Kisilak[1]	[1]Arhel d.o.o., Slovenija [2] Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija
8	**Optimizacija diafiltracije in koncentriranja proteinskih elucij kot priprava na sušenje**	Marko Kete[1], Blaž Grilc[2], Nika Osel[2], Mateja Frančeškin Krapež[1], Alja Kisilak[1]	[1]Arhel d.o.o., Slovenija [2] Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija

	Naslov posterja	Avtorji	Ustanova
9	**Filtracija in diafiltracija sirotke - optimizacija masnega izkoristka za laktoferin in laktoperoksidazo**	Marko Kete[1], Nika Osel[2], Jernej Oberčkal[3], Mateja Frančeškin Krapež[1]	[1]Arhel d.o.o., Slovenija [2]Univerza v Ljubljani, Fakulteta za farmacijo, Slovenija [3]Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija
10	**Primerjava učinkovitosti selektivne precipitacije α-laktalbumina iz različnih virov sirotke**	Polona Zabukovec, Tjaša Prevc, Maja Zupančič Justin	ARHEL d.o.o., Slovenija
11	**Spremljanje rasti laktokokov in tvorbe nizina z uporabo različnih virov sirotke kot gojišča**	Polona Zabukovec[1], Diana Paveljšek[2], Tjaša Prevc[1], Maja Zupančič Justin[1]	[1]ARHEL d.o.o., Slovenija, [2]Univerza v Ljubljani, Biotehniška fakulteta, Inštitut za mlekarstvo in probiotike, Slovenija
12	**Optimizacija gojenja kefirnih zrn v sirotki**	Maja Čič, Tinkara Rozina, Špela Palčar, Tinkara Vardjan, Tjaša Prevc, Maja Zupančič Justin	Arhel d.o.o., Slovenija
13	**Proizvodnja bioplina iz sirotke skozi daljše obdobje bi lahko bila motena zaradi pomanjkanja mikrohranil**	Leon Deutsch[1], Sabina Kolbl Repinc[2], Marko Blagojević[2], Katarina Vogel Mikuš[3,] Blaž Stres[1,2,4]	[1] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za zootehniko, Slovenija [2] Univerza v Ljubljani, Fakulteta za gradbeništvo, Slovenija [3] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Slovenija [4] Inštitut Jožef Stefan, Odsek za avtomatiko, biokibernetiko in robotiko, Slovenija
14	**Možnosti uporabe kisle sirotke v proizvodnji bioplina v kombinaciji z odpadnim biološkim blatom**	Lijana Fanedl, Blaž Petek, Romana Marinšek Logar	Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za mikrobiologijo, Slovenija
15	**Ločevanje sirotkinih frakcij z uporabo membranskih filtracij**	Irena Petrinič, Hermina Bukšek, Marjana Simonič	Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, Slovenija
16	**Izbor mikrofiltracijskih keramičnih membran s ciljem doseganja minimalnega prehoda mikroorganizmov in maksimalnega prehoda laktoferina**	David Farčnik, Matevž Koršič, Špela Palčar, Tinkara Vardjan, Marko Kete	Arhel d.o.o., Slovenija

	Naslov posterja	Avtorji	Ustanova
17	**Povečanje izplena laktoferina iz kisle sirotke s postopkom diafiltracije**	David Fračnik, Blaž Lokar, Marko Kete	Arhel d.o.o., Slovenija
18	**Proučevanje možne rabe zgoščenega preostanka kisle sirotke po mikrofiltraciji**	Maja Čič, Tinkara Rozina, Alja Kisilak, David Farčnik, Maja Zupančič Justin	Arhel d.o.o., Slovenija
19	**Izdelava posipa z aktivnimi mikroorganizmi iz ostankov sirotke za pospeševanje fermentacije organskih odpadkov in pripravo kompostne osnove**	Maja Čič, Tinkara Rozina, Maja Zupančič Justin	Arhel d.o.o., Slovenija
20	**Protimikrobna aktivnost peptidov, pridobljenih z encimsko hidrolizo laktoferina**	Špela Gruden[1], Petra Mohar Lorbeg[2], Bojana Bogovič Matijašić[2], Nataša Poklar Ulrih[1]	[1]Biotehniška fakulteta, Oddelek za živilstvo, Katedra za biokemijo in kemijo živil, Slovenija [2]Biotehniška fakulteta, Inštitut za mlekarstvo in probotike, Slovenija

CLOSURE CONFERENCE OF THE PROJECT LIFE for Acid Whey

Advanced ways to exploit the potential of whey: the transition from an environmental problem to a valuable natural resource

February 15th, 2022, Four Pionts by Sheraton Ljubljana Mons, Ljubljana
In the organization of Arhel d.o.o. and Institute for Dairy Science and Probiotics, Slovenia

LECTURE SCHEDULE

	Lecturer	Institution	Lecture Title
8:00-9:00			Registration and coffee
9:00-9:10	Nataša Polkar Ulrih	University of Ljubljana, Biotechnical Faculty, Slovenia	**Welcome speech**
INTRODUCTION, SITUATION IN SLOVENIA, TECHNOLOGICAL SOLUTIONS			
9:10-9:25	Maja Zupančič Justin	Arhel d.o.o., Slovenia	**Whey: an environmental problem or a valuable natural resource - the starting points of the LIFE for Acid Whey and LAKTIKA project**
9:30-9:50	Barbara Rupnik	Chamber of Commerce and Industry of Slovenia, Slovenia	**Whey in Slovenian Dairy industry**
9:55-10:10	Svetlana Vidovič, Robert Berce	Bering d.o.o., Slovenia	**Process technologies for whey processing: challenges and solutions for the dairy industry, raw material producers and biotechnology companies**
10:15-10:30	Irena Petrinič	University of Maribor, Faculty of Chemistry and Chemical Engineering, Slovenia	**Testing of different draw solutions in forward osmosis process for concentration of acid whey**
10:35-10:50	Sara Drmota Prebil	BIA Separations d.o.o., Slovenia	**Application of chromatographic procedures on monolithic columns for isolation of proteins and other bioactive compounds in food industry**
10:55-11:10			Coffee break
WHEY PROTEINS, INDUSTRIAL ISOLATION AND EXTRACTION			
11:10-11:30	Jernej Oberčkal	University of Ljubljana, Biotechnical Faculty, Institute of Dairy Science and Probiotics, Slovenia	**Nutritional, functional and bioactive properties of whey proteins and related use**
11:35-11:55	Marko Kete	Arhel d.o.o., Slovenia	**Development of a method and optimized industrial process for lactoferrin isolation**
12:00-12:15	Nika Osel	University of Ljubljana, Faculty of Pharmacy, Slovenia	**Development of analytical methods for the evaluation of lactoferrin and selected whey proteins**

	Lecturer	Institution	Lecture Title
12:20-12:35	Blaž Grilc	University of Ljubljana, Faculty of Pharmacy, Slovenia	**Development of a dosage form containing lactoferrin to maintain a healthy balance of the intestinal microbiota**
12:40-12:55	Špela Gruden	Biotechnical Faculty, Department of Food Science and Technology, Slovenia	**Antimicrobial activity of peptides generated by enzymatic hydrolysis of lactoferrin**
13:00-14:30			Lunch break, poster session
BIOTECHNOLOGICAL USE OF WHEY			
14:30-14:50	Bojana Bogovič Matijašić	University of Ljubljana, Biotechnical Faculty, Institute of Dairy Science and Probiotics, Slovenia	**Biotechnological utilization of whey**
14:55-15:10	Diana Paveljšek	University of Ljubljana, Biotechnical Faculty, Institute of Dairy Science and Probiotics, Slovenia	**Utilization of acid whey for the cultivation of lactic acid bacteria and extraction of beneficial metabolites**
15:15-15:30	Romana Marinšek Logar	University of Ljubljana, Biotechnical Faculty, Microbiology Dept., Slovenia	**Biogas production from whey**
15:35-15:50	Irena Barukčić	University of Zagreb, Faculty of Food Technology and Biotechnology, Croatia	**Whey as a valuable source of functional beverages**
15:55-16:10	Maja Bjelošević	University of Ljubljana, Faculty of Pharmacy, Slovenia	**Use of whey in cosmetology: examples of cosmetic products**
16:10 – 17:00			Networking, poster session, closure

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POSTER SESSION

	Poster title	Authors	Institution
1	**Analytical approach for stability evaluation of lactoferrin**	Nika Osel, Timeja Planinšek Parfant, Jurij Trontelj, Albin Kristl, Robert Roškar	University of Ljubljana, Faculty of Pharmacy, Slovenia
2	**Overview of Lactoferrin Stability in Aqueous Solutions**	Nika Osel, Timeja Planinšek Parfant, Albin Kristl, Robert Roškar	University of Ljubljana, Faculty of Pharmacy, Slovenia
3	**Analytical method for vitamin B12 determination in fermented acid whey**	Timeja Planinšek Parfant[1], Nika Osel[1], Albin Kristl[1], Diana Paveljšek[2], Bojana Bogovič Matijašić[2], Robert Roškar[1]	[1]University of Ljubljana, Faculty of Pharmacy, Slovenia [2]University of Ljubljana, Biotechnical faculty, Department of Animal Science, Institute of Dairy Science and Probiotics, Slovenia
4	**Stability Study of Enteric-Coated Pellets Containing Lactoferrin**	Nika Osel, Blaž Grilc, Timeja Planinšek Parfant, Albin Kristl, Robert Roškar	University of Ljubljana, Faculty of Pharmacy, Slovenia
5	**Purification of immunoglobulin G from whey and its stabilization**	Jernej Oberčkal, Bojana Bogovič Matijašić	University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia
6	**Production of nisin and vitamin B by acid whey bioconversion**	Diana paveljšek[1], Jernej Oberčkal[1], Timeja Planinšek Parfant[2], Nika Osel[2], Robert Roškar[2], Bojana Bogovič Matijašić[1]	[1]University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia [2]University of Ljubljana, Faculty of Pharmacy, The Chair of Biopharmaceutics and Pharmacokinetics, Slovenia
7	**Development and optimization of an industrial process for the isolation of LPO from sweet whey using CIM chromatographic column**	Marko Kete[1], Jernej Oberčkal[2], Mateja Frančeškin Krapež[1], Alja Kisilak[1]	[1]Arhel d.o.o., Slovenia [2]University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia

	Poster title	Authors	Institution
8	**Optimization of diafiltration and concentration of protein elutions as preparation for drying**	Marko Kete[1], Blaž Grilc[2], Nika Osel[2], Mateja Frančeškin Krapež[1], Alja Kisilak[1]	[1]Arhel d.o.o., Slovenija [2]University of Ljubljana, Faculty of Pharmacy, Slovenia
9	**Whey filtration and diafiltration – optimization of mass yield for lactoferrin and lactoperoxidase**	Marko Kete[1], Nika Osel[2], Jernej Oberčkal[3], Mateja Frančeškin Krapež[1]	[1]Arhel d.o.o., Slovenia [2]University of Ljubljana, Faculty of Pharmacy, Slovenia [3]University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia
10	**Comparison of the efficiency of selective precipitation of α-lactalbumin from different whey sources**	Polona Zabukovec, Tjaša Prevc, Maja Zupančič Justin	ARHEL d.o.o., Slovenia
11	**Monitoring lactococcal growth and nisin formation using different whey sources as cultivation medium**	Polona Zabukovec[1], Diana Paveljšek[2], Tjaša Prevc[1], Maja Zupančič Justin[1]	[1]ARHEL d.o.o., Slovenia, [2]University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia
12	**Optimization of kefir grain cultivation in whey**	Maja Čič, Tinkara Rozina, Špela Palčar, Tinkara Vardjan, Tjaša Prevc, Maja Zupančič Justin	Arhel d.o.o., Slovenija
13	**Gas production from cheese whey over longer periods may be interrupted by micronutrient restriction**	Leon Deutsch[1], Sabina Kolbl Repinc[2], Marko Blagojevič[2], Katarina Vogel Mikuš[3,] Blaž Stres[1,2,4]	[1]University of Ljubljana, Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia [2]University of Ljubljana, Faculty of civil and geodetic engineering, Slovenia [3]University of Ljubljana, Biotechnical faculty, Department of biology, Slovenia [4]Jožef Stefan Institute, Department of Automation, Biocybernetics and Robotics, Slovenia
14	**Possibilities of using acid whey in biogas production in combination with sewage sludge**	Lijana Fanedl, Blaž Petek, Romana Marinšek Logar	[1]University of Ljubljana, Biotechnical Faculty, Microbiology Dept., Slovenia
15	**Separation of whey fractions using membrane filtrations**	Irena Petrinič, Hermina Bukšek, Marjana Simonič	University of Maribor, Faculty of Chemistry and Chemical Engineering, Slovenia
16	**Selection of microfiltration ceramic membranes to achieve minimum passage of microorganisms and maximum passage of lactoferrin**	David Farčnik, Matevž Koršič, Špela Palčar, Tinkara Vardjan, Marko Kete	Arhel d.o.o., Slovenia

	Poster title	Authors	Institution
17	**Increase of lactoferrin yield from acid whey by the diafiltration process**	David Fračnik, Blaž Lokar, Marko Kete	Arhel d.o.o., Slovenia
18	**Studying the possible use of concentrated acid whey residue after microfiltration**	Maja Čič, Tinkara Rozina, Alja Kisilak, David Farčnik, Maja Zupančič Justin	Arhel d.o.o., Slovenia
19	**Production of starter with active microorganisms from whey residues to promote fermentation of organic waste and preparation of compost base**	Maja Čič, Tinkara Rozina, Maja Zupančič Justin	Arhel d.o.o., Slovenia
20	**Antimicrobial activity of peptides generated by enzymatic hydrolysis of lactoferrin**	Špela Gruden[1], Petra Mohar Lorbeg[2], Bojana Bogovič Matijašić[2], Nataša Poklar Ulrih	[1]Biotechnical faculty, Department of Food Science and Technology, Slovenia [2]Biotechnical faculty, Institute of Dairy Science and Probiotics, Slovenia

KAZALO

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EDITORIAL

Uvodnik

Konferenca predstavlja zaključni dogodek projekta LIFE for Acid Whey (Ponovna uporaba odpadne kisle sirotke za ekstrakcijo bioaktivnih proteinov z visoko dodano vrednostjo), sofinanciranega s pomočjo evropskega finančnega inštrumenta LIFE. Osrednja tema projekta je bila kisla sirotka, ki preostaja po mlečnokislinski fermentaciji mleka, s katero pridobivamo produkte kot sta skuta in grški tip jogurta. Zaradi vsebnosti mlečne kisline je predelava v praškaste produkte, kot so sirotkin koncentrat in sirotkini izolati, v primeru kisle sirotke s tehnološkega vidika zahtevnejša in ta pogosteje v mlekarnah konča kot odpadek. V projektu smo se osredotočili na izolacijo sirotkinega proteina laktoferina. Le-ta se v kisli sirotki običajno pojavlja v višjih koncentracijah kot v sladki sirotki. Zaradi njegove bioaktivne vloge v organizmu pa povpraševanje po tem proteinu narašča, in sicer na trgu mlečnih formul, prehranskih dopolnil, premium mlečnih izdelkov, kozmetike in zdravil. Cilj projekta je bil vzpostavitev demonstracijskega objekta za pridobivanje laktoferina, testiranje primernosti monolitne kromatografske kolone kot glavnega orodja za izolacijo laktoferina, optimizacija industrijskega postopka ter predelava preostanka sirotke v bioplinarni. Del aktivnosti je bil namenjen testiranju uporabnosti preostanka kisle sirotke kot gojišča za gojenje koristnih mlečnokislinskih organizmov in njihovih metabolitov. V projektu je podjetje Arhel sodelovalo z Inštitutom za mlekastvo in probiotike Biotehniške fakultete Univerze v Ljubljani.

V času izvajanja projekta in testiranja sirotk različnih slovenskih in tujih mlekarn, sta prišla projektna partnerja do zaključka, da je v slovenskem prostoru podobno neučinkovito izrabljena tudi sladka sirotka, ki pa ima velik potencial drugih sirotkinih proteinov, med katerimi so ravno tako proteini z bioaktivno vlogo. Tako je nastal nov projekt, LAKTIKA (Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil) v okviru Operativnega programa za izvajanje evropske kohezijske politike 2014 – 2020. V okviru projekta LAKTIKA je v koordinaciji podjetja Arhel d.o.o. sodelovalo še več partnerjev iz akademske sfere, Univerze v Ljubljani in Univerze v Mariboru. V projektu smo se prvenstveno osredotočili na selektivno izolacijo preostalih sirotkinih proteinov. Sirotka je sestavljena iz približno 90 % vode, preostalo suho snov sestavlja laktoza (4,9 %), minerali (0,5 %), maščobe (0,1 %) in proteini (0,7 %), med katerimi so najbolj zastopani β-laktoglobulin, α-laktalbumin, goveji serumski albumin, imunoglobulini, laktoferin, laktoperoksidaza ter nekateri manjši proteini. Kljub nizkemu deležu, predstavljajo sirotkini proteini 20% vseh proteinov mleka. Proteini sirotke so bogat vir aminokislin, hkrati pa imajo številne funkcije. Nekateri delujejo protimikrobno, imunomodulatorno in/ali antikancerogeno in so kot taki zanimivi za uporabo v živilski in farmacevtski industriji. Farmacevtsko industrijo pa zanimajo predvsem čiste oblike omenjenih proteinov, katerih izolacijam smo se posvečali. Nadalje, smo se v projektu LAKTIKA posvetili optimizacijam membranskih tehnik zgoščevanja in separacije, specifičnim biotehnološkim izrabam sirotke kot gojišča za mlečnokislinske organizme, nadgradnji surovine laktoferina v končni produkt ter opredelitvi drugih končnih produktov z uporabo sirotkinih frakcij. V okviru konference tako predstavljamo tudi rezultate projekta LAKTIKA, ki dopolnjujejo in nadgrajujejo aktivnosti projekta LIFE for Acid whey.

Skupna točka obeh projektov je bila nadgradnja uporabe sirotke, kot stranskega produkta mlekarske industrije, v produkte z višjo dodano vrednostjo ob čim manjšem negativnem vplivu na okolje. Oba projekta sta tako v tesni povezavi z evropsko strategijo »od vil do vilic«, ki celovito obravnava izziv trajnostnih prehranskih sistemov, pri čemer gre za neločljivo povezavo med zdravimi ljudmi, zdravimi družbami in zdravim planetom. Je tudi osrednji del agende »Zelenega dogovora« in trajnostnega razvoja.

V tehnoloških postopkih predelave hrane nastane veliko stranskih produktov, ki imajo številne neugodne okoljske, družbene in gospodarske učinke. Na tak moment naletimo tudi v mlekarski industriji. V zadnjih letih pridelava in predelava mleka ter povpraševanje potrošnikov po mlečnih izdelkih strmo narašča, kar pomeni tudi porast količine sirotke, kot glavnega stranskega produkta ali sekundarne surovine. V proizvodnji sira in skute predstavlja sirotka približno 90 % procesiranega mleka. Ocenjujejo, da svetovna produkcija sirotke znaša okrog 160 milijonov ton na leto, pri čemer je celotna proizvodnja sirotke v EU ocenjena na 40 milijonov ton letno. Kljub temu, da se okrog 70 % sladke sirotke predela v različne prehranske izdelke, preostanek predstavlja resen okoljski problem. Ob neustrezni obravnavi, predstavlja sirotka enega največjih živilskih onesnaževalcev z biološko potrebo po kisiku (BPK) >35 000 ppm in kemično potrebo po kisiku (KPK)> 60 000 ppm za njeno razgradnjo. S tega vidika je sirotko v EU prepovedano odvajati na biološke čistilne naprave.

Zaradi visokega energetskega potenciala pa po drugi strani predstavlja zanimiv substrat za anaerobno konverzijo v bioplin. Tudi tej temi je na konferenci namenjenih nekaj prispevkov.

Sirotka kot sekundarna surovina predstavlja bogati vir biološko zanimivih spojin. Po tej plati je zato sirotko in njene spojine smiselno v največji možni meri uporabiti kot živilo in šele v zadnjih korakih kot energetsko bogat odpadek za anaerobno konverzjo v bioplin. Možnosti izrabe in pretvorb sirotke in njenih sestavin so številne. Z rezultati obeh projektov smo uspeli opredeliti nekaj novih kaskadnih pristopov izrabe sirotke. S predavanji in posterji udeležencev obeh projektov ter gostujočimi predavatelji smo na konferenci predstavili nekaj inovativnih tehnoloških pristopov za koncentriranje sirotke ter izolacijo in čiščenje sirotkinih proteinov s poudarkom na laktoferinu. Med drugim je v zborniku predstavljen razvoj farmacevtske formulacije z laktoferinom za ohranjanje zdravega ravnovesja črevesne mikroflore, pridobivanjem protimikrobno aktivnih peptidov z encimsko hidrolizo, uporabo kisle in sladke sirotke za gojenje mlečnokislinskih bakterij in pridobivanje metabolitov, pridobivanje funkcionalnih napitkov, uporabo sirotke v kozmetiki in nenazadnje za pridobivanje bioplina.

Organizatorji konference se zahvaljujemo vsem avtorjem in udeležencem, ki so prispevali k uspešni izvedbi konference.

Kot posameznikom in kot družbi nam je zaupana prihodnost bodočih generacij in prihodnost našega planeta!

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Editorial

The conference was a closing event of the LIFE for Acid Whey project (Reuse of waste acid whey for the extraction of bioactive proteins with high added value), co-financed by the European Financial Instrument LIFE. The project's central theme was acid whey, which remains after the lactic acid fermentation of milk, with which we obtain products such as cottage cheese and Greek type of yoghurt. Due to lactic acid content, acid whey processing into powdered products, such as whey concentrate and whey isolates, is more technologically demanding and more often ends up in dairies as waste. The project focused on the isolation of the whey protein lactoferrin. It usually occurs in higher concentrations in acid whey than in sweet whey. Due to its bioactive role in the body, the demand for this protein is growing, namely in the market of baby formulas, food supplements, premium dairy products, cosmetics and medicines. The project aimed to establish a demonstration facility for lactoferrin production, test the suitability of monolithic chromatographic columns as the main tool for lactoferrin isolation, optimise the industrial process, and process whey residue in a biogas plant. Part of the activities was dedicated to testing residual acid whey's applicability as a culture medium for the cultivation of beneficial lactic acid organisms and their metabolites. In the project, the company Arhel cooperated with the Institute of Dairy Science and Probiotics of the Biotechnical Faculty of the University of Ljubljana.

During the implementation of the project and testing of whey from various Slovenian and foreign dairies, the project partners concluded that sweet whey is similarly inefficiently used in Slovenia but has the excellent potential of other whey proteins, including proteins with a bioactive role. Thus, a new project was created, LAKTIKA (Fractionation and processing of whey proteins and exploitation of the residue for the formulation of new functional foods and food supplements) within the Operational Program for the Implementation of European Cohesion Policy 2014-2020. Several partners from the academic sphere, the University of Ljubljana and the University of Maribor, coordinated by Arhel d.o.o., participated. The project focused primarily on the selective isolation of residual whey proteins. Whey consists of about 90% water. The remaining whey dry matter consists of lactose (4.9%), minerals (0.5%), fats (0.1%) and proteins (0.7%), among which are the most represented β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin, lactoperoxidase and some minor proteins. Despite their low proportion, whey proteins make up 20% of all milk proteins. Whey proteins are a rich source of amino acids and, at the same time, have many functions. Some have antimicrobial, immunomodulatory and/or anti-cancer effects and are of interest in the food and pharmaceutical industries. The pharmaceutical industry is mainly interested in pure forms of these proteins, the isolation of which we have focused on. Furthermore, the LAKTIKA project focused on optimising membrane concentration and separation techniques, specific biotechnological uses of whey as a culture medium for lactic acid organisms, upgrading the lactoferrin raw material into the final product and defining other end-products using whey fractions. Therefore, within the conference, we present the results of the LAKTIKA project, which complement and upgrade the LIFE activities for Acid whey project.

The common point of both projects was to upgrade the use of whey, as a by-product of the dairy industry, into products with higher added value while minimising the negative impact on the environment. Both projects are thus closely linked to the European Farm to Fork Strategy, which comprehensively addresses the challenge of sustainable food systems, with an inseparable link between healthy people, healthy societies and a healthy planet. It is also at the heart of the Green Deal and sustainable development agenda.

Many by-products are produced in food processing processes, which have adverse environmental, social and economic effects. We also encounter such a moment in the dairy industry. In recent years, milk production and processing and consumer demand for dairy products have risen sharply, which means an increase in whey as the main by-product or secondary raw material. Whey accounts for about 90% of processed milk in cheese and cottage cheese production. World whey production is estimated to be around 160 million tonnes per year, with total whey production in the EU estimated at 40 million tonnes per year. Even though about 70% of sweet whey is processed into various food products, the rest is a severe environmental problem. In case of inadequate treatment, whey represents one of the most significant food pollutants with a biological oxygen demand (BOD) >35,000 ppm and a chemical oxygen demand (COD) >60,000 ppm for its degradation. From this point of view, whey is banned in the EU from biological treatment plants. On the other hand, due to its high energy potential,

it is an attractive substrate for anaerobic conversion to biogas. There were also some contributions to this topic at the conference.

As a secondary raw material, whey is a rich source of biologically interesting compounds. In this respect, it is therefore sensible to use whey and its compounds as much as possible as food and only in the last steps as energy-rich waste for anaerobic conversion to biogas. The possibilities of using and converting whey and its ingredients are numerous. With the results of both projects, we were able to identify some new cascading approaches to the use of whey. With lectures and posters of participants of both projects and guest lecturers, we presented at the conference some innovative technological processes for whey concentration and isolation and purification of whey proteins with emphasis on lactoferrin. Among others, the development of a pharmaceutical formulation with lactoferrin to maintain a healthy balance of intestinal microflora is presented in the proceedings. In addition, the papers focus on the production of active peptides with antimicrobial function by enzymatic hydrolysis, the use of acid and sweet whey to grow lactic acid bacteria and production of metabolites, the formulation of functional drinks, the use of whey in cosmetics and last but not least, for the production of biogas.

The conference organisers would like to thank all the authors and participants who contributed to the successful implementation of the conference.

As individuals and as a society, we are entrusted with the future of the next generations and our planet!

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PRISPEVKI

CONTRIBUTIONS

Predavanja

Lectures

Sirotka: okoljski problem ali dragocen naravni vir – izhodišča projekta LIFE for Acid Whey in LAKTIKA

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Povzetek
Prispevek predstavlja ključna izhodišča, na katerih je temeljila prijava razvojnih projektov LIFE for Acid Whey in LAKTIKA. Cilji projektov so bili usmerjeni v razvoj kaskadnih pristopov obravnave sirotke za pridobivanje produktov z višjo dodano vrednostjo, kot jo predstavlja sirotka kot stranski produkt predelave mleka.

Ključne besede: laktoferin, sirotkini proteini, sirotka, sirotkine sestavine, kaskadna raba sirotke

Uvod

V prispevku predstavljamo izhodišča za prijavo in ključne pridobitve izvajanja projektov LIFE for Acid Whey s polnim naslovom Ponovna uporaba odpadne kisle sirotke za ekstrakcijo bioaktivnih beljakovin z visoko dodano vrednostjo in projekta LAKTIKA s polnim naslovom Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil. Projekta sta bila sofinancirana s strani evropskega finančnega instrumenta LIFE ter Operativnega programa za izvajanje evropske kohezijske politike v obdobju 2014 – 2020. Izhodišča in rezultati nakazani v nadaljevanju so podrobneje predstavljeni v prispevkih zbornika, ki sledijo.

Povod za prijavo projektov LIFE in LAKTIKA

Odločitev za proizvodnjo določenega izdelka mora biti vedno vezana na povpraševanje s strani trga. V našem primeru smo zaznali naraščajoče povpraševanje po laktoferinu, zanimivem bioaktivnem sirotkinem proteinu, ki je v sirotki prisoten v nizkih koncentracijah. Običajno ga je najti več v kisli sirotki, ki je z vidika nadaljnje predelave v mlekarnah bolj problematična in pogosteje predstavlja odpadek in s tem okoljski problem v mlekarski industriji. Seznanjeni smo bili tudi z odličnim orodjem za separacijo laktoferina iz sirotke, monolitnimi kromatografskimi kolonami, s katerimi smo na preliminarnih testiranjih pridobili visoko čiste frakcije laktoferina in v okviru nadaljnjih raziskav opredelili natančen postopek izolacije (Kete s sod., 2020).
Kljub doseganju visoke čistosti ločbe proteina na kromatografski koloni ter ohranjanju njegovih odličnih bioaktivnih lastnosti, je bilo potrebno optimizirati celoten tehnološki postopek pridobivanja proteina. To je zajemalo iskanje rešitev za zmanjševanje izgub dragocenega produkta, rabe energije ter procesnih surovin v vseh korakih procesa. Ob načrtovanju možne industrijske proizvodnje laktoferina smo hkrati prišli do spoznanja, da se moramo obravnave sirotke lotiti celostno, z vidika vseh njenih sestavin in potencialov in ne le z vidika izolacije omenjenega proteina. Za cilj smo si zadali proučiti nekaj izbranih poti kaskadne rabe sirotke kot živila, vira bioaktivnih proteinov ali surovine za biotehnološke pretvorbe v produkte z dodano vrednostjo. Izraba preostanka obravnave sirotke v obliki substrata za pridobivanje energije v bioplinarnah, je bila predvidena kot zadnji korak v kaskadi. Za malo podjetje je bilo financiranje tako obsežne naloge iz lastnih sredstev prezahtevno in smo zato pristopili k iskanju nepovratnih sredstev in bili uspešni v omenjenih dveh razpisih.

Z novimi spoznanji se lahko približamo težavam mlekarn

Potrebe po razvoju na področju celostne in kaskadne rabe sirotke se kažejo tudi v težavah, ki pestijo mlekarne. V slovenskih mlekarnah se soočajo ne le z viški kisle sirotke, katere uporabnost za nadaljnjo predelavo naj bi bila bolj problematična, temveč tudi z viški sladke sirotke, ki ni optimalno izkoriščena.
Ena izmed težav, ki pestijo mlekarne, je soočanje z veliko porabo vode, ki se v svetu gibljejo med 0,2 – 11 litri vode na kilogram predelanega mleka (Lingaard-Jørgensen et al. 2018). Hkrati z veliko porabo vode nastajajo velike količine odpadne vode, katere čiščenje in odvoz ravno tako predstavlja velik strošek. Eden od načinov zmanjšanja porabe vode in s tem povezanih stroškov je ponovna uporaba vode iz sirotke, ki predstavlja okrog 94% sirotke. S tem se občutno zmanjša količina in s tem stroški odvoza in predelave preostanka, ki se tretira kot odpadek, primeren za odvoz v anaerobno predelavo za pridobivanje bioplina. S takim pristopom pa zanemarimo velik hranilni potencial sirotkinih sestavin, ki ga predstavljajo predvsem laktoza in drugi oligosaharidi, sirotkini proteini, mineralne snovi in vitamini.
V zadnjih desetih letih je prišlo do velikih napredkov v ponudbi procesne opreme za separacijo in sušenje sirotkinih sestavin. Kljub razvoju na področju opreme in tehnoloških postopkov pa le-ta v okviru mlekarn še vedno ostaja v precejšnji meri neizkoriščen vir, ki se prodaja kot surovina z nizko dodano vrednostjo za

nadaljnjo predelavo ali kot odpadek. Naslednja težava, na katro naletijo predvsem male in srednje velike mlekarne, je namreč pomanjkanje sredstev za vlaganje v posodobitev opreme za naprednejšo izrabo sirotke. Investicijski stroški so visoki, hkrati pa novi postopki zahtevajo določeno mero razvoja, skupaj z vrednotenjem dejanskih obratovalnih stroškov uvajanja novih tehnoloških postopkov. Postopki nadaljnje predelave sirotke v veliki meri obsegajo različne membranske in kromatografske postopke separacije ter postopke sušenja, ki so občutljivi postopki in zahtevajo natančne nastavitve.
Omahovanje pri odločitvah za vlaganja v nove tehnološke postopke predelave sirotke je tudi posledica nepoznavanje trga za prodajo novo pridobljenih sirotkinih sestavin in izdelkov, kot so prehranska dopolnila in v nekaterih primerih pomanjkanje poznavanja novih produktov s strani potrošnikov.
Na drugi strani pesti mlekarne velika konkurenca v okviru ponudbe klasičnih mlečnih izdelkov, kot so fermentirani mlečni izdelki in siri, zato je prodor na trg z inovativnimi izdelki na osnovi sestavin pridobljenih iz preostankov predelave mleka, kot je sirotka, dobrodošel. Na trg si vedno bolj utirajo pot izdelki oplemeniteni z različnimi funkcionalnimi sestavinami sirotke, kot so bioaktivni proteini in njihovi peptidi, oligosaharidi, fosfolipidi, vitamini, minerali in koristni mikroorganizmi in njihovi metabolni produkti. Dodane sestavine povzdignejo običajna živila v tako imenovana funkcionalna živila. Funkcionalna živila opredeljujemo kot živila, za katere je zadovoljujoče izkazano, da koristno vplivajo na eno ali več ciljnih funkcij v telesu, na način, ki izboljša zdravje in počutje oziroma zmanjšuje tveganje za bolezen (NIJZ, 2010). Funkcionalna živila obogatena s sestavinami sirotke in produkti biotehnološke pretvorbe sirotke izkazujejo med drugim učinke kot so probiotično in prebiotično delovanje, obogatitev z beljakovinami in esencialnimi aminokislinami, minerali in vitamini, antioksidativno, imunostimulatorno in protimikrobno delovanje proti škodljivim mikroorganizmom. Poleg funkcionalnih živil narašča povpraševanje in ponudba prehranskih dopolnil in posebnih dietetičnih proizvodov z dodanimi sestavinami sirotke za skupine potrošnikov s posebnimi prehranskimi zahtevami kot so dojenčki, športniki in starostniki s potrebami po specifičnih hranilih.
S sodelovanjem z mlekarnami v okviru obeh razvojnih projektov smo prišli do mnogih uvidov na področju lastnosti sirotke iz različnih tehnoloških postopkov in uspeli opredeliti nekaj kaskadnih poti rabe sirotke s ciljem njene izrabe z večjo dodano vrednostjo.

S povezovanjem slovenskih raziskovalnih inštitucij in podjetij smo poglobili znanja na več področjih možne izrabe sirotke

Podjetje Arhel se je v razvojnih aktivnostih povezal z Biotehniško fakulteto, Inštitutom za mlekarstvo in probiotike, Fakulteto za farmacije Univerze v Ljubljani, Fakulteto za kemijo in kemijsko tehnologijo Univerze v Mariboru ter številnimi slovenskimi mlekarnami. Združili smo znanja s področja načrtovanja procesne opreme in avtomatizacije, separacijskih tehnik, sodobnih analitičnih metod, farmacevtske tehnologije, biofarmacije in farmakokinetike, znanj s področja mlečnokislinskih bakterij in probiotikov ter oblikovanja funkcionalnih izdelkov in prehranskih dopolnil.
Zaključimo lahko, da smo s projektnimi aktivnostmi prišli do rezultatov, s katerimi smo v precejšni meri optimizirali tehnološki postopek izolacije laktoferina in se približali lastnemu oblikovanju končnih produktov na osnovi sirotke. Dosežki na posameznih področjih razvoja so podrobno predstavljeni v prispevkih sodelujočih raziskovalcev na obeh projektih v nadaljevanju zbornika.

Zahvala

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Whey: an environmental problem or a valuable natural resource - the starting points of the LIFE for Acid Whey and LAKTIKA project

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Abstract
The paper presents the key starting points on which the application for the LIFE for Acid Whey and LAKTIKA development projects was based. The projects' objectives were to develop cascade approaches to whey treatment to obtain products with higher added value than presents whey itself as a by-product of milk processing.

Keywords: lactoferrin, whey proteins, whey, whey ingredients, cascade use of whey

Introduction
The paper presents the starting points for application and critical achievements of the LIFE for Acid Whey project with the full title Reuse of waste acid whey for the extraction of bioactive proteins with high added value and the LAKTIKA project with the title Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements. The projects were co-financed by the European Financial Instrument LIFE and the Operational Program for the Implementation of European Cohesion Policy in 2014-2020. The starting points and results described below are presented in more detail in the following articles of the proceedings.

Reasons for applying for the LIFE and LAKTIKA projects
The decision to produce a particular product must always be tied to market demand. In our case, we detected a growing demand for lactoferrin, an interesting bioactive whey protein present in whey in low concentrations. However, it is usually present in higher concentrations in acid whey, which is considered more problematic from the point of view of further processing in dairies and more often represents waste and thus a problem for dairies. We were also acquainted with an excellent tool for separating lactoferrin from whey, monolithic chromatographic columns. We obtained high-purity fractions of lactoferrin in preliminary tests and defined a precise isolation procedure (Kete et al., 2020).
Despite achieving highly pure protein isolate on the chromatographic column and preserving its excellent bioactive properties, it was necessary to optimise the entire technological process of protein isolation. This included finding solutions to reduce valuable product losses, energy consumption and consumption of process raw materials at all stages of the process. Besides, when planning the possible industrial production of lactoferrin, we realised that we must approach the treatment of whey holistically, in terms of all its components and potentials and not only in terms of isolation of this protein. The aim was to study some selected routes of cascade use of whey as a food-stuff, a source of bioactive proteins or raw material for biotechnological conversions into value-added products. Production of energy from the whey residuals in biogas plants was envisaged as the last step in the cascade. For a small company, financing such an extensive task from its own resources was too demanding. Therefore, we approached the search for grants and were successful in the two tenders.

With new insights, we can get closer to the problems of dairies
The need for development in integrated and cascade use of whey is also reflected in the difficulties threatening dairies. Slovenian dairies are facing not only surpluses of acid whey, the usefulness of which for further processing is said to be more problematic, but also a surplus of sweet whey, which is not optimally utilised.
One of the many problems plaguing dairies is facing high water consumption, ranging from 0.2 to 11 litres of water per kilogram of processed milk worldwide (Lingaard-Jørgensen et al., 2018). Along with increased water consumption, large amounts of wastewater are generated, which are also very expensive to clean and dispose of. One way to reduce water consumption and the associated costs is to reuse water from whey, accounting for around 94%. This significantly reduces the amount and thus the costs of removal and processing of the residue, which is treated as a waste suitable for disposal in anaerobic processing for biogas production. However, with such an approach, we neglect the tremendous nutritional potential of whey ingredients, which is mainly represented by lactose and other oligosaccharides, whey proteins, minerals and vitamins.
Over the last ten years, significant progress has been made in supplying process equipment to separate and dry whey ingredients. Despite developments in equipment and technological processes, whey remains a largely untapped resource within dairies, sold as a raw material with low added value for further processing or as

waste. In particular, another problem faced by small and medium-sized dairies is the lack of funds to invest in upgrading equipment for more advanced whey use. Investment costs are high, and at the same time, new procedures require a certain degree of development, together with the evaluation of the actual operating costs of the new technological approaches introduced. The processing systems of whey primarily involve various membrane and chromatographic separation and drying processes, which are delicate and require precise adjustments.

The hesitation in investing in new technological processes for whey processing is also due to the lack of knowledge of the market for the sale of newly acquired whey ingredients and products such as food supplements and, in some cases, the lack of knowledge about the new products by consumers.

On the other hand, dairies face fierce competition in offering classic dairy products such as fermented milk and cheeses, so market penetration with innovative products based on ingredients derived from milk processing residues such as whey is welcome. Products enriched with various functional whey components, such as bioactive proteins and their peptides, oligosaccharides, phospholipids, vitamins, minerals and beneficial microorganisms and their metabolic products, are increasingly making their way to the market. The added ingredients elevate ordinary foods into so-called functional foods. Functional foods are defined as foods that have satisfactorily shown to have a beneficial effect on one or more target functions in the body that improve health and well-being or reduce the risk of disease (NIJZ, 2010). Functional foods enriched with whey ingredients and metabolites from biotechnological whey transformation show effects such as probiotic and prebiotic activity, antioxidant, immunostimulatory and antimicrobial action against harmful microorganisms, and enrichment with proteins, essential amino acids, minerals and vitamins. In addition to functional foods, there is a growing demand and supply of dietary supplements and special dietary products with added whey ingredients for consumers with special dietary requirements such as infants, athletes, and the elderly with specific nutrients.

By cooperating with dairies in both development projects, we gained many insights into the properties of whey from various technological processes. In addition, we managed to identify some cascade routes of whey processing to use it with greater added value.

By connecting Slovenian research institutions and companies, we have deepened our knowledge in several areas of the possible use of whey

In its development activities, the Arhel company connected with the Biotechnical Faculty, the Institute of Dairy Science and Probiotics, the Faculty of Pharmacy of the University of Ljubljana, the Faculty of Chemistry and Chemical Technology of the University of Maribor and numerous Slovenian dairies. As a result, we combined knowledge in process equipment design and automation, separation techniques, modern analytical methods, pharmaceutical technology, biopharmacy and pharmacokinetics, knowledge in the field of lactic acid bacteria and probiotics and the design of functional products and food supplements.

We can conclude that the project activities led to results that significantly optimised the technological process of lactoferrin isolation and brought us closer to our own design of whey-based end products. Achievements in individual areas of development are presented in detail in the papers of participating researchers on both projects in the continuation of the proceedings.

Acknowledgements

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Sirotka v slovenski mlekarski industriji

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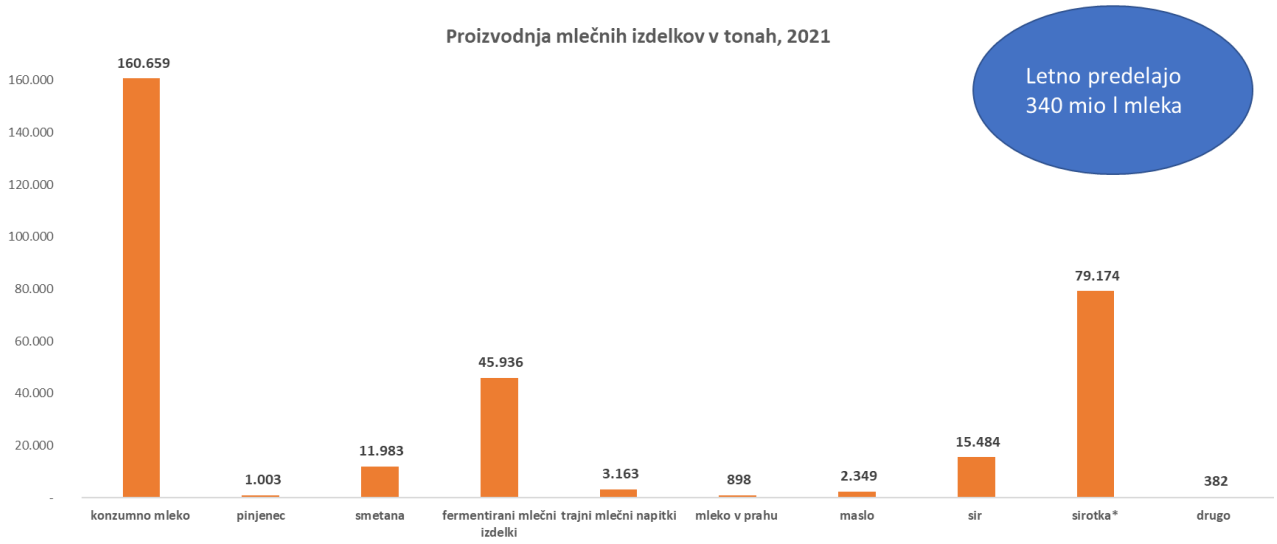
Povzetek
Slovenska mlekarska industrija pri proizvodnji sira in skute na leto proizvede okrog 80 mio litrov sladke in 30 mio litrov kisle sirotke. Tako iz okoljskega kot ekonomskega vidika je izziv čim boljša koncentracija sirotke in obenem učinkovitejši izkoristek njenega hranilnega in energetskega potenciala.

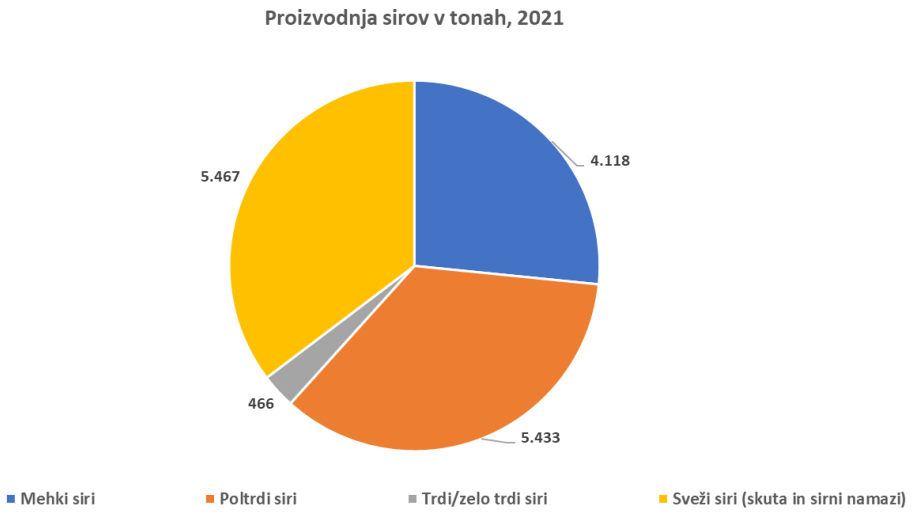
Ključne besede: sirotka, slovenska mlekarska industrija

Uvod

Slovenske mlekarne so v letu 2021 skupno predelale 340 mio litrov mleka, večinoma v konzumno mleko, fermentirane mlečne izdelke in sire. Na leto pri proizvodnji okrog 16 tisoč ton sira in skute nastane znatna količina stranskega proizvoda – sladke in kisle sirotke, ki pa ni v celoti zajeta v uradno statistiko.



Slika 1: Proizvodnja mlečnih izdelkov v tonah, 2021 (*Sirotka: v ekvivalentih tekoče sirotke). Vir: GZS-ZKŽP in SURS (Ljubljanske mlekarne, Mlekarna Celeia, Pomurske mlekarne, Mlekarna Ekolat, Mlekarna Planika, Mlekarna Krepko, Loška mlekarna).



Slika 2: Proizvodnja sirov v tonah, 2021 (Vir: GZS-ZKŽP in SURS (Ljubljanske mlekarne, Mlekarna Celeia, Pomurske mlekarne, Mlekarna Ekolat, Mlekarna Planika, Mlekarna Krepko, Loška mlekarna).

Proizvodnja in načini uporabe sirotke v slovenskih mlekarnah

Slovenske mlekarne so v letu 2021 skupno proizvedle okrog 80 mio litrov sladke sirotke in 30 mio litrov kisle sirotke. Kljub manjšim količinam kisla sirotka predstavlja večji problem, saj jo je težje predelati in tržno ni tako zanimiva. Največji del sirotke se v mlekarnah predela v 3 – 6 kratni koncentrat, ki ga prodajo večinoma v tujino za namen nadaljnje predelave. Še vedno se pomemben del sirotke uporabi za krmo prašičev, kar pa za mlekarne zaradi prevoza večinoma predstavlja strošek. Poleg tega v primeru pojava bolezni živali ni dolgoročno zagotovljenega odjema sirotke s strani rejcev. Tudi pri oddaji v bioplinarne imajo mlekarne strošek, sirotka pa je obravnavana kot odpadek. Manjši del sirotke se v mlekarnah predela v končne proizvode kot so različni sirotkini napitki, nekaj pa se jo predela tudi v albuminsko skuto.

Stanje in izzivi na področju ravnanja s sirotko v Sloveniji

Ravnanje s sirotko za slovenske mlekarne v veliki meri še vedno predstavlja strošek. Tako iz okoljskega kot ekonomskega vidika je pomemben izziv čim boljša koncentracija sirotke ter obenem učinkovitejši izkoristek njenega hranilnega in energetskega potenciala. Potrebno bi bilo proučiti ustreznost obstoječe zakonodaje in možnosti sodelovanja mlekarn z lokalnimi kmetijami ali drugimi proizvodnimi obrati pri zbiranju odpadkov za proizvodnjo bioplina. Za celovito in trajnostno predelavo sirotke v izdelke višje dodane vrednosti bi bila potrebna tudi večja vlaganja v nove tehnologije.

Whey in Slovenian Dairy Industry

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Abstract
The Slovenian dairy industry produces around 80 million litres of sweet and 30 million litres of acid whey in cheese and cottage cheese production. From both the environmental and economic point of view, the challenge is to concentrate the whey as much as possible and at the same time make more efficient use of its nutritional and energy potential.

Keywords: whey, Slovenian dairy industry

Introduction
In 2021, Slovenian dairies processed 340 million litres of milk, mostly into drinking milk, fermented milk products and cheeses. In addition, production of around 16,000 tons of cheese and cottage cheese produces a significant amount of by-products per year - sweet and acid whey, which is not fully included in official statistics.

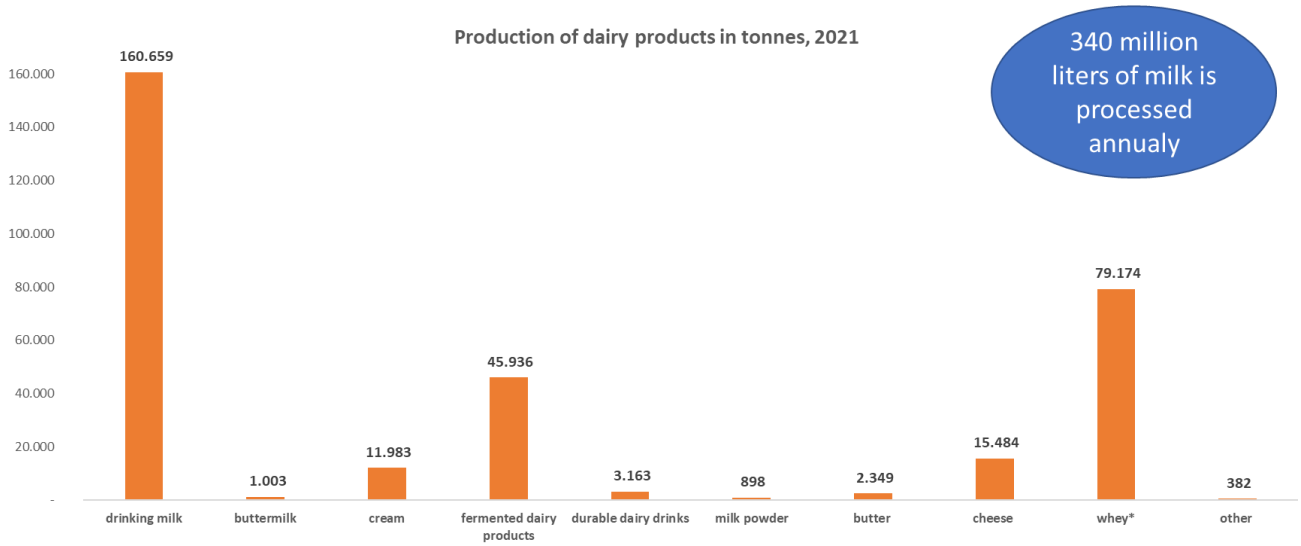

Figure 1: Dairy production in tonnes, 2021 (* Whey: in equivalents of liquid whey). Source: GZS-ZKŽP and SURS (Ljubljanske mlekarne, Mlekarna Celeia, Pomurske mlekarne, Mlekarna Ekolat, Mlekarna Planika, Mlekarna Krepko, Loška mlekarna).

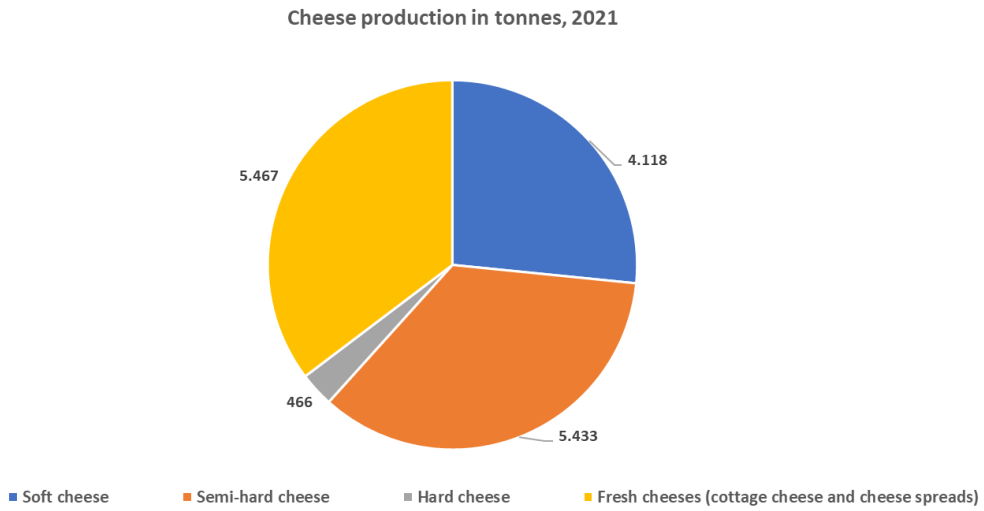

Figure 2: Cheese production in tonnes, 2021. Source: GZS-ZKŽP in SURS (Ljubljanske mlekarne, Mlekarna Celeia, Pomurske mlekarne, Mlekarna Ekolat, Mlekarna Planika, Mlekarna Krepko, Loška mlekarna.

Production and methods of using whey in Slovenian dairies

In 2021, Slovenian dairies produced around 80 million litres of sweet whey and 30 million litres of acid whey. Despite the smaller quantities, acid whey is a bigger problem, as it's harder to process and not as interesting for the market. The largest part of whey is processed in dairies into 3 - 6 x concentrate, which is mainly exported for further processing. An essential amount of whey is still used to feed pigs, which is mostly a cost for dairies due to transport. In addition, there is no long-term guarantee of whey take over by breeders in the case of animal disease. When dairies deliver whey to biogas plants, it's treated as waste, and this is a cost for dairies as well. A small part of whey is processed in dairies into finished products such as various whey beverages, and some whey is also processed into albumin curd.

Situation and challenges in the field of whey management in Slovenia

Whey management is still mainly a cost for Slovenian dairies. From both an environmental and economic point of view, an important challenge is the best possible concentration of whey and the more efficient use of its nutritional and energy potential. The adequacy of existing legislation and the possibility of cooperation with local farms or other processing plants in waste collection for biogas production should be examined. Comprehensive and sustainable whey processing into higher value-added products would also require greater investments in new technologies.

Tehnologija za predelavo sirotke: izzivi in rešitve za mlekarsko industrijo, proizvajalce surovin in biotehnološka podjetja

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Povzetek
Tradicionalno se je sirotko obravnavalo kot stranski proizvod predelave mleka. Sirotka in izdelki pridobljeni z njenim frakcioniranjem v zadnjih letih pridobivajo pomembno vlogo v živilski, zdravstveni in kozmetični industriji, biotehnologiji in drugih panogah kot surovine ali končni izdelki. Znanje o tehnologiji predelave in frakcioniranja sirotke zato postaja ključnega pomena za te industrije.
Večno vprašanje vseh omenjenih industrijskih panog pa je, kako takšno proizvodnjo organizirati, da bo proizvodnja donosna in trajnostna.
Bering d.o.o., Slovenija v sodelovanju z Milk & More Dairy Consulting, Nemčija ima dolgoletne izkušnje na področju predelave sirotke, od pridobivanja laktoze in proizvodnje koncentrata sirotkinih beljakovin (WPC), do ekstrakcije visoko cenjenih beljakovin in posebnih izdelkov, kot so laktuloza, laktitol, laktoferin, laktoperoksidaza in fosfolipidi.

Ključne bedsede: sirotka, predelava sirotke, frakcioniranje sirotke, sirotkine beljakovine, razbarvanje laktoze, koncentrat sirotkinih beljakovin (WPC), laktuloza, laktitol, laktoferin, laktoperoksidaza, fosfolipidi

Uvod

- Opis sestavin sirotke
- Tehnologija frakcioniranja sirotke in pričakovana donosnost
- Glavna oprema za frakcioniranje sirotke
- Frakcioniranje sirotke – splošni diagram predelave in izdelki
- Tehnologije za proizvodnjo različnih izdelkov iz sirotke
- Beringove rešitve na ključ

Slika 1: Glavna oprema za obdelavo sirotke.

Slika 2: Produkti frakcioninja sirotke

Whey processing technologies: challenges and solutions for the dairy industry, raw material producers, and biotechnology companies

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Abstract
Traditionally whey used to be recognized as a by-product of dairy processing. However, in the last years, whey and products of its fractionation are taking an important role in food, healthcare, cosmetics, biotechnology, and other industries as raw materials or final products. Consequently, the knowledge and technology of whey processing and fractionation is becoming vital for these industries.
Of course, the eternal question of the industry is how to organize such production and make it work in a profitable and sustainable way.
Bering Ltd. from Slovenia in cooperation with Milk & More Dairy Consulting, Germany has long years of experience in this area ranging from the extraction of lactose and whey protein concentrate (WPC) production to extracting highly valued proteins and special products such as lactulose, lactitol, lactoferrin, lactoperoxidase, phospholipids.

Keywords: whey, whey processing, whey fractioning, whey proteins, lactose decoloring, whey protein concentrate (WPC), lactulose, lactitol, lactoferrin, lactoperoxidase, phospholipids

Introduction / Further subtitles
- Description of whey ingredients
- Whey fractionation technology and expected profitability
- Main equipment for whey fractioning
- Whey fractioning – general processing diagram and products
- Technologies for the production of different products out of whey
- Turnkey solutions from Bering

Figure 1: Main equipment for the whey processing

Figure 2: Product of whey fractionation

Preizkušanje različnih gonilnih raztopin s procesom osmoze za koncentriranje kisle sirotke

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Povzetek
Ena izmed obetavnih tehnologij za koncentriranje živilskih proizvodov je uporaba procesa osmoze. Ta tehnologija ima več prednosti pred drugimi membranskimi tehnologijami, kot je spontanost transporta vode brez delovanja z visokimi hidravličnimi tlaki in s tem priložnost za varčevanje z energijo. V okviru raziskave smo preizkušali različne gonilne raztopine (natrijev klorid, magnezijev klorid, kalcijev klorid, kalijev laktat, natrijev citrat dihidrat, amonijev acetat) za namen koncentriranja osiromašene kisle sirotke ob uporabi votlo-vlaknastih osmoznih membran. Ugotovili smo, da z vsemi raztopinami soli z enakim izhodiščem (primerljiv začetni osmotski tlak) dosežemo podobno učinkovitost. Ob uporabi 0,5 L sirotke in 1 L gonilne raztopine, ki so dale razliko osmotskega tlaka 32,2 - 38,2 bar, je koncentriranje sirotke bilo končano v štirih urah z doseženo 66,0 - 70,8 % učinkovitostjo procesa.

Ključne besede: kisla sirotka, osmoza, gonilna raztopina, osmotski tlak, votlo-vlaknaste membrane

Uvod
Na področju obdelave odpadne vode, priprave pitne vode ter čiščenja vode za njeno ponovno uporabo, je na voljo več vrst membranskih separacijskih procesov. Ena izmed izpopolnjenih membranskih tehnologij je proces osmoze. Gre za proces, ki zadržuje tako delce kot tudi ione, in sicer na energetsko učinkovit način brez uporabe zunanjega tlaka (v primerjavi z reverzno osmozo), zaradi česar osmoznim procesom zadnja leta posvečajo veliko pozornosti. Prenos vode poteka skozi polprepustno membrano na spontan način na osnovi naravnih zakonitosti. Gonilno silo procesa predstavlja razlika v osmotskem tlaku med osnovno in gonilno raztopino, zaradi česar voda prehaja skozi membrano iz manj koncentrirane (osnovne) raztopine v bolj koncentrirano (gonilno) raztopino. Bistvena prednost osmoznih procesov je zmanjšanje mašenja membran zaradi neuporabe zunanjega tlaka. Proces osmoze se odlično obnese tudi v kombinaciji z drugimi vrstami membranskih separacijskih procesov. Na primeru obdelave izcednih voda, odpadnih oljnih emulzij, proizvodnje pitne vode in celo pri koncentriranju živilskih proizvodov, kjer je osmozni sistem bil umeščen pred reverzno-osmoznim (RO) sistemom kot pred-obdelava, se je izkazalo, da proces dobro deluje, ker obratuje v zaprtem krogu, kjer se gonilna raztopina ponovno koncentrira z RO postopkom. V takšnih aplikacijah lahko osmozni sistem zaščiti RO sistem, saj vpliva na zmanjšanje koncentracijske polarizacije in podaljša življenjsko dobo membrane. V svetovnem merilu so osmozo uspešno uporabili za obdelavo procesne vode v mlekarski industriji. V manjšem obsegu (Chen in sod., 2019; Aydiner in sod., 2013; Aydiner in sod., 2013(a); Aydiner in sod., 2014; Wang in sod., 2017) lahko zasledimo uporabo osmoznih procesov tudi pri obdelavi samega mleka oz. sirotke. Osmoza je nov postopek, ki pri nas v Sloveniji še ni v uporabi.

Eksperimentalni del
Za osmozne filtracije smo uporabili 0,5 L osiromašene kisle sirotke (permeat mikrofiltracije, ki mu je bil odvzet del proteinov) kot osnovne raztopine in 1 L raztopine soli kot gonilne raztopine. V Tabeli 1 so prikazane gonilne raztopine z enakim osmotskim tlakom, ki smo jih uporabili pri eksperimentih. Začetna razlika v osmotskih tlakih med osnovno in gonilno raztopino je bila v razponu od 32,2 do 38,2 bar.

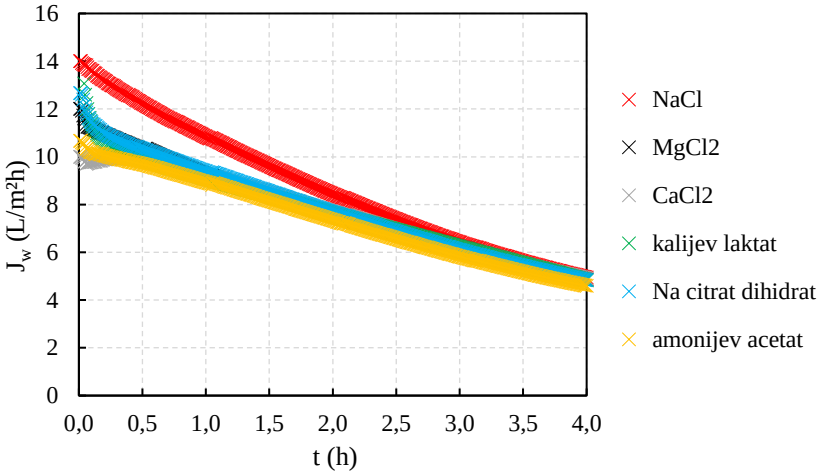
Tabela 1: Osmotski tlak uporabljenih gonilnih raztopin.

Vrsta gonilne raztopine	Osmotski tlak (bar)
1 M NaCl (natrijev klorid)	41-42 bar
0,7 M $MgCl_2$ (magnezijev klorid)	41-42 bar
0,7 M $CaCl_2$ (kalcijev klorid)	41-42 bar
1,4 M $KC_3H_5O_3$ (kalijev laktat)	41-42 bar
0,67 M $Na_3C_6H_5O_7$ x 2 H_2O (natrijev citrat dihidrat)	41-42 bar
0,88 M $NH_4CH_3CO_2$ (amonijev acetat)	41-42 bar

Proces osmoze je potekal z uporabo laboratorijske izvedbe votlo-vlaknastega osmoznega membranskega modula (Aquaporin A/S, Kgs. Lyngby, Danska) s skupno površino membrane 180 cm^2 in s premerom posameznega vlakna 195 µm.

Rezultati in diskusija

Ob uporabi šestih različnih gonilnih raztopin smo izvedli osmozne filtracije (Slika 1) za namen koncentriranja osiromašene kisle sirotke.



Slika 1: Fluks vode skozi membrano v odvisnosti od časa ob uporabi različnih gonilnih raztopin pri koncentriranju osiromašene kisle sirotke.

Iz Slike 1 vidimo, da se začetni fluks vode, kljub primerljivi gonilni moči raztopin, nekoliko razlikuje glede na vrsto uporabljene gonilne raztopine. Ne glede na to, se fluks po štirih urah obratovanja v vseh primerih ustali pri 4,6 – 4,8 $Lm^{-2}h^{-1}$. Razlika v osmotskih tlakih med obema raztopinama pade na 0,9 – 2,8, zaradi česar se prenos vode skozi membrano ustavlja. Z vsemi gonilnimi raztopinami smo prišli do podobnega rezultata. Sirotka se je skoncentrirala za faktor 2,9 - 3,4.

Zaključek

Na podlagi izvedenih filtracij lahko zaključimo, da smo iz osiromašene kisle sirotke z uporabo različnih gonilnih raztopin s primerljivo začetno gonilno močjo v štirih urah uspeli odvesti 66,0 - 70,8 % vode. To pomeni, da se je sirotka skoncentrirala za faktor 2,9 - 3,4. Pri tem je potrebno poudariti, da bi višji koncentracijski faktor lahko bil dosežen z uporabo večje začetne količine gonilne raztopine, kar bi zmanjšalo vpliv redčenja gonilne raztopine tekom procesa, s čimer bi gonilna moč raztopin soli slabela počasneje. Še optimalnejši pristop pa bi bil vzdrževanje konstantne gonilne moči raztopin soli tekom obratovanja s kontinuirnim dodajanjem koncentrata, s čimer moč gonilne raztopine tekom procesa sploh ne bi pojenjala. Z raziskavo smo pokazali, da vse gonilne raztopine dajejo podobne rezultate. Vsekakor pa bi z vidika dostopnosti in cene bila raztopina NaCl najprimernejša izbira.

Zahvala

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Testing of different draw solutions in forward osmosis process for concentration of acid whey

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Abstract
One of the promising technologies for concentrating food products is the use of the forward osmosis processes. This technology has several advantages over other membrane technologies, such as the spontaneity of water transport without operating with high hydraulic pressures and thus the opportunity to save energy. As part of the study, various draw solutions (sodium chloride, magnesium chloride, calcium chloride, potassium lactate, sodium citrate dihydrate, ammonium acetate) were tested for the purpose of concentrating depleted acid whey using hollow fiber forward osmosis membranes. We found that all tested salt solutions with the same starting point (comparable initial osmotic pressure) resulted to similar efficiency. When using 0.5 L of whey and 1 L of draw solution, which gave a difference in osmotic pressure of 32.2 - 38.2 bar, the whey concentrating was completed in four hours with achieved efficiency of 66.0 - 70.8%.

Keywords: acid whey, forward osmosis, draw solution, osmotic pressure, hollow fibre membranes

Introduction
In the field of the wastewater treatment, the preparation of drinking water and the purification of water for its re-use, a lot of different membrane separation processes are available. One of the advanced membrane technologies is the Forward Osmosis. By definition, it is a separation process that keeps both particles and ions in an energy efficient way without using external pressure (as it is the case in reverse osmosis). Therefore, in recent years, osmosis has gained much attention. Forward osmosis is carried out spontaneously, based on natural laws. Osmosis processes exploit the osmotic pressure difference between feed and draw solution as driving force, which causes the water to pass through the semipermeable membrane from a less concentrated (feed) solution to a more concentrated (draw) solution. The main advantage is the reduction of membrane fouling because of working without additional energy consumption. In the case of leachate, waste oil emulsions, drinking water production, and even the concentration of food products, where the forward osmosis system was installed as pre-treatment prior to the reverse osmosis system, it turned out that the process worked well, because it operated in a closed circle, where the 'draw solution' is again concentrated by the reverse osmosis process. In such applications, forward osmosis can protect the reverse osmosis system, affect the reduction of the concentration polarisation, and prolong the life of the membrane. On the global scale, osmosis was successfully used to treat process water in the dairy industry. To a lesser extent (Chen et al., 2019; Aydiner et al., 2013; Aydiner et al., 2013 (a); Aydiner et al., 2014; Wang et al., 2017) we can trace the use of osmotic processes also in the processing of milk/whey itself. Osmosis is a new process and is not widely used in Slovenia yet.

Experimental part
For forward osmosis filtrations, 0.5 L of depleted acid whey (permeate of microfiltration, from which some proteins were extracted) was used as the feed solution and 1 L of the salt solution as the draw solution. Table 1 shows the salt solutions with the same osmotic pressure, which were used as draw solutions in the experiments. The initial difference in osmotic pressures between the feed and draw solutions ranged from 32.2 to 38.2 bar.

Table 1: Draw solutions and their osmotic pressures.

Type of the draw solution	Osmotic pressure (bar)
1 M NaCl (sodium chloride)	41-42 bar
0,7 M $MgCl_2$ (magnesium chloride)	41-42 bar
0,7 M $CaCl_2$ (calcium chloride)	41-42 bar
1,4 M $KC_3H_5O_3$ (potassium lactate)	41-42 bar
0,67 M $Na_3C_6H_5O_7$ x 2 H_2O (sodium citrate dihydrate)	41-42 bar
0,88 M $NH_4CH_3CO_2$ (ammonium acetate)	41-42 bar

The forward osmosis process was performed using a hollow fiber forward osmosis membrane lab-scale module (Aquaporin A/S, Kgs. Lyngby, Denmark) with a total membrane surface area of 180 cm^2 and a single fiber diameter of 195 µm.

Results and discussion

Forward osmosis filtrations (Figure 1) were performed using six different draw solutions to concentrate depleted acid whey.

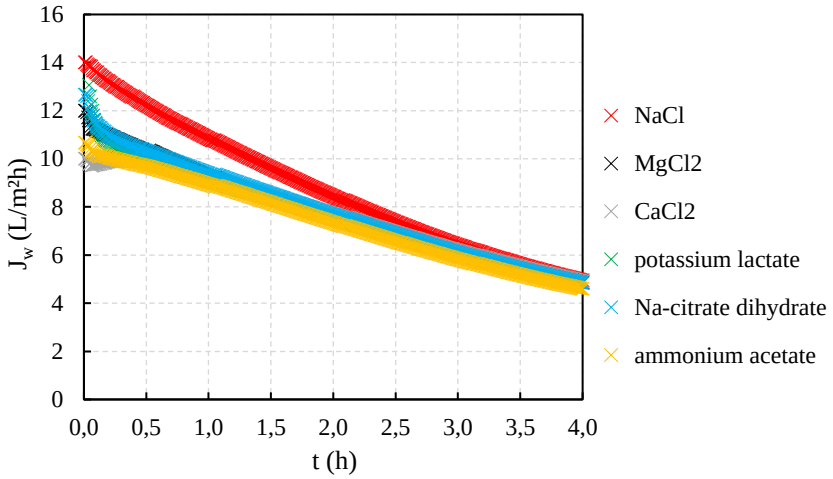

Figure 1: Water flux through the membrane as a function of time using different draw solutions in the concentrating of depleted acid whey.

From Figure 1 we can see that the initial water flux, despite the comparable driving power of the solutions, differs slightly depending on the type of driving solution used. Nevertheless, the flux stabilizes at 4.6 - 4.8 $Lm^{-2}h^{-1}$ after four hours of operation. The difference in osmotic pressures between the two solutions drops to 0.9 - 2.8, which stops the transfer of water through the membrane. A similar result was obtained with all draw solutions. Whey was concentrated by a factor of 2.9 - 3.4.

Conclusion

Based on the performed filtrations, we can conclude that we managed to remove 66.0 - 70.8% of water from the depleted acid whey using different draw solutions with comparable initial drawing power in four hours. This means that the whey was concentrated by a factor of 2.9 - 3.4. It should be noted that a higher concentration factor could be achieved by using a larger initial amount of draw solution, which would reduce the effect of diluting the draw solution during the process, thus weakening the driving power of salt solutions more slowly. An even more optimal approach would be to maintain a constant driving power of the salt solutions during operation by continuous adding of stock solution, so that the power of the draw solution would not decrease at all during the process. Research has shown that all draw solutions give similar results. However, in terms of affordability and price, NaCl solution would be the most appropriate choice.

Acknowledgements

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Uporaba kromatografskih postopkov na monolitnih kolonah za izolacijo proteinov in drugih bioaktivnih molekul v živilski industriji

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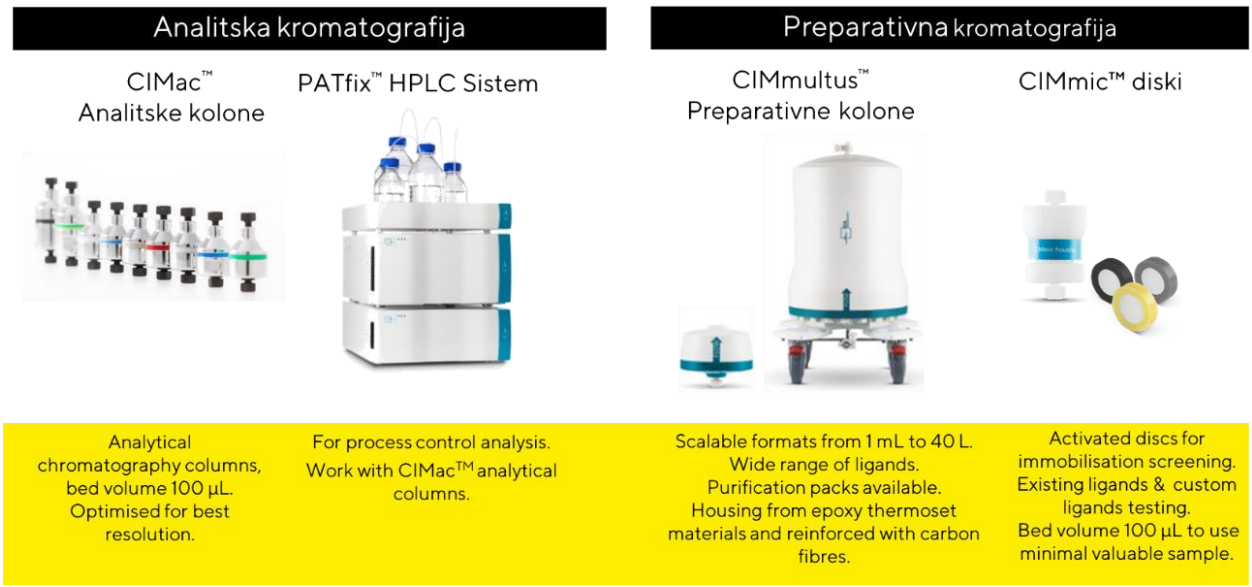
Povzetek
Za izolacijo in čiščenje različnih kemijskih in bioloških spojin je ena izmed najpogosteje uporabljenih metod tekočinska kromatografija, kjer se komponente v vzorcu ločujejo na podlagi razporeditve med mobilno in stacionarno fazo. BIA Separations je vodilno podjetje v razvoju monolitne tehnologije in edini proizvajalec monolitnih kromatografskih kolon CIM (angl. Convective Interaction Media) za proizvodnjo, čiščenje in analizo večjih bioloških molekul, kot so virusi, plazmidna DNK, eksosomi, protitelesa, bakteriofagi, večji proteini itd. Prav kromatografija je trenutno najpomembnejša metoda, ki omogoča očiščenje ciljnih makromolekul do stopnje, ki je potrebna za uporabo v terapevtske namene (Štrancar in sod., 2002). Poleg omenjenih področij pa različne metode kromatografije uporabljamo tudi v živilski industriji za izolacijo in ločevanje različnih bioaktivnih spojin iz hrane (Cavaliere in sod., 2018).

Ključne besede: kromatografija, monolitne kolone CIM, bioaktivne spojine, kisla sirotka

Uvod

Poenostavljeno lahko rečemo, da monolitne kolone CIM delujejo kot pametni filtri. Pri monolitni koloni stacionarno fazo predstavlja porozen polimer iz enega kosa. Kanali v monolitu so med seboj povezani in odprti, za razliko od delčnih kolon, kjer večina interakcij poteka v ti. slepih porah. Glavna prednost monolitov pred standardnimi delčnimi kolonami je masni prenos analita med mobilno in stacionarno fazo, ki poteka preko konvekcije in omogoča ločbo neodvisno od pretoka. Tehnologija omogoča majhen padec tlaka na koloni, kar vpliva na hitro ločbo, pri čemer se ohranjajo visoke vezavne kapacitete in kromatografske resolucije (Barut in sod., 2008). So zelo preprosti za uporabo in tako omogočajo hitre procese, z njimi dosegamo veliko čistost končnih produktov, omogočajo doseganje visoke kapacitete in visoke izkoristke. V primerjavi s tradicionalnimi kromatografskimi sistemi imajo monolitni sistemi CIM mnogo prednosti, ki pa so najbolj izrazite pri ločevanju in koncentriranju večjih bioloških makromolekul. Izjemen potencial za imobilizacijo ligandov na monolite je privedel do uporabe te tehnologije v analitske in preparativne namene. BIA Separations ima na razpolago različne kolone linije CIMac, ki ustrezajo analitskim potrebam in kolone preparativne linije CIMmultus, kjer je izbor velikosti kolon zelo širok (od 1 mL pa vse do 40 L) (Slika 1).



Slika 3: Nabor vseh monolitnih kolon CIM™ in analitskih sistemov podjetja BIA Separations.

Kromatografija v živilski industriji

Zadnje čase se vse več pozornosti posveča nutricevtikom in drugim bioaktivnim spojinam, ki so naravno prisotne v hrani. Ohranjanje zdravja in preprečevanje bolezni z uživanjem zdrave hrane je privlačno tako za potrošnike kot tudi za živilsko industrijo. Prav zaradi tega je zelo pomembno okarakterizirati bioaktivne spojine in jim pripisati specifično delovanje. Kemična raznolikost in prisotnost v kompleksnih mešanicah predstavlja velik izziv tako za izolacijo kot tudi za karakterizacijo teh spojin. Prav zaradi tega so potrebni učinkoviti sistemi za ločevanje, večina teh pa temelji ravno na kromatografiji. Najpogostejše bioaktivne spojine v hrani so polifenoli, fenoli, karotenoidi in različni peptidi oz. proteini (med te spadajo tudi encimi). Za izolacijo teh spojin se uporablja različne kromatografske postopke. Uporaba monolitnih kolon je prevladujoča predvsem na področju izolacije večjih proteinov, kjer se običajno izkorišča načela ionsko-izmenjevalne kromatografije (Cavaliere in sod., 2018).

Monolitne kolone in kisla sirotka

V zadnjem času so vse bolj iskani tudi posamezni proteini iz sirotke, ki je načeloma stranski produkt mlekarske industrije. Posamezni proteini sirotke, kot so na primer laktoferin, laktoperoksidaza in imunoglobulini, ne predstavljajo samo vira aminokislin, temveč so pomembni regulatorni dejavniki s široko biološko vlogo. Iz kisle sirotke lahko s pomočjo kationske-izmenjevalne kromatografije izoliramo več različnih proteinov. V projektu LIFE for Acid Whey je bila raziskana uporaba inovativne tehnologije monolitnih kromatografskih kolon, ki so omogočile veliko hitrejšo separacijo sirotkinih proteinov v primerjavi z delčnimi kolonami, saj omogočajo konvekcijski tok skozi kolone, kjer transport proteinov do ligandov ni omejen z difuzijo. Prednost je vsekakor tudi večkratna uporaba monolitnih kolon, saj le-te prenesejo zelo rigorozno čiščenje z 1 M NaOH in so tako ustrezno očiščene in pripravljene za ponovno uporabo.

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Application of chromatographic procedures on monolithic columns for isolation of proteins and other bioactive compounds in food industry

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Abstract
For the isolation and purification of various chemical and biological compounds, one of the most commonly used methods is liquid chromatography, where the components in the sample are separated based on the distribution between the mobile and stationary phase. BIA Separations is a leading company in the development of monolithic technology and the only manufacturer of CIM (Convective Interaction Media) monolithic chromatographic columns for the production, purification and analysis of bigger biological molecules such as viruses, plasmid DNA, exosomes, antibodies, bacteriophages, bigger proteins, etc. Chromatography is currently the most important method that allows the purification of target macromolecules to the extent necessary for use in therapeutic purposes (Štrancar et al., 2002). In addition to the mentioned fields, various chromatography methods are also used in the food industry for the isolation and separation of different bioactive compounds from food (Cavaliere et al., 2018).

Keywords: chromatography, CIM monolithic columns, bioactive compounds, acid whey

Introduction
We can say that CIM monolithic columns act as smart filters. In the case of a monolithic column, the stationary phase is represented by a porous polymer in one piece. The channels in the monolith are interconnected and open, unlike porous particle columns, where most of the interactions take place in the so-called dead-end pores. The main advantage of monoliths over standard porous particle columns is the mass transfer of the analyte between the mobile and stationary phases, which takes place via convection and allows separation independent of the flow. The technology allows for a small drop in column pressure, which results in rapid separation while maintaining high binding capacities and chromatographic resolutions (Barut et al., 2008). They are very easy to use and thus enable fast processes, high purity of the final products, high capacity and high efficiency. Compared to traditional chromatographic systems, monolithic CIM systems have many advantages, which are especially pronounced in the separation and concentration of larger biological macromolecules. The remarkable potential for immobilizing ligands on monoliths has led to the use of this technology for analytical and preparative purposes. BIA Separations offer various CIMac columns for analytical needs and CIMmultus preparative columns, where the choice of column sizes is very wide (from 1 mL up to 40 L) (Figure 1).

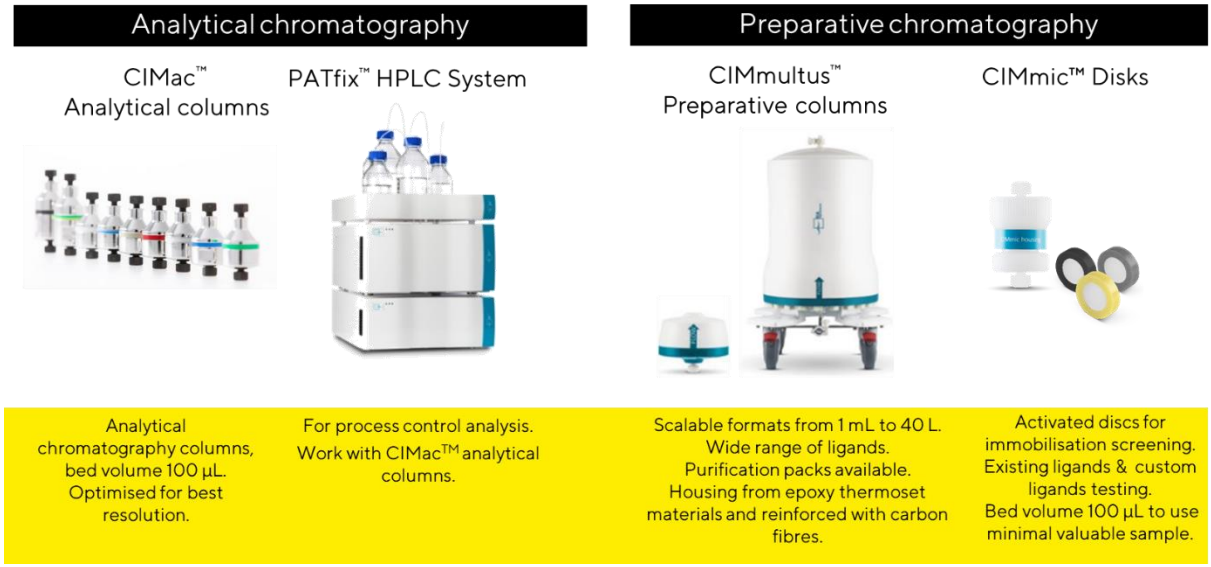

Figure 3: Portfolio of all CIM™ monolithic columns and analytical systems of BIA Separations.

Chromatography in food industry

Recently, a lot of attention is dedicated to nutraceuticals and other bioactive compounds naturally present in food. Maintaining health and preventing disease by eating healthy food is attractive for both consumers and food industry. This is also the reason why it is very important to characterize bioactive compounds and ascribe a specific activity to them. Chemical diversity and presence in complex mixtures or matrices is very challenging for both the isolation and characterization of these compounds. Because of that efficient separation systems are needed and most of them are based on chromatography. The most common bioactive compounds in food are polyphenols, phenols, carotenoids and various peptides or proteins (these also include enzymes). Various chromatographic procedures are used to isolate these compounds. The use of monolithic columns is predominant in the field of larger proteins isolation, where the principles of ion-exchange chromatography are usually used (Cavaliere et al., 2018).

Monolithic columns and acid whey

Nowadays, demand for individual whey proteins, which are in principle a by-product of the dairy industry, is increasing. Individual whey proteins, such as lactoferrin, lactoperoxidase and immunoglobulins, are not only a source of amino acids, but are also important regulatory factors with a broad biological role. Several different proteins can be isolated from acid whey by cation exchange chromatography. The LIFE for Acid Whey project investigated the use of innovative monolithic chromatographic column technology, which enabled much faster separation of whey proteins compared to porous particle columns, as they allow convective flow through columns where protein transport to ligands is not limited by diffusion. The multiple use of monolithic columns is certainly an advantage, as they can withstand very rigorous cleaning with 1 M NaOH and are thus properly cleaned and ready for reuse.

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Hranilne, funkcionalne in bioaktivne lastnosti sirotkinih proteinov ter z njimi povezane rabe

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Povzetek
Sirotka predstavlja bogat vir proteinov z visokim komercialnim potencialom. Poleg odlične zastopanosti esencialnih aminokislin izkazujejo tudi biološke aktivnosti, kot so protimikrobno in protirakavo delovanje ter vpliv na razvoj črevesja in možganov. Pri razgradnji s prebavnimi encimi nastanejo bioaktivni peptidi s protimikrobnim in antihipertenzičnim delovanjem. Uporabljajo se v prehrani, kot sestavina živil, živil za posebne zdravstvene namene, mlečnih formul ali kot prehranska dopolnila, pa tudi v kozmetični in farmacevtski industriji, kot stabilizatorji ali protimikrobna sredstva. Pridobivanje sirotkinih proteinov iz sirotke je posebej zanimivo tudi zato, ker sirotka predstavlja najpomembnejši stranski produkt v mlekarski industriji in je zato lahko dostopna ter je njena ponovna uporaba zaželjena, tako z vidika varovanja okolja, kot tudi z ekonomskega vidika.

Ključne besede: sirotkini proteini, bioaktivni proteini, bioaktivni peptidi

Uvod
Kravje mleko vsebuje približno 3,5 % proteinov, od česar 80 % predstavljajo kazeini, 20 % pa sirotkini proteini (6,5 g/L). Večina slednjih, odvisno od tehnološkega postopka, ob koagulaciji kazeinov preide v sirotko. Narava je z evolucijo poskrbela, da mleko, posledično pa tudi sirotka kot stranski produkt predelave mleka, vsebujeta snovi, ki so za človeka koristne. Razen hranilne vrednosti so bile opisane številne bioaktivne lastnosti sirotkinih proteinov in / ali peptidov, ki nastanejo z razgradnjo s prebavnimi encimi. Nekateri sirotkini proteini pa izkazujejo tudi tehnološko zanimive lastnosti, ki se s pridom uporabljajo v industriji. V živilih in krmi sirotkine proteine uporabljajo kot skupne izolate proteinov (npr. WPI – izolat sirotkinih proteinov, ki se uporablja predvsem v športni prehrani) ali kot očiščene formulacije posameznega proteina (npr. v medicinski prehrani).

Hranilne in bioaktivne lastnosti sirotkinih proteinov ter njihova uporaba
Sirotkini proteini imajo visoko vsebnost esencialnih aminokislin (AK), med katerimi je pomemben predvsem levcin, ki uravnava izgradnjo novih proteinov in predstavlja 9 % do 12 % AK v sirotkinih proteinih (Banaszek et al., 2018, Auestad et al., 2021). Poleg tega služijo sirotkini proteini kot prenašalci hranil (vitaminov, maščobnih kislin in kovinskih ionov), imajo pa tudi širok nabor bioaktivnega delovanja.

Preglednica 1 prikazuje najbolj poznane sirotkine proteine, njihovo aktivnost in uporabo. V kravjem mleku je najbolj zastopan sirotkin protein β-laktoglobulin (β-LG), ki predstavlja približno 60 % vseh sirotkinih proteinov. V humanem mleku tega proteina ni, vsebuje pa zato višje koncentracije skoraj vseh ostalih (z izjemo serumskega albumina, SA, ki je enako zastopan v obeh vrstah mleka): α-laktalbumina (α-LA, 13% vseh sirotkinih proteinov v kravjem mleku), imunoglobulinov (Ig), laktoferina (Lf), laktoperoksidaze (LPO), osteopontina (OPN) in lizocima. V sladko sirotko se med sirjenjem sprosti glikomakropeptid, ki ga zaradi narave nastanka ne pričakujemo v humanem mleku.

Preglednica 1: Hranilne in bioaktivne lastnosti sirotkinih proteinov ter njihova uporaba

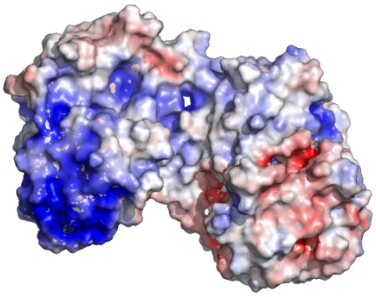
Sirotkini proteini	**Koncentracija v kravjem (humanem) mleku (g/L)**	**Biološka aktivnost (Haschke et al., 2016, Auestad et al., 2021)**	**Uporaba (Auestad et al., 2021, Kukovics et al., 2013, Kitts et al., 2003)**
β-LG	2,9 – 5,5 (/)	Prenašalec hranil (maščobne kisline, vitamini), protimikrobna aktivnost, protirakavo delovanje	Uporaba v prehrani (esencialne AK), lastnosti penjenja in želiranja
α-LA	1,2 (1,9)	Prenašalec hranil (Ca, maščobne kisline, vitamini), protivnetno delovanje, protimikrobna aktivnost	Uporaba v prehrani (esencialne AK), dodatek v mlečnih formulah
GMP*	1,2 (/)	Uravnavanje prebave in metabolizma, protirakavo delovanje, uravnavanje	Ne vsebuje Phe: prehrana ljudi s fenilketonurijo

		imunskega sistema, protimikrobno delovanje, prebiotično delovanje.	
Ig	0,7 (1,3)	Uravnavanje imunskega sistema, protimikrobna aktivnost	Protimikrobno delovanje: krma, prehranska dopolnila
Lf	0,1-0,5 (1,5 – 4,0)	Uravnavanje imunskega sistema, protimikrobna aktivnost, razvoj črevesja, protirakavo delovanje, prenašalec železa.	Protimikrobno delovanje: prehrana; dodatek v mlečne formule (esencialne AK, bioaktivnost), krma, medicina, kozmetika
SA	0,4 (0,4)	Prenašalec hranil (kovinski ioni, maščobne kisline)	Kot stabilizator v znanosti in medicini.
LPO	0,03 (1,5)	Protimikrobna aktivnost, degradacija karcinogenov, zaščita pred peroksidativnimi učinki	Protimikrobno delovanje: prehrana, medicina, kozmetika, podaljšanje obstojnosti mleka
OPN	0,018 (0,14)	Uravnavanje imunskega sistema, razvoj črevesja, možganov, kosti	Dodatek v mlečnih formulah, prehranska dopolnila

*GMP nastane s cepitvijo κ-kazeina in je prisoten le v sirotki, ki nastane z encimsko koagulacijo.

Lf kot modelni bioaktivni protein

Slika 1 prikazuje Lf kot modelni bioaktivni protein v sirotki. Modro označen N-konec Lf je močno pozitivno nabit in je odgovoren za protimikrobno aktivnost, ki ni odvisna od vezave železa. Ta del Lf po cepitvi s pepsinom predstavlja laktofericin, peptid s protimikrobno aktivnostjo, ki je lahko tudi višja od protimikrobne aktivnosti izvirnega Lf. Laktofericin se veže na negativno nabito zunanjo bakterijsko membrano, se internalizira in povzroči večjo permeabilnost citoplazemske membrane, s tem pa porušenje transmembranskega potenciala (Gifford et al., 2005).



Slika 1: Prostorski model laktoferina s pobarvanimi AK ostanki glede na naboj.

Zaključek

Zanimanje za izrabo sirotkinih proteinov nenehno narašča. Zaradi širokega nabora aktivnosti, hranilne vrednosti in možne uporabe predstavljajo vir, ki ga ob rasti prebivalstva v naslednjih desetletjih ne bi smeli prezreti. Sirotka kot stranski produkt predstavlja idealni vir za pridobivanje sirotkinih proteinov.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey (št. pogodbe LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (št. pogodbe OP20.03521) Operativnega programom EKP 2014 – 2020.

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Nutritional, functional and bioactive properties of whey proteins and related use

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Abstract
Whey is a rich source of proteins with high commercial potential. In addition to being an excellent source of essential amino acids, they also exhibit biological activities such as antimicrobial and anticancer activity and they influence the development of the intestine and the brain. When degraded by digestive enzymes, bioactive peptides with antimicrobial and antihypertensive activity are generated. They are used in nutrition, as food ingredients, in foods for special medical purposes, milk formulas or as dietary supplements and also in the cosmetics and pharmaceutical industries, as stabilizers or antimicrobial agents. The extraction of whey proteins from whey is particularly interesting because whey is the main by-product in the dairy industry and is therefore easily accessible and reusable, both in terms of environmental protection and from an economic point of view.

Keywords: whey proteins, bioactive proteins, bioactive peptides

Introduction
Bovine milk contains approximately 3.5% protein, of which 80% is casein and 20% is whey protein (6.5 g / L). Most of the latter, depending on the technological process, are released into whey during the coagulation of caseins. With evolution, nature has ensured that milk, and therefore also whey as a by-product of milk processing, contains substances that are beneficial to humans. In addition to their nutritional value, a number of bioactive properties of whey proteins and/or peptides resulting from degradation by digestive enzymes, have been described. However, some whey proteins also exhibit technologically interesting properties that make them useful in industry. In food and feed, whey proteins are used as whey protein isolates (e.g. WPI - used especially in sports nutrition) or as purified formulations of individual proteins (e.g. in medical nutrition).

Nutritional and bioactive properties of whey proteins and their use
Whey proteins have a high content of essential amino acids (AA), of which leucine is particularly important as it regulates the synthesis of new proteins and represents 9% to 12% of all AA in whey proteins (Banaszek et al., 2018, Auestad et al., 2021). In addition, whey proteins serve as carriers of nutrients (vitamins, fatty acids and metal ions) and have a wide range of biological activities.

Table 1 shows the most common whey proteins, their bioactivity and uses. The most common whey protein in bovine milk is β-lactoglobulin (β-LG), which accounts for about 60% of all whey proteins. β-LG is not present in human milk. Human milk, however, contains higher concentrations of almost all other common whey proteins (with the exception of serum albumin, SA, which is present in equal amounts in both types of milk): α-lactalbumin (α-LA, 13% of all whey proteins in bovine milk), immunoglobulins (Ig), lactoferrin (Lf), lactoperoxidase (LPO), osteopontin (OPN) and lysozyme. Glycomacropeptide is released into sweet whey during casein coagulation, and is not expected in human milk due to the nature of its formation.

Table 1: Nutritional and bioactive properties of whey proteins and their use

Whey protein	**Concentration in bovine (human) milk (g / L)**	**Biological activity (Haschke et al., 2016, Auestad et al., 2021)**	**Use (Auestad et al., 2021, Kukovics et al., 2013, Kitts et al., 2003)**
β-LG	2,9 – 5,5 (/)	Nutrient transporter (fatty acids, vitamins), antimicrobial activity, anticancer activity	Dietary use (essential AA), foaming and gelling properties
α-LA	1,2 (1,9)	Nutrient transporter (Ca, fatty acids, vitamins), anti-inflammatory action, antimicrobial activity	Dietary use (essential AA), additive in milk formulas
GMP*	1,2 (/)	Regulation of digestion and metabolism, anticancer activity, regulation of the immune system, antimicrobial activity, prebiotic activity	Does not contain Phe: medical nutrition in people with phenylketonuria

Ig	0,7 (1,3)	Modulation of the immune system, antimicrobial activity	Antimicrobial activity: animal feed, dietary supplements
Lf	0,1-0,5 (1,5 – 4,0)	Modulation of the immune system, antimicrobial activity, intestinal development, anticancer activity, iron transporter.	Antimicrobial action: nutrition; additive in milk formulas (essential AA, bioactivity), animal feed, use in medicine and cosmetics
SA	0,4 (0,4)	Nutrient transporter (metal ions, fatty acids)	As a stabilizing protein in science and medicine
LPO	0,03 (1,5)	Antimicrobial activity, carcinogen degradation, protection against peroxidative effects	Antimicrobial activity: nutrition, medicine, cosmetics, prolonging shelf life of milk
OPN	0,018 (0,14)	Modulation of the immune system, development of the intestine, brain, bones	Additive in milk formulas, dietary supplements

* GMP is formed by cleavage of κ-casein and is present only in whey formed by enzymatic coagulation.

Lf as a model bioactive protein

Figure 1 shows Lf as a model bioactive protein in whey. The blue-colored N-terminus of Lf is strongly positively charged and is responsible for its iron-independent antimicrobial activity. This portion of Lf after cleavage with pepsin represents lactoferricin, a peptide with antimicrobial activity that can also be higher than the antimicrobial activity of the original Lf. Lactoferricin binds to the negatively charged outer bacterial membrane, internalizes, and causes greater permeability of the cytoplasmic membrane, disrupting the transmembrane potential (Gifford et al., 2005).

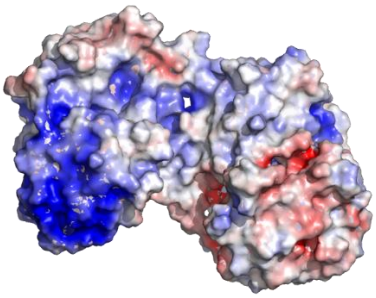

Figure 1: Space-filling model of lactoferrin with residues colored with respect to charge.

Conclusion

Interest in the use of whey proteins is constantly increasing. Due to the wide range of biological activities, nutritional value and possible uses, they represent a resource that should not be overlooked in the coming decades as the population grows. Whey as a waste material represents an ideal source for obtaining whey proteins.

Acknowledgements

This research was co-funded by the project LIFE for Acid Whey (contract No. LIFE16 ENV/SI/000335) of the European financial instrument LIFE and the project LAKTIKA (contract No. OP20.03521) in the frame of the Operational Programme ECP 2014 – 2020.

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Razvoj metode in optimiziranega industrijskega postopka izolacije laktoferina

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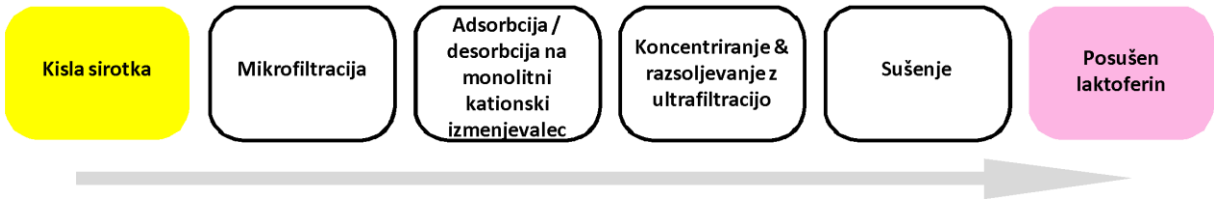
Povzetek
V začetni fazi raziskav smo razvili metodo izolacije laktoferina (LF) iz kisle sirotke (KS) na laboratorijskem nivoju, pri čemer smo uporabljali CIMac™ SO3-0.1 analitsko in CIMmultus™ SO3-1mL (BiaSeparations, Sartorius) kromatografsko kolono. Nato smo izvedli več optimizacij metode na manjšem preparativnem nivoju, pri čemer smo uporabljali 80 in 800 mL kolone. Rezultati testov so pokazali, da je prenos metode izolacije LF na višjo raven relativno enostaven, kar se je izkazalo tudi ob prehodu na polindustrijsko raven, kjer smo uporabljali 8L kromatografsko kolono. Na tem nivoju smo nato dodatno testirali različno kemijsko sestavo pufrov in izolacijo LF v kombinaciji z laktoperoksidazo (LPO) iz sladke sirotke (SS). Rezultat je mednarodna patentna prijava postopka izolacije LF. Nadalje smo za potrditev industrijske uporabnosti testirali ponovljivost kromatografske izolacije LF ter stabilnost delovanja 8L kolone. V ta namen smoopravili 138 kromatografskih ciklovna dveh 8L kolonah. Zaradi potrebe po racionalizaciji rabe surovin na industrijski ravni in zmanjševanju okoljskega odtisa proizvodnje, je bilo potrebno optimirati tudi količine vode, fosfatov in soli v pufernih raztopinah. V ta namen smo optimirali koncentracije pufernih raztopin in testirali različne pristope ponovne uporabe in recikliranja pufrov in njihovih sestavin. Preizkušali smo tudi različne pristope priprave sirotke na kromatografski postopek za povečanje vezavne kapacitete LF na SO3 monolitni kromatografski koloni. Pri tem nam je uspelo za 60-90% povečati kapaciteto kolone za vezavo LF iz sirotke v primerjavi z izhodiščno metodo. Rezultat optimizacije industrijskega postopka sta dve slovenski patentni prijavi.

Ključne besede: laktoferin, CIM monolitne kromatografska kolone, kisla sirotka, izolacija

Uvod
Kisla sirotka se velikokrat obravnava kot odpadek, vendar vsebuje proteine (npr. laktoferin – LF in laktoperoksidazo– LPO), ki izkazujejo protimikrobne, protivirusne in antioksidativne lastnosti in lahko nudijo zaščito pred rakavimi obolenji, boleznimi srca ter pomagajo pri krepitvi imunskega sistema (Gonzalez-Chavezin sod.,2009, Macwan in sod., 2016). Proteini, ki predstavljajo večinski delež v sirotki so β-laktoglobulin (β-LG), α-laktalbumin (α-LA), goveji serumski albumin (BSA) in imunoglobulini (npr. IgG). Poleg omenjenih sta prisotna tudi LPO in LF, a predstavljata občutno nižji delež celokupnih proteinov (<1%). Njuna nizka koncentracija in visoka bazičnost zahtevata specifičen pristop k izolaciji, pri čemer se najpogosteje uporablja ionsko izmenjevalna kromatografija (IEX).CIM monolitne kromatografske kolone (Bia Separations, Sartorius) se uporablja na širokem področju izolacije različnih bioloških molekul in virusov (Nesterenko, 2018). Njihove prednosti so predvsem v visoki ločljivosti, hitrosti ločbe in enostavnem prenosu metod iz laboratorijske na industrijsko raven. Za izolacijo LF iz KS smo uporabili kolono z močnim kationskim izmenjevalcem ($SO3^-$). Zaradi prisotnosti mikroorganizmov, proteinskih in maščobnih agregatov, drugih proteinov, sladkorjev in mlečne kisline, izolacija LF ni enostavna. To zahteva ustrezno predpripravo sirotke na samo kromatografijo, uporabo ustrezne metode, pufernih raztopin in več spremljajočih postopkov (**Slika 1**).



Slika 1. Shema poteka procesa izolacije laktoferina iz kisle sirotke.

Izolacija LF iz sirotke
Razvoj metode izolacije LF smo pričeli na laboratorijskem nivoju in pri tem uporabljali CIMac™ SO3-0.1 analitsko in CIMmultus™ SO3-1mL kromatografsko kolono. Omenjeno metodo smo nato optimirali na manjšem preparativnem nivoju, pri čemer smo uporabljali 80 in 800 mL kolone. Rezultati testov so pokazali, da je prenos metode izolacije LF na višjo raven relativno enostaven, kar se je izkazalo tudi ob prehodu na polindustrijsko raven, kjer smo uporabljali 8L kromatografsko kolono. Na tem nivoju smo dodatno testirali različno sestavo pufrov na osnovi fosfata, karbonata ali citrata in izolacijo LF/LPO iz sladke sirotke (SS). LF izolat smo z uporabo HPLC analitske kromatografije, SDS PAGE analize ter merjenjem bioaktivnosti LF

primerjali z drugimi na trgu prisotnimi LF. Rezultati karakterizacije z uporabo omenjenih metod so pokazali, da v primerjavi z ostalimi produkti dosegamo primerljivo, večkrat celo višjo čistost (>98%), ter bistveno višjo celokupno bioaktivnost (C + A vrednost =74,9%) v primerjavi z vsemi analiziranimi vzorci (C+A vrednost <66%, **Tabela 1**). Metodo za izolacijo LF smo v obliki patentne prijavevložili v več državah po svetu (Kete in sod., 2018).

Tabela 1. Bioaktivnost komercialno dostopnih LF in lastnega izolata LF, izoliranega s kromatografijo na monolitnih kolonah (Arhel d.o.o.). Celokupna bioaktivnost je izražena s seštevanjem C- in A-vrednosti, ki teoretično poda delež aktivnega proteina v vzorcu.

Vzorci laktoferina	Life Extension (Lactoferrin caps - Bioferrin; 95% Apolactoferrin)	Ingredia Nutritional (Prodiet Lactoferrin, >95%)	NRL Pharma	Sigma goveji laktoferin; ≥85%	Arhel d.o.o.
C-vrednost [%]	48,6* (31,1 – vz.)	52,1	49,2	7,5	70,0 -74,0
A-vrednost [%]	11,7* (7,5 – vz.)	7,7	9,0	58,5	2 - 4,9
Skupaj (A+C) [%]	60,3	59,8	58,2	66,0	74,9 (pov.)

Vzporedno z optimizacijo metode izolacije LF smo izvajali tudi testiranja predpriprave sirotke na samo izolacijo in ponovljivost kromatografskega cikla izolacije. Tako smo na polindustrijskem nivoju na dveh CIMmultus™ SO3-8000 (Bia Separations, Sartorius) monolitnih kromatografskih kolonah opravili 138 kromatografskih ciklov izolacije LF. Izkazalo se je, da postopek zagotavlja visoko stopnjo ponovljivosti, ohranjanje funkcionalnosti kromatografske kolone in izolacijo visoko čistega produkta LF z visoko bioaktivnostjo.

Optimizacija porabe surovin

Na polindustrijskem nivoju smo za dodatno optimizacijo pufernih raztopin (uporabljenih soli) testirali več načinovnjihoveponovneuporabe v postopku kromatografske izolacije,možnost recikliranja surovinin ponovne uporaberaztopin z visoko slanostjo v procesih zgoščevanja preostankov sirotke. Z rešitvami dosegamo polno odstranitev fosfatov iz pufernih raztopin in znatno zgostitev preostankov sirotke (Zupančič Justin s sod, 2021a).

Razvoj metode za povečanje vezavne kapacitete kromatografske kolone na LF

Z ciljem po višjem izkoristku posameznega kromatografskega cikla in s tem povečanjem kapacitete proizvodnje, smo preizkušali tudi različne možnostipovečanja vezavne kapacitete LF na SO3 monolitnokromatografskokolono.Z razvojem metode nam je uspelo povečati kapaciteto vezave LF za 60 do 90% v primerjavi z izhodiščnometodo izolacije (Zupančič Justin s sod., 2021).

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey (št. pogodbe LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (št. pogodbe OP20.03521) OP EKP 2014 – 2020. Za dobave sirotke se zahvaljujemo mlekarnam Planika, Celeia in Pomurske mlekarne. Za pomoč in usmeritve v fazah razvoja metode izolacije LF se zahvaljujemo dr Alešu Štrancarju, BIA Separations.

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Development of a method and an optimized industrial process for the isolation of lactoferrin

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Abstract
At the laboratory level of research, we developed an isolation method for lactoferrin (LF) from acid whey (AW), using CIMac ™ SO3-0.1 analytical and CIMmultus ™ SO3-1mL (Bia Separations, Sartorius) chromatographic columns. Several optimizations of the method were then performed at a lower preparative level, using 80 and 800 mL columns. The tests showed that transferring the method to a higher level is relatively easy, which was also confirmed bya transition to the semi-industrial level, where an8L chromatographic column was used. At this level, we then further tested the different chemical compositions of the buffers and the isolation of LF in combination with lactoperoxidase (LPO) from sweet whey (SW). The result is an international patent application for the LF isolation process. Furthermore, to confirm the industrial applicability, we tested the repeatability of the LF chromatographic isolation method and the operation stability of the 8L column. For this purpose, we performed 138 chromatographic cycles on two 8L columns. Due to the need to rationalize the use of raw materials at the industrial level and reduce the environmental footprint of production, it was also necessary to optimize the amount of water, phosphates and salts in buffer solutions. For this purpose, we optimized the concentrations of buffer solutions and tested different approaches to reuse and recycle buffers and their components. We also tested different approaches of preparation of AW before the isolation process to increase the LF binding capacity of SO3 monolithic chromatographic columns. In doing so, we managed to significantly increase the capacity (60-90%) compared to the initial method. Results of industrial process optimization are two Slovenian patent applications.

Keywords: lactoferrin, CIM monolithic chromatographic column, acid whey, isolation

Introduction
Acid whey (AW) is often considered a waste. Still, it contains proteins (lactoferrin - LF and lactoperoxidase - LPO), which have antimicrobial, antiviral and antioxidant properties and can provide protection against cancer, heart disease and help strengthen the immune system (Gonzalez-Chavez et al., 2009, Macwan et al., 2016). The most common proteins present are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (e.g., IgG). In addition to those, LPO and LF are also present, but they represent a significantly lower share of total proteins (<1%). Their low concentration and high alkalinity require a specific approach to isolation, with ion-exchange chromatography (IEX) being the most commonly used. CIM monolithic chromatographic columns (Bia Separations, Sartorius) are used in a wide range of isolation of various biological molecules and viruses (Nesterenko, 2018). Their advantages are mainly high resolution, separation speed and easy transfer of methods from laboratory to industrial level. For isolation of LF from AW,a column with a strong cation exchanger (SO3-) was used. However, isolation of LF is not easy due to the presence of microorganisms, protein and fat aggregates, other proteins, sugars, and lactic acid. Itrequires proper preparation of the whey for the chromatography itself, the use of the appropriate method, buffer solutions and several accompanying procedures (**Figure 1**).

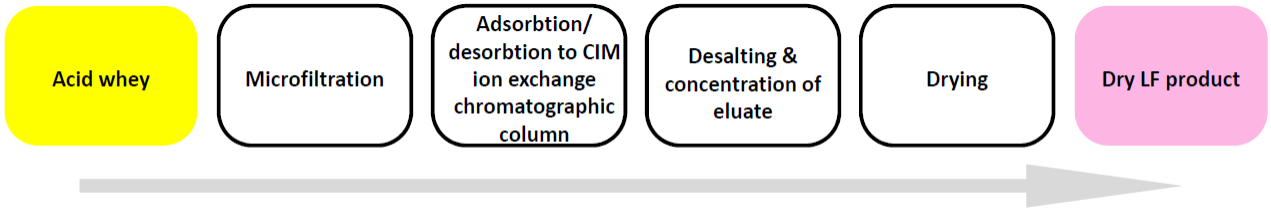

Figure 1. Process scheme of lactoferrin isolation from acid whey.

Isolation of LF from whey
The development of the LF isolation method was started at the laboratory level using CIMac ™ SO3-0.1 analytical and CIMmultus ™ SO3-1mL chromatographic column. After that, the obtained method was optimized at a lower preparative level using 80 and 800 mL columns. The test results showed that the transfer of the LF isolation method to a higher level is relatively easy, which was also demonstrated during the transition to the semi-industrial level, where an 8L chromatographic column was used. At this level, we also tested different buffers compositions based on phosphate, carbonate or citrate and isolation of LF/LPO from

sweet whey (SS). LF isolates were compared with other LFs on the market using HPLC chromatography, SDS PAGE analysis and measurement of LF bioactivity. The results of characterization methods showed that in comparison with other products, we achieve comparable, often even higher purity (> 98%) and significantly higher overall bioactivity (C + A value = 74.9%) compared to all analyzed samples (C + A value <66%, **Table 1**). A patent application for the LF isolation method has been filed in several countries worldwide (Kete et al., 2018).

Table 1. Bioactivity of commercially available LF and LF isolates isolated using CIM monolithic chromatography (Arhel d.o.o.). Total bioactivity is expressed by summing the C- and A-values, which theoretically give the proportion of active protein in the sample

Lactoferrin samples	Life Extension (Lactoferrin caps - Bioferrin; 95% Apolactoferrin)	Ingredia Nutritional (Prodiet Lactoferrin, >95%)	NRL Pharma	Sigma bovine lactoferrin; ≥85%	Arhel d.o.o.
C-value [%]	48,6* (31,1 – vz.)	52,1	49,2	7,5	70,0 -74,0
A-value [%]	11,7* (7,5 – vz.)	7,7	9,0	58,5	2 - 4,9
Sum (A+C) [%]	60,3	59,8	58,2	66,0	74,9 (avg.)

In parallel with the optimization of the LF isolation method, we also performed tests on whey pretreatment before chromatographic isolation and tested the repeatability of the chromatographic isolation cycles. Thus, 138 chromatographic cycles of LF isolation were performed on a semi-industrial level on two CIMmultus ™ SO3-8000 (Bia Separations, Sartorius) monolithic chromatographic columns. The tests proved a high degree of repeatability, maintenance of the functionality of the chromatographic column and isolation of highly pure LF product with high bioactivity.

Optimization of raw material consumption

At the semi-industrial level, for additional optimization of buffer solutions (salts used), we tested several ways of reusing them in the chromatographic isolation process, the possibility of recycling raw materials and reusing highly salty solutions in the process of concentration of whey residues. With the solutions, we achieve complete removal of phosphates from buffer solutions and significant removal of water from whey (Zupančič Justin et al., 2021a).

Development of a method for increasing the LF binding capacity of a chromatographic column

To increase the efficiency of each chromatographic cycle and thus increase the production capacity, we also tested various possibilities to increase the binding capacity of LF on a monolithic SO3 chromatographic column. As a result, with the development of the new method, we managed to increase the binding capacity of LF by 60 to 90% compared to the initial isolation method (Zupančič Justin et al., 2021b).

Acknowledgements

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Razvoj analiznih metod za vrednotenje laktoferina in izbranih sirotkinih proteinov

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Povzetek
Vrednotenje vsebnosti in stabilnosti sirotkinih proteinov, uspešnosti postopkov izolacije in čiščenja ter razvoj formulacij zahtevajo razvoj ustrezne analitske podpore, kar predstavlja poseben analizni izziv. Zato je bil glavni namen raziskovalnega dela razviti analizno metodologijo, ki omogoča vrednotenje laktoferina in izbranih sirotkinih proteinov v različnih vzorcih. Osredotočili smo se na komplementarne kromatografske metode, pristop pa smo dodatno razširili s sofisticirano dvodimenzionalno tekočinsko kromatografijo in enostavnejšimi spektroskopskimi tehnikami. Razvite metode smo uporabili za določanje vsebnosti in stabilnosti sirotkinih proteinov v različnih vzorcih, s tem pa smo omogočili razvoj in izboljšave postopkov pridobivanja in nadaljnje uporabe sirotkinih proteinov.

Ključne besede: laktoferin, sirotkini proteini, analizne metode, HPLC, 2D-LC

Uvod
Sirotka je bila dolga leta obravnavana kot odpadni material, vendar pa zaradi bogate sestave, predvsem zaradi vsebnosti proteinov, pridobiva na pomembnosti (Smithers 2015). Vrednotenje uspešnosti postopkov izolacije in čiščenja posameznih sirotkinih proteinov, ovrednotenje njihove stabilnosti in razvoj formulacij z izbranimi sirotkinimi proteini zahtevajo razvoj ustrezne analitske podpore, kar pa zaradi strukturne kompleksnosti proteinov predstavlja poseben analizni izziv (ICH Q5C, 1995).

Namen
Primarni namen raziskovalnega dela je bil razviti analizno metodologijo za vrednotenje laktoferina (Lf) in izbranih sirotkinih proteinov v različnih vzorcih.

Materiali in metode
Standarde Lf, α-laktalbumina, β-laktoglobulina, govejega serumskega albumina (BSA), IgG in laktoperoksidaze (LPO) smo dobili od Sigme-Aldrich. Osredotočili smo se na razvoj komplementarnih kromatografskih metod. Preizkusili smo različne reverzno-fazne (RP), izključitvene (SEC) in ionsko-izmenjevalne (IEC) kromatografske kolone. Uporabljali smo Agilentov 1100/1200 HPLC sistem z detektorjem z nizom diod. Analizni pristop smo dodatno razširili z dvodimenzionalno tekočinsko kromatografijo (2D-LC; Waters) in preizkusili možnost uporabe enostavnejših spektroskopskih tehnik (fluorimetrija, UV spektroskopija, metode za določanje koncentracije celokupnih proteinov).

Rezultati in diskusija
Razvoj analiznih metod za vrednotenje sirotkinih proteinov smo pričeli z Lf, saj je aktualen zaradi svojih številnih pozitivnih učinkov na zdravje ljudi. Najbolj zahteven je bil razvoj RP-HPLC metode, saj je mehanizem ločbe pri tem tipu kromatografije kompleksen. Razvoj metode je potekal v treh stopnjah, preizkusili pa smo štiri RP kolone (C3, C4, C8 in C18). Najbolj optimalna z vidika oblike kromatografskega vrha za Lf in ločbe Lf od njegovih razpadnih produktov je bila metoda, ki smo jo razvili na koloni BioZen™ Intact XB-C8 150×4,6 mm, 3,6 µm (Phenomenex). Metodo smo validirali v skladu s smernico ICH. V stresnih vzorcih smo zaznali do 3 razpadne produkte in ugotovili, da lahko opazimo razlike v mehanizmih razpada Lf v različnih vzorcih (Osel 2021).
Druga kromatografska metoda je bila SEC-HPLC, pri kateri ločba temelji na razlikah v velikosti in obliki analitov. Pri razvoju te metode smo preizkusili 2 različni koloni. Izkazalo se je, da je bila bolj optimalna metoda, ki smo jo razvili na koloni XBridge Protein BEH SEC 150×7,8 mm, 3,5 µm (Waters). Metodo smo validirali v skladu s smernico ICH. V stresnih vzorcih smo s SEC-HPLC metodo zaznali agregate in fragmente Lf ter opazili razlike v mehanizmu razpada Lf v različnih vzorcih. Vendar pa smo SEC-HPLC metodo uporabljali predvsem kot komplementarno metodo za kvalitativne namene, saj je bila slabša kot RP-HPLC metoda z vidika validacijskih parametrov, vpliva vzorčnega medija na kromatografski odziv ter zahtevnejšega rokovanja s kolono (Osel 2021).
Kromatografski analizni pristop smo dodatno razširili še z vključitvijo IEC-HPLC, pri kateri proteine ločujemo glede na njihov naboj. Pri razvoju metode smo uporabili šibko kationsko izmenjevalno kolono CiMAC 0,1 mL (BIA Separations). Na elucijo Lf je bistveno vplival pH mobilne faze, način elucije, koncentracija soli v elucijski mobilni fazi ter gradientni programi, medtem ko so imeli izbira puferskega sistema, temperatura kolone in pretoka mobilne faze manjši ali zanemarljiv vpliv.

V nadaljevanju smo razvite metode razširili in prilagodili za izbrane sirotkine proteine. V nabor analitov smo vključili sirotkine proteine, ki so v sirotki prisotni v največjih koncentracijah ali pa so zanimivi zaradi pozitivnih učinkov na zdravje ljudi. Z IEC-HPLC metodo lahko selektivno določamo Lf in LPO v prisotnosti ostalih sirotkinih proteinov, ne moremo pa s to metodo vrednotiti vseh sirotkinih proteinov. Tudi s SEC-HPLC metodo ne moremo ločiti vseh izbranih sirotkinih proteinov, saj imajo nekateri (LPO, Lf, BSA) podobno velikost. Kljub temu pa lahko s to metodo selektivno določamo LPO pri drugi valovni dolžini detekcije (405 nm). Razvoj RP-HPLC metode za vse sirotkine proteine je bil zahteven, saj je bilo težko doseči primerno obliko kromatografskih vrhov (IgG) ali ločbo (Lf in BSA) izbranih analitov. Z optimiziranimi RP-HPLC metodami smo lahko vrednotili izbrane sirotkine proteine v relativno enostavnih vzorcih. Zapletlo pa se je pri kompleksnih vzorcih, kar smo rešili z uporabo 2D-LC, ki je temeljila na kombinaciji SEC-HPLC in RP-HPLC metode. Z razvito 2D-LC smo uspeli ločiti vse izbrane sirotkine proteine. Ker pa gre za relativno sofisticirano tehniko, je v praksi nismo uporabljali. Poskusili smo tudi z uporabo enostavnejših, hitrejših in dostopnejših spektroskopskih tehnik, vendar se je izkazalo, da je njihova uporabnost zaradi pomanjkanja selektivnosti omejena.

Razvite metode smo uporabili na številnih raziskovalnih področjih v okviru projekta LAKTIKA. Določali smo vsebnost Lf in izbranih sirotkinih proteinov v različnih vzorcih in tako omogočali vrednotenje uspešnosti izolacije posameznih proteinov iz sirotke in postopkov čiščenja. Določali smo tudi vsebnost Lf v komercialno dostopnih prehranskih dopolnilih. Izvedli smo stabilnostne študije z različnimi vzorci pri različnih pogojih shranjevanja. Z razvitimi metodami smo nudili analitsko podporo pri razvoju formulacije z Lf. Z izdelano formulacijo smo izvedli tudi stabilnostno študijo, pri kateri smo preverjali vpliv ovojnine in pogojev shranjevanja na stabilnost Lf.

Zaključek

Razvoj ustrezne analizne metodologije je nujen za ovrednotenje vsebnosti oziroma stabilnosti sirotkinih proteinov v različnih vzorcih. Razvite kromatografske metode smo uporabili na številnih raziskovalnih področjih v okviru projekta LAKTIKA in s tem nudili potrebno analizno podporo. V prihodnosti bomo pristop dodatno razširili z masno spektroskopijo, s katero bomo sirotkine proteine lahko bolj zanesljivo in selektivno vrednotili v kompleksnih vzorcih.

Zahvala

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Development of analytical methods for the evaluation of lactoferrin and selected whey proteins

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Abstract
The development of an analytical methodology that allows the evaluation of content and stability of whey proteins, the evaluation of isolation and purification processes, and the development of formulations, presents a special analytical challenge. Therefore, the main purpose of this work was to develop an analytical methodology for the evaluation of lactoferrin and selected whey proteins in various samples. We were focused on complementary chromatographic methods. The analytical approach was additionally extended with sophisticated two-dimensional liquid chromatography and simple spectroscopic techniques. The developed methods were used to evaluate the content and stability of whey proteins in various samples, thus enabling the development and improvement of procedures for the production and further processing of whey proteins.

Keywords: lactoferrin, whey proteins, analytical methods, HPLC, 2D-LC

Introduction
Whey has been considered a waste material for many years. However, due to its rich composition with emphasis on proteins, it is gaining in importance (Smithers 2015). The evaluation of the isolation and purification processes of individual whey proteins, the evaluation of their stability, and the development of formulations with selected whey proteins require the development of appropriate analytical support, which presents a special analytical challenge due to the structural complexity of proteins (ICH Q5C, 1995).

Objective
The main purpose of this research was to develop an analytical methodology for the evaluation of lactoferrin (Lf) and selected whey proteins in various samples.

Materials and Methods
Reference standards of Lf, α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), IgG and lactoperoxidase (LPO) were obtained from Sigma-Aldrich. We were focused on the development of complementary chromatographic methods. Various reversed-phase (RP), size-exclusion (SEC) and ion-exchange (IEC) chromatographic columns were tested. An Agilent 1100/1200 HPLC system, equipped with a diode array detector was used. The analytical approach was further extended by two-dimensional liquid chromatography (2D-LC; Waters). The possibility of using simpler spectroscopic techniques (fluorimetry, UV spectroscopy, methods for total protein concentration determination) was tested as well.

Results and Discussion
The chromatographic methods for the evaluation of Lf were developed since the interest in this whey protein is growing due to its beneficial effects on human health. RP-HPLC method development was quite challenging due to the complexity of the separation mechanism. In the three stages of RP-HPLC method development, four RP columns were tested (C3, C4, C8, and C18). The most optimal in terms of Lf peak shape and the separation between Lf and its degradation products was the method developed on the BioZen ™ Intact XB-C8 150 × 4.6 mm, 3.6 µm column (Phenomenex). This method was validated according to the ICH guideline**.** Up to three degradation products were detected in stress samples. The differences in the degradation mechanisms of Lf in various samples were observed by using the RP-HPLC method (Osel 2021).
The separation in the SEC-HPLC is based on differences in analyte size and shape. Two SEC columns were tested during the method development. A more optimal method was developed on the XBridge Protein BEH SEC 150 × 7.8 mm, 3.5 µm column (Waters). This method was also validated according to the ICH guideline. Aggregates and fragments of Lf were detected in various stress samples. Differences in the Lf degradation mechanisms could also be observed with the SEC-HPLC method. However, the SEC-HPLC method was only used as a complementary method since it was inferior to the RP-HPLC method in terms of validation parameters, sample media influence on the chromatographic response, and more demanding column handling (Osel 2021).
The chromatographic analytical approach was further extended by the inclusion of IEC-HPLC, which separates proteins according to their charge. A weak cation exchange column CiMAC 0.1 mL (BIA Separations) was

used in the method development. Elution of Lf was significantly affected by mobile phase pH, elution mode, salt concentration in elution mobile phase, and gradient programs, while the choice of a buffer system, column temperature, and mobile phase flow rate had less or negligible impact.

Later, the developed methods were optimized for the other selected whey proteins. The analytes were selected according to their concentration in whey or their potential use due to their positive effects on human health. The IEC-HPLC method can be used to selectively determine Lf and LPO in the presence of other whey proteins. However, it does not allow the evaluation of all selected whey proteins. Similarly, not all whey proteins can be separated using the SEC-HPLC method as some of them (LPO, Lf, BSA) have a similar molecular weight. However, the developed SEC-HPLC method can be used for the evaluation of LPO since it can be selectively determined at a different detection wavelength (405 nm). The development of the RP-HPLC method for the selected whey proteins was challenging as the separation (Lf and BSA) or the appropriate chromatographic peak shape (IgG) of selected analytes was difficult to achieve. The optimized RP-HPLC methods were used for the evaluation of the selected whey proteins in relatively simple samples. For more complex samples, a 2D-LC method which was based on a combination of SEC-HPLC and RP-HPLC method was developed. The 2D-LC method allowed the separation of all selected whey proteins. However, the 2D-LC method was not used in routine practice since it is a relatively sophisticated technique. The feasibility of using fast, simple and accessible spectroscopic techniques was also considered; however, their applicability is limited due to a lack of selectivity.

The developed methods were used in various research areas within the project LAKTIKA. The content of Lf and selected whey proteins in various samples was determined, thus enabling the evaluation of the isolation and purification processes of individual whey proteins. The Lf content in commercially available dietary supplements was also evaluated. Several stability studies with various samples under different storage conditions were conducted. The analytical support for the development of the formulation with Lf was provided. Moreover, a stability study with the prepared Lf formulation was carried out, where the effects of the packaging and storage conditions were evaluated.

Conclusion

The development of an appropriate analytical methodology is necessary for the evaluation of the content or stability of whey proteins in various samples. The developed chromatographic methods were used in various research areas within the project LAKTIKA and provided the necessary analytical support. In the future, the analytical approach will be extended with mass spectroscopy, which will allow a more reliable and selective evaluation of whey proteins in complex samples.

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Razvoj farmacevtske formulacije z laktoferinom za ohranjanje zdravega ravnovesja črevesne mikrobiote

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Povzetek
Laktoferin (LF) je mlečni protein, ki izkazuje veliko pozitivnih učinkov na delovanje človeškega organizma. LF izkazuje selektivno bakteriostatično delovanje proti patogenim mikroorganizmom. V tej študiji smo razvili prehransko dopolnilo z vgrajenim visokim odmerkom LF (200 mg). Farmacevtska oblika nudi zaščito LF pred ostrimi pogoji v želodcu in omogoča njegovo dostavo na mesto delovanja v aktivni obliki ter izboljšuje stabilnost izdelka med shranjevanjem.

Ključne besede: laktoferin, stabilnost, prehransko dopolnilo, oblaganje pelet

Uvod
Laktoferin je mlečni protein, ki izkazuje veliko pozitivnih učinkov na delovanje človeškega organizma (1). Številni znanstveniki ga uvrščajo med proteine z visokim, a neizkoriščenim potencialom. LF izkazuje selektivno bakteriostatično delovanje proti patogenim mikroorganizmom (2). Zato bi LF lahko uporabili za uravnavanje razmerja med koristnimi bakterijami in patogeni v črevesni mikrobioti. Ravnovesje med mikroorganizmi je namreč pogoj za ustrezno delovanje prebavil. Za dostavo aktivnega proteina v tanko črevo pa potrebujemo primerno farmacevtsko obliko. Ta mora nuditi zaščito pred pogoji v želodcu in zagotavljati primerno sproščanje proteina na mestu delovanja.

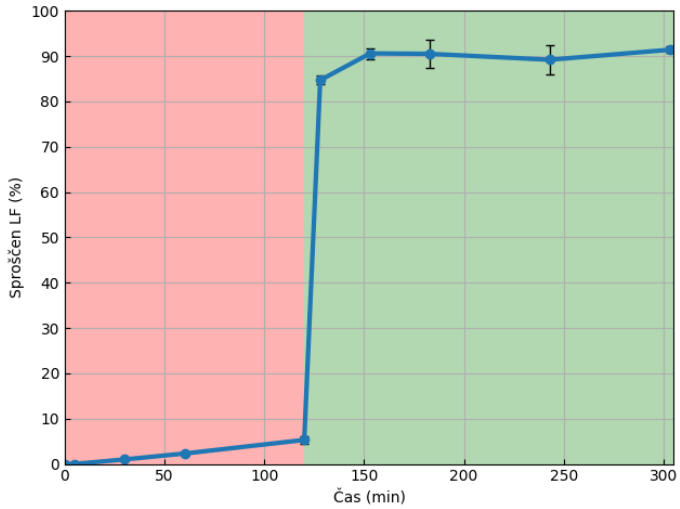
Namen
Cilj raziskovalnega dela je bil razviti peroralno farmacevtsko obliko, in sicer trdo želatinsko kapsulo z LF. Vanjo smo želeli vgraditi vsaj 200 mg LF in ga zaščititi pred vplivi želodca.

Oblaganje pelet z LF in zaščitno oblogo
Formulacijo z LF smo pripravili z dvostopenjskim postopkom oblaganja pelet v vrtinčnoslojni komori Glatt GPCG1. Peletna jedra (Cellets 200) smo oblagali v vrtinčnoslojnem sistemu z razprševanjem od spodaj. Uporabili smo koaksialno šobo za razprševanje s premerom 1,2 mm. Razmejitveni valj je bil nameščen 15 mm nad razdelilno ploščo. Peletna jedra smo v prvi stopnji obložili z disperzijo, ki vsebuje LF, vezivo (hipromeloza Pharmacoat 606), mehčalo (polietilen glikol 6000) in prečiščeno vodo. Za oblaganje smo uporabili LF v trdni obliki ali vodno raztopino LF, izolirano iz kisle sirotke. Disperzijo za oblaganje pelet z LF smo pripravili postopoma. Najprej smo na 80 °C segreli prečiščeno vodo in v njej dispergirali Pharmacoat 606. Suspenzijo smo ohladili pod 35 °C, da se je polimer raztopil. Nato smo dodali mehčalo PEG 6000 in LF. Disperzijo smo razprševali na peletna jedra pri pogojih, ki so predstavljeni v preglednici 1. Med procesom oblaganja smo pazili, da temperatura produkta ni presegla 40 °C, saj bi to lahko vplivalo na aktivnost LF. Na obložene pelete smo v naslednjem koraku nanesli zaščitno oblogo, ki je vsebovala polimer Eudragit L, sredstvo proti sprijemanju glicerol monostearat, mehčalo trietil citrat in površinsko aktivno snov Tween 80. Med oblaganjem nismo zaznali nekompatibilnosti obeh oblog. Adhezija zaščitne obloge na sloj LF je bila zadostna in ob koncu oblaganja skoraj ni bilo prašnih delcev. Tudi delež aglomeriranih pelet je bil manjši od 1 %, zato sklepamo, da je bil proces oblaganja ustrezen. V povprečju smo določili 93 % izkoristek procesa izdelave pelet. Pelete smo polnili v trde želatinske kapsule in določili, da je vsebnost LF v kapsuli 200 mg.

Preglednica 1: Pogoji oblaganja pelet v vrtinčnoslojni komori.

Procesna stopnja	Vstopna temperatura (°C)	Temperatura produkta (°C)	Izstopna temperatura (°C)	Pretok zraka (m^3/h)	Tlak razprševanja (bar)	Pretok disperzije (g/min)
LF obloga	70	35	35	65	1.8	14
GR obloga	50	30	31	65	1.8	8

Sproščanje LF iz pelet

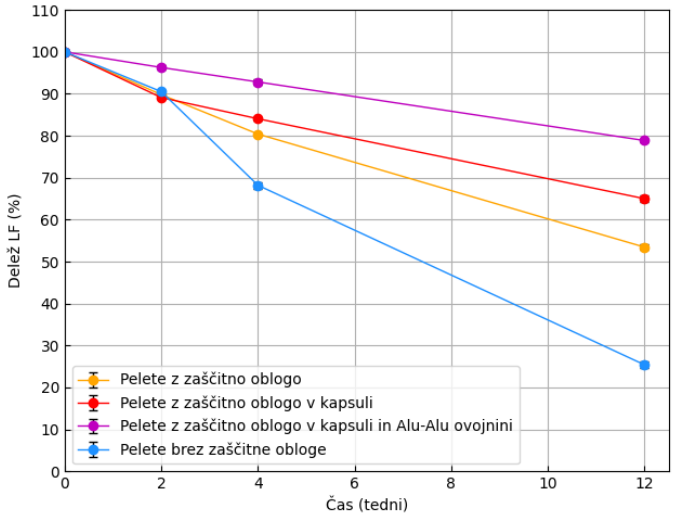


Sproščanje LF iz pelet smo testirali z dobro uveljavljeno napravo z vesli USP II. Sproščanje smo preverjali v šestih ponovitvah s standardno metodo sproščanja (Ph. Eur. 2.9.3.). Hitrost vrtenja mešal smo nastavili na 100 min^{-1}. Vzorce smo odvzeli po 5, 30, 60, 120, 125, 150, 180, 240 in 300 min. Pet mililitrov vsakega vzorca smo filtrirali skozi PVDF membranske filtre z velikostjo por 0,2 µm in jih analizirali z reverznofazno HPLC metodo (3). Rezultati sproščanja so predstavljeni na sliki 1. Ugotovili smo, da pelete ustrezajo zahtevam za farmacevtske oblike z zakasnjenim sproščanjem. V kislem mediju se je v dveh urah sprostilo manj kot 10 % celotnega odmerka LF.

Slika 4: Sproščanje LF iz pelet v kislem (rdeče) in nevtralnem mediju (zeleno).

Stabilnost kapsul z LF

Pri farmacevtskih oblikah s proteini je vprašanje stabilnosti poglavitnega pomena za določitev roka uporabnosti izdelka, zato smo preverili stabilnost LF pri pospešenih pogojih shranjevanja (40 °C in 75 % relativne vlažnosti). Vzorce smo shranjevali v klimatski komori in jih analizirali v vnaprej določenih časovnih točkah (0, 2, 4 in 12 tednov) s stabilnostno-indikativno reverznofazno HPLC metodo (3). Vzorce smo raztopili v 50 mM fosfatnem pufru s pH 6,8, jih mešali na magnetnem mešalu 2 h in filtrirali skozi membranski filter. Pripravili smo tri ponovitve vsakega vzorca. Rezultati, prikazani na sliki 2, kažejo, da zaščitna obloga stabilizira LF. Dodatno lahko rok uporabnosti izdelka podaljšamo s polnjenjem pelet v trdo kapsulo in pakiranjem v Alu-Alu ovojnino.



Slika 5: Stabilnost pelet z LF pri pospešenih pogojih shranjevanja.

Sklep

Izdelava prehranskega dopolnila v obliki obloženih pelet z visokim odmerkom LF v vrtinčnoslojni komori je izvedljiva. Profili sproščanja LF iz pelet ustrezajo zahtevam za farmacevtske oblike z zakasnjenim sproščanjem. Farmacevtska oblika vsebuje pelete, obložene z LF in zaščitno oblogo, ki ščiti protein in omogoča njegovo dostavo v tanko črevo. Ustrezna primarna ovojnina predstavlja ključni sestavni del farmacevtske oblike za izboljšanje stabilnosti LF in podaljšanja roka uporabnosti prehranskega dopolnila.

Zahvala

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Development of a dosage form containing lactoferrin to maintain a healthy balance of the intestinal microbiota

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Abstract
Lactoferrin (LF) is a milk protein that has various beneficial effects on human body function. LF exhibits selective bacteriostatic activity against pathogenic microorganisms. In this study, a pharmaceutical formulation with a built-in high dose of LF (200 mg) was developed. The pharmaceutical form protects LF from harsh gastric conditions and allows the delivery of active LF to the site of action. Additionally, the protective coating improves the stability of LF in the product during storage.

Keywords: lactoferrin, stability, food supplement, pellet coating

Introduction
Lactoferrin (LF) is a milk protein that has various beneficial effects on human body function (1). Many scientists classify it as a protein with high, but unfortunately, unused potential. LF exhibits selective bacteriostatic activity against pathogenic microorganisms (2). Therefore, LF could be used to regulate the ratio of beneficial bacteria and pathogens in the intestinal microbiota. The balance between microorganisms is a prerequisite for the proper functioning of the gastrointestinal tract. To deliver the active protein to the small intestine, a suitable delivery system is required. It should provide protection from gastric conditions and ensure adequate release of the protein at the site of action.

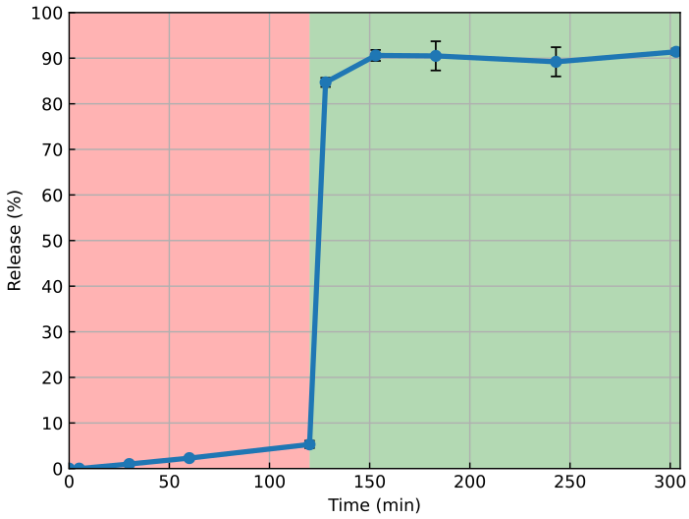
Aim
The objective of the research was to develop an oral dosage form with LF in the form of a hard capsule. The dosage form should contain at least 200 mg of LF into capsule and protect it from the harsh gastric environment.

Pellet coating with LF and enteric coating
The formulation with LF was prepared in a two-step pellet coating process in a Glatt GPCG1 fluidized bed chamber. Pellet cores (Cellets 200) were coated in a bottom spray fluid bed system. A coaxial spray nozzle with a diameter of 1.2 mm was used. The limiting cylinder was located 15 mm above the distribution plate. In the first step, the pellet cores were coated with a dispersion containing LF, a binder (Hypromellose Pharmacoat 606), a plasticizer (Polyethylene Glycol 6000) and purified water. LF in solid form or aqueous solution of LF isolated from acid whey was used for coating. The dispersion for coating of the pellets with LF was prepared step by step. First, Pharmcoat 606 was dispersed in purified water at 80 °C. The suspension was cooled to below 35 °C to dissolve the polymer. Then the plasticizer PEG 6000, and LF were added. The dispersion was sprayed onto the pellet cores under the conditions listed in Table 1. During the coating process, care was taken to ensure that the product temperature did not exceed 40 °C, as this could affect LF activity. In the next step, an enteric coating containing the polymer Eudragit L, glycerol monostearate, the plasticizer triethyl citrate, and the surfactant Tween 80 was applied to the coated pellets. No incompatibilities between the two coatings were observed during the coating process. The adhesion of the protective coating to the LF layer was sufficient and there were almost no dust particles at the end of the coating. The percentage of agglomerated pellets was less than 1%, from which we conclude that the coating process was adequate. On average, the efficiency of pellet production was 93%. The pellets were filled into hard gelatin capsules and the content of LF in the capsule was 200 mg.

Table 1: Pellet coating conditions in the fluid bed coater

Process step	Inlet temperature (°C)	Product temperature (°C)	Outlet temperature (°C)	Air flow rate (m^3/h)	Nozzle pressure (bar)	Dispersion flow rate (g/min)
LF layer	70	35	35	65	1.8	14
Enteric layer	50	30	31	65	1.8	8

Release of LF from pellets.



The release of LF from pellets was tested using a USP II apparatus. Release was tested in six replicates using the standard procedure (Ph. Eur. 2.9.3.). A stirrer speed of 100 rpm was set. Samples were taken after 5, 30, 60, 120, 125, 150, 180, 240, and 300 min. Samples of 5 mL were filtered through 0.2 µm PVDF membrane filters. The samples were analyzed by a reversed-phase HPLC method (3). The results presented in Figure 1 show that the pellets met the requirements for sustained release dosage forms. Less than 10% of the total LF was released in the acidic medium within two hours.

Figure 1: Release of the pellets in acidic (red) and neutral medium (green).

Stability of the capsules with LF

In protein formulations, the stability is of utmost importance as it determines the shelf life of the product. Therefore, the stability of LF was tested under accelerated storage conditions (40 °C and 75% relative humidity). Samples were stored in a climate chamber and analyzed at predetermined time points (0, 2, 4, and 12 weeks), using a stability-indicating reversed-phase HPLC method (3). Pellets were dissolved in a 50 mM phosphate buffer pH 6.8, stirred on a magnetic stirrer for 2 hours, and filtered through a membrane filter. Three replicates of each sample were prepared. The results shown in Figure 2 indicate that the protective layer has a stabilizing effect on the LF. In addition, the shelf life of the product can be extended by filling the pellets into a hard capsule and packing them in an Alu-Alu package.

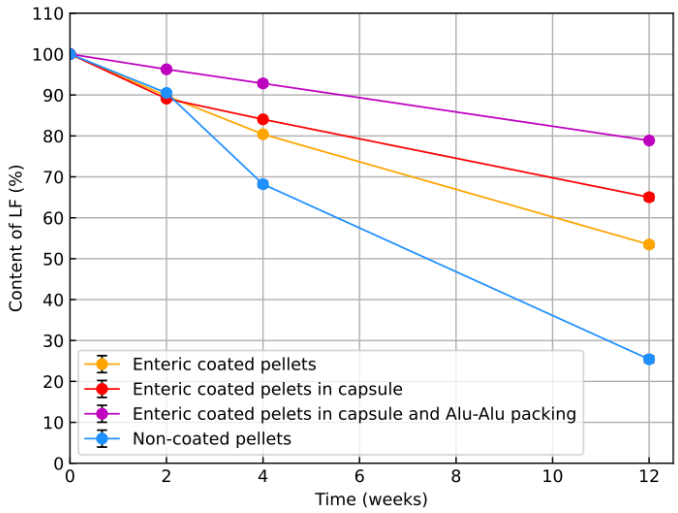


Figure 2: Stability of LF in pellets at accelerated storage conditions.

Conclusion

The production of a high-dose LF dietary supplement in a fluidized bed chamber is feasible. The protective-coated pellets allow LF to safely pass through the stomach and deliver the active protein to the small intestine. The release profile of the pellets meets the requirements for sustained release dosage forms. Appropriate primary packaging has proven to be a key component of the pharmaceutical form to improve the stability of LF and extend the shelf life of the dietary supplement.

Acknowledgements

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Protimikrobna aktivnost peptidov, pridobljenih z encimsko hidrolizo laktoferina

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Povzetek
Laktoferin je glikoprotein, ki ima v telesu sesalcev številne funkcije in ima posledično pomembno vlogo v primarnem imunskem sistemu. Ena od pomembnih funkcij laktoferina je njegovo protibakterijsko delovanje. Laktoferin deluje protibakterijsko proti številnim po Gramu-pozitivnim in po Gramu-negativnim bakterijam. Študije so pokazale, da encimska hidroliza laktoferina vodi v nastanek laktoferinskih peptidov, ki imajo boljšo protibakterijsko aktivnost proti po Gramu-pozitivnim in po Gramu-negativnim bakterijam, kot nativen protein. Zaradi naraščajoče odpornosti bakterij proti antibiotikom predstavljajo laktoferinski peptidi velik potencial kot naravni antibiotiki. V naši raziskavi smo se ciljno osredotočili v pridobivanje novih protibakterijskih peptidov iz laktoferina, ki bi imeli boljšo protibakterijsko aktivnost kot nativen protein. Le-to smo skušali doseči s preskušanjem različnih encimov in pogojev encimske hidrolize, kot so pH, temperatura in čas encimske hidrolize. Tako smo pridobili peptide, ki so imeli boljšo protimikrobno aktivnost kot laktoferin.

Ključne besede: hidroliza laktoferina, laktoferinski peptidi, laktofericin, protimikrobna aktivnost

Uvod
Laktoferin je 80 kDa velik glikoprotein s sposobnostjo vezave železovih ionov. Najdemo ga v granulah polimorfonuklarnih neutrofilcev ter v različnih telesnih izločkih sesalcev, vključno z mlekom. Laktoferin ima pomembno vlogo v primarnem imunskem sistemu in je posledično pomembna molekula pri naravni obrambi telesa gostitelja. Le-to je povezano z njegovimi številnimi funkcijami v telesu, ki vključujejo protimikrobno (protibakterijska, protivirusna, protiglivična in protiparazitarna), antikancerogeno, protivnetno, imunomodulatorno in antioksidativno funkcijo (Ward in sod. 2005). Protibakterijska aktivnost je bila med prvimi funkcijami, ki so jo pripisali laktoferinu in je posledično tudi najbolj raziskana. Laktoferin deluje proti številnim po Gramu-pozitivnim in po Gramu-negativnim bakterijam bakteriostatično ali baktericidno.

Laktoferinski peptidi
Encimska hidroliza laktoferina s pepsinom je vodila v izolacijo protibakterijskega peptida katerega protibakterijsko aktivnost je bila višja kot pri nativem proteinu (Tomita in sod. 1991). Peptid, poimenovan laktofericin (Lf-cin), ima prav tako protibakterijsko aktivnost proti številnim po Gramu-pozitivnim in po Gramu-negativnim bakterijam, poleg tega pa se ponaša tudi z drugimi pomembnimi funkcijami, kot so protivirusna, protiparazitarna, protiglivična in antikancerogena (Grifford in sod. 2005). Odkritju laktofericina so sledile številne študije različnih raziskovalnih skupin in odkrivanje novih laktoferinskih peptidov z boljšo protibakterijsko aktivnostjo. Številne raziskave laktoferina in drugih laktoferinskih peptidov so omogočile boljše razumevanje samega mehanizma protibakterijske aktivnosti naravnih peptidov ter povezave med strukturo in funkcijo protibakterijske aktivnosti peptidov. Posledično ni presenečenje, da so se kmalu pojavili tudi sintetični laktofericinski peptidi z boljšo protibakterijsko aktivnostjo kot laktoferin, kot sta naprimer laktoferampin (van der Kraan in sod. 2004) in Lf-himera (Bolscher in sod. 2009).

Proučevanje protibakterijske aktivnosti laktoferinskih peptidov je pomembno, saj se nekateri izmed teh peptidov tvorijo naravno v procesu prebave in vplivajo na raznovrstnost bakterij v črevesju (Kuwata in sod. 1998). Poleg tega se v zadnjem času srečujemo s porastom odpornosti bakterij proti antibiotikom in je potreba po novih antibiotikih vse večja. Laktoferinski peptidi s svojim protibakterijskim delovanjem predstavljajo velik potencial kot naravni antibiotiki, ki bi jih lahko uporabili tako v humani kot v veterinarski medicini (Bruni in sod.. 2016). Dodatno pa je bilo v primeru Lf-himere dokazano tudi sinergistično delovanje z antibiotiki proti bakterijam (Leon Sicairos in sod. 2009).

Encimska hidroliza laktoferina in peptidi s protimikrobnim delovanjem
V naši študiji smo izvedli encimsko hidrolizo laktoferina s ciljem pridobivanja novih laktoferinskih peptidov, ki bi imeli boljšo protibakterijsko aktivnost kot laktoferin sam. Za doseganje zastavljenih ciljev smo encimsko hidrolizo izvedli z različnimi encimi, kot so pepsin, tripsin in kimotripsin. Optimizirali pa smo tudi druge dejavnike, kot so temperatura, pH in čas encimske hidrolize. Peptide, pridobljene z encimsko hidrolizo

laktoferina, smo analizirali na protibakterijsko aktivnost z metodo mikrodilucije z indikatorskim sevom *Latilactobacillus sakei* IM 108 (ATCC 15522), tako da smo določali minimalno inhibitorno koncentracijo (MIC). Kljub temu, da so laktoferinski hidrolizati pokazali protibakterijsko aktivnost, pa je bila le-ta običajno enaka oziroma slabša v primerjavi z protibakterijsko aktivnostjo laktoferina (Tabela 1). Nadalje smo želeli proučiti tudi, če velikost peptidov (kDa) vpliva na njihovo protibakterijsko aktivnost. Laktoferinske hidrolizate smo z uporabo ultrafiltracijskih filtrov (z velikostjo por 10 kDa ali 50 kDa) ločili po velikosti na posamezne frakcije. Tripsinski peptidi in kimotripsinski peptidi z velikostjo 10-50 kDa, pridobljeni pri pH 8,0 (37°C/4h), so pokazali boljšo protibakterijsko aktivnost kot laktoferin (manjšo MIC), medtem ko so ostali laktoferinski peptidi izkazovali enako ali višjo vrednost MIC.

Tabela 1: Protimikrobna aktivnost hidrolizatov laktoferina in p osameznih frakcij po ultrafiltraciji, pridobljenih z encimsko hidrolizo s pepsinom, tripsinom ali kimotripsinom pod različnimi pogoji encimske hidrolize. Vrednosti MIC, ki so bile nižje od tistih za laktoferin, so označene rdeče.

Vzorci	Pogoji encimske hidrolize	MIC (mg/ml)
Laktoferin	/	0,016
Pepsinski hidrolizati	pH 3,0; 37°C/4h	0,08
Pepsinski peptidi (10-50 kDa)	pH 3,0; 37°C/4h	0,016
Tripsinski hidrolizati	pH 3,0; 37°C/4h	0,08
Tripsinsksi peptidi (10-50 kDa)	pH 8,0; 37°C/4h	0,0032

Vzorci	Pogoji encimske hidrolize	MIC (mg/ml)
Laktoferin	/	2,5
Tripsinski peptidi (10-50 kDa)	pH 8,0; 37°C/4h	1,25
Tripsinski peptidi (10-50 kDa)	pH 7,0; 37°C/4h	>10
Tripsinski peptidi (10-50 kDa)	pH 7,8; 37°C/4h	>10
Tripsinski peptidi (10-50 kDa)	pH 9,0; 37°C/4h	>10
Tripsinski peptidi (10-50 kDa)	pH 8,0; 37°C/2h	>10
Tripsinski peptidi (10-50 kDa)	pH 8,0; 37°C/24h	>10

Vzorci	Pogoji encimske hidrolize	MIC (mg/ml)
Lactoferin	/	0,63
Kimotripsinski peptidi (10-50 kDa)	pH 8,0; 37°C/4h	0,08
Kimotripsinski peptidi (10-50 kDa)	pH 9,0; 37°C/4h	>10
Kimotripsinski peptidi (10-50 kDa)	pH 8,0; 50°C/4h	0,63
Kimotripsinski peptidi (10-50 kDa)	pH 9,0; 50°C/4h	>10
Kimotripsinski peptidi (10-50 kDa)	pH 8,0; 60°C/1h	0,63

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Antimicrobial activity of peptides generated by enzymatic hydrolysis of lactoferrin

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Abstract
Lactoferrin is a glycoprotein that has numerous functions in the mammalian body and therefore plays an important role in the innate immune system. One of the most important lactoferrin functions is its antibacterial activity, as lactoferrin is effective against a wide range of Gram-positive and Gram-negative bacteria. In addition, enzymatic hydrolysis of lactoferrin has been shown to result in the generation of lactoferrin peptides that have even more potent antibacterial activity against various Gram-positive and Gram-negative bacteria than the native protein. Due to the increasing resistance of bacteria to antibiotics, these lactoferrin-derived peptides show great potential as natural antimicrobials. The aim of our study was to produce new lactoferrin-derived peptides with better antibacterial activity than native protein by selecting different enzymes for enzymatic hydrolysis and using different enzymatic conditions such as pH, temperature and duration of enzymatic hydrolysis. This allowed us to generate lactoferrin peptides with better antibacterial activity than lactoferrin.

Keywords: lactoferrin hydrolysis, lactoferrin-derived peptides, lactoferricin, antimicrobial activity

Introduction
Lactoferrin is an 80 kDa, iron-binding glycoprotein found in the granules of polymorphonuclear neutrophils and various mammalian body secretions, including milk. Lactoferrin plays an important role in the innate immune system. It is considered an important host defence molecule due to its numerous body functions, including antimicrobial (antibacterial, antiviral, antifungal, and antiparasitic), antitumor, anti-inflammatory, immunomodulatory, and antioxidant properties (Ward et al. 2005). Antibacterial activity was one of the first attributed to lactoferrin and is also the most studied. Lactoferrin has bacteriostatic and bactericidal antibacterial activity against various Gram-positive and Gram-negative bacteria.

Lactoferrin derived peptides
Enzymatic digestion of lactoferrin by pepsin led to the isolation of an antibacterial peptide, antibacterial activity of which was even greater than that of the native protein (Tomita et al. 1991). This peptide, named lactoferricin (Lf-cin), also has antibacterial activity against a variety of Gram-positive and Gram-negative bacteria and several other functions attributed to lactoferrin, such as antiviral, antiparasitic, antifungal, and antitumor (Grifford et al. 2005). The discovery of lactoferricin was followed by numerous studies by various research groups and the discovery of new lactoferrin peptides with better antibacterial activity. These studies on lactoferricin and other lactoferrin-derived peptides help us to better understand the mechanisms behind the antibacterial activity of the naturally occurring peptides and the structure-function relationship of peptide antibacterial activity. Therefore, it is not surprising that synthetic lactoferrin peptides such as lactoferampin (Lf-ampin) (van der Kraan et al. 2004) and Lf-chimaera (Bolscher et al. 2009) with better antimicrobial activity than lactoferrin have been produced.
Investigating the antibacterial activity of peptides derived from lactoferrin is important since some of these peptides are naturally produced in the body during digestion and may have a great impact on bacterial diversity in the gut (Kuwata et al. 1998). Moreover, given the increasing bacterial resistance to antibiotics and the need for new antibiotics, these lactoferrin-derived peptides represent great potential as natural antibiotics that can be used in human and veterinary medicine (Bruni et al. 2016). Furthermore, Lf-chimaera has been shown to act in synergy with antibiotics against bacteria (Leon Sicairos et al. 2009).

Enzymatic hydrolysis of lactoferrin and peptides antibacterial activity
In our study, we performed enzymatic hydrolysis of lactoferrin to generate new lactoferrin-derived peptides with greater antibacterial activity than lactoferrin. For this purpose, different enzymes such as pepsin, trypsin, and chymotrypsin were used for the enzymatic digestion of lactoferrin. We also optimize other parameters of enzymatic hydrolysis, such as the temperature, pH of the solution and duration. These peptides/hydrolysates generated by the enzymatic digestion of lactoferrin were tested for antibacterial activity against *Latilactobacillus sakei* IM 108 (ATCC 15522) using the microdilution method, and the minimal inhibitory

concentration (MIC) was determined. Although lactoferrin hydrolysates exhibited antibacterial activity, this activity was generally equal to or even lower than that of the native protein (Table 1). Therefore, we also tested whether peptide size (kDa) affected the antibacterial activity of lactoferrin peptides. Lactoferrin hydrolysates were separated into fractions based on their molecular weight using ultrafiltration filters (pore size 10 kDa in 50 kDa). Tryptic and chymotryptic peptides, size from 10 to 50 kDa, generated by enzymatic hydrolysis at pH 8,0 (37°C/4h) showed higher antibacterial activity than lactoferrin against *Latilactobacillus sakei* IM 108 (lower MIC values). In contrast, other lactoferrin peptides had the same or even higher MIC values (Table 1).

Table 1: Antimicrobial activity of lactoferrin hydrolysates obtained by enzymatic digestion by pepsin, trypsin, or chymotrypsin under different enzymatic hydrolysis conditions, and of individual UF fractions against *Latilactobacillus sakei* IM 108. MIC values that were lower than those for lactoferrin are marked in red.

Samples	Enzymatic conditions	MIC (mg/ml)
Lactoferrin	/	0,016
Pepsin hydrolisates	pH 3,0; 37°C/4h	0,08
Pepsin peptides (10-50 kDa)	pH 3,0; 37°C/4h	0,016
Trypsin hidrolisates	pH 3,0; 37°C/4h	0,08
Tryptic peptides (10-50 kDa)	pH 8,0; 37°C/4h	0,0032

Samples	Enzymatic conditions	MIC (mg/ml)
Lactoferrin	/	2,5
Tryptic peptides (10-50 kDa)	pH 8,0; 37°C/4h	1,25
Tryptic peptides (10-50 kDa)	pH 7,0; 37°C/4h	>10
Tryptic peptides (10-50 kDa)	pH 7,8; 37°C/4h	>10
Tryptic peptides (10-50 kDa)	pH 9,0; 37°C/4h	>10
Tryptic peptides (10-50 kDa)	pH 8,0; 37°C/2h	>10
Tryptic peptides (10-50 kDa)	pH 8,0; 37°C/24h	>10

Samples	Enzymatic conditions	MIC (mg/ml)
Lactoferrin	/	0,63
Cymotryptic peptides (10-50 kDa)	pH 8,0; 37°C/4h	0,08
Cymotryptic peptides (10-50 kDa)	pH 9,0; 37°C/4h	>10
Cymotryptic peptides (10-50 kDa)	pH 8,0; 50°C/4h	0,63
Cymotryptic peptides (10-50 kDa)	pH 9,0; 50°C/4h	>10
Cymotryptic peptides (10-50 kDa)	pH 8,0; 60°C/1h	0,63

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Biotehnološka uporaba sirotke

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Povzetek
Sladka sirotka, ki je stranski produkt v proizvodnji sira, in kisla sirotka, ki nastane predvsem pri proizvodnji grškega tipa jogurta in skute, sta najpomembnejša stranska produkta mlekarske industrije. Nadaljnja predelava sirotke z naprednimi biotehnološkimi pristopi omogoča proizvodnjo inovativnih izdelkov z visoko dodano vrednostjo, namesto obravnave kot odpadka. V prispevku izpostavljamo predvsem biotehnološke pristope k pretvorbi sirotke s pomočjo fermentacije. Veliko zanimanja je predvsem za proizvodnjo biopolimerov kot surovine za bioplastiko, probiotikov in postbiotikov. Pri teh procesih sodeluje cela vrsta industrijsko zanimivih mikroorganizmov iz vrst mlečnokislinskih bakterij, ocetnokislinskih bakterij, kvasovk, plesni in drugih. Številnim izdelkom iz sirotke pripisujejo pozitivne učinke na zdravje in jih zato tržijo kot funkcionalna živila, prehranska dopolnila ali veterinarske pripravke. Razvoj inovativnih izdelkov iz sirotke tako lahko prispeva k trajnostni proizvodnji, zaščiti okolja, posredno pa tudi k izboljšanju javnega zdravja.

Ključne besede: sirotka, valorizacija, biotehnološka pretvorba, fermentacija, bioplastika, probiotiki, postbiotiki

Uvod
Sirotka je najpomembnejši stranski produkt mlekarske industrije. Sladka sirotka, ki je je največ, nastane pri proizvodnji sira, kisla pa predvsem pri proizvodnji grškega tipa jogurta in skute. V Evropi še vedno preveč sirotke, predvsem kisle, obravnavajo kot odpadek, čeprav obstajajo številne možnosti za njeno nadaljnjo uporabo. Sirotko ali sirotkine frakcije (koncentrat sirotkinih beljakovin – WPC, izolat sirotkinih beljakovin – WPI) je mogoče neposredno uporabiti v živilski industriji. Razen tega je mogoče iz sirotke izločiti posamezne sestavine (laktozo, beljakovine, polipeptide) in jih uporabiti za prehrano ali nadalje predelati. V tem pregledu pa predstavljamo predvsem možnosti biotehnološke pretvorbe sirotke ali njenih sestavin, da bi proizvedli izdelke z visoko dodano vrednostjo in hkrati zmanjšali negativni vpliv odpadne sirotke na okolje (biološka potreba po kisiku - BPK in kemična potreba po kisiku - KPK).

Biotehnološki pristopi k uporabi sirotke
Biotehnološka pretvorba sirotke vključuje biokemijsko ali encimsko obdelavo, pri čemer nastanejo oligosaharidi (npr. galakto-oligosaharidi iz laktoze – prebiotiki), hidrolizirati z bioaktivnimi peptidi, aminokisline in sladkorji (npr. glukoza, galaktoza), ali pa mikrobno fermentacijo, s katero dobimo različne produkte. V zadnjem času veliko pozornosti posvečajo sintezi biopolimerov iz sirotke, saj je zanimanje za bioplastiko zaradi njene biorazgradljivosti termoplastičnosti, biokompatibilnosti in netoksičnosti zelo veliko. Številnim drugim produktom iz sirotke pa pripisujejo pozitivne učinke na zdravje, zlasti probiotikom in postbiotikom, pridobljenim v gojiščih na osnovi sirotke.

Primeri mikrobiološke pretvorbe sirotke, zanimivi za industrijo
V Preglednici 1 so predstavljeni primeri pretvorbe sirotke z mikrobno fermentacijo, kot so proizvodnja polihidroksialkanoatov (PHA) – bioplastike (Peydayesh et al., 2021; Chang et al., 2021), postbiotikov (Park et al., 2021), biosurfaktantov (Kachrimanidou et al., 2021), etanola (Carvalho et al., 2021), bakterijske celuloze (Nguyen et al., 2022) in drugi. Med novejšimi izdelki velja omeniti postbiotike, stranske produkte procesa fermentacije sirotke, ki ga izvajajo probiotiki (Park et al., 2021). Postbiotični izdelki, ki vsebujejo bioaktivne presnovke (bakteriocine, vitamine, organske kisline, SCFA, eksopolisaharide EPS itd.) same ali skupaj z neživimi mikroorganizmi oziroma njihovimi komponentami, si uspešno utirajo pot na trg funkcionalnih živil, farmacevtskih izdelkov in veterinarskih pripravkov, čeprav zakonodaja v EU za to skupino izdelkov še ni usklajena.

Table 1: Komercialno zanimivi izdelki, pridobljeni z mikrobiološko pretvorbo sirotke.

Produkt	**Produkcijski organizem**	**Uporaba**
bioetanol	*Kluyveromyces, Saccharomyces*	napitki, biogoriva
polihidroksialkanoati (PHA)	*Hydorgenophaga pseudoflava* *Cupriavidus necator* rekombinantne *Escherichia coli* *Pseudomonas hydrogenovora*	bioplastika
organske kisline (mlečna, ocetna, propionska, citronska, jantarna, laktobionska, glukonska..)	laktobacili, ocetnokislinske bakterije, *Propionibacterium, Candida, Aspergillus niger*..	živilska in kemična industrija
celični proteini	*Kluyverimyces* sp., kefirna zrna	hrana za živali, živilska industrija
celično olje	*Fusarium, Mucor*..	živilska in farmacevtska industrija
encimi (β-galaktozidaza, α-amilaza, proteaze..)	mlečnokislinske bakterije, *Bacillus*, *Kluyveromyces, Aspergillus*..	živilska in kemična industrija
glicerol	*Kluyveromyces, Aspergillus*	biogorivo, kemična industrija
celuloza	ocetnokislinske bakterije	biocompositni materijali
biosurfaktanti	laktobacili, *Pseudomonas*, etc.	živilska in farmacevtska industrija, kozmetika
probiotiki	mlečnokislinske bakterije, bifidobakterije, etc.	funkcionalna hrana, prehranska dopolnila
postbiotiki (bakteriocini, eps, cla..)	mlečnokislinske bakterije, bifidobacterije, *Bacillus*	prehranska dopolnila
vitamins (B6, B12..)	propionibakterije	živilska in farmacevtska industrija

Zaključki

Obstaja veliko zanimanje za biotehnološko pretvorbo sirotke v različne izdelke, saj pomaga zmanjšati obremenilni učinek na okolje, dobimo pa različne izdelke z visoko dodano vrednostjo. Proizvajalci se pri tem srečujejo z ovirami, kot so visoki proizvodni stroški (prevoz, nizke koncentracije nekaterih sestavin, stranski proizvodi, regulativa). Razvoj novih izdelkov iz sirotke lahko prispeva tudi k okoljski trajnosti mlekarske industrije in posredno tudi k izboljšanju javnega zdravja.

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The biotechnological utilization of whey

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Abstract
Sweet whey, which is a by-product of cheese production, and acid whey, which is mainly produced in the manufacture of yoghurt and Greek-style cottage cheese, are the main by-products of the dairy industry. Further processing of whey using advanced biotechnological approaches allows producers to create innovative, value-added products instead of treating it as waste. In this paper, we highlight biotechnological approaches to the conversion of whey by fermentation. There is great interest in the production of biopolymers as raw materials for bioplastics, probiotics and postbiotics. A whole range of industrially interesting microorganisms from lactic acid bacteria, acetic acid bacteria, yeasts, moulds and others are involved in these processes. Many whey products are considered to have health benefits and are therefore marketed as functional foods, dietary supplements or veterinary preparations. The development of innovative whey products can thus contribute to sustainable production, environmental protection and, indirectly, to improving public health.

Keywords: whey, valorization, biotechnological conversion, fermentation, bioplastics, probiotic, postbiotic

Introduction / Further subtitles
Whey is the most important byproduct of the dairy industry. Sweet whey, which is the most abundant, comes from the production of cheese, while acid whey comes mainly from the production of Greek-type yoghurt and cottage cheese. In Europe, too much whey, especially acid whey, is still treated as waste, although there are many possibilities for its further use. Whey or whey fractions (whey protein concentrate - WPC, whey protein isolate - WPI) can be used directly in the food industry. In addition, individual components can be recovered (lactose, proteins, polypeptides) and used as food or further processed. However, in this review we addressed the possibilities of biotechnological conversion of whey or its components to produce high value-added products while reducing the pollutant potential (biological oxygen demand BOD and chemical oxygen demand COD) of waste whey.

Biotechnological approaches for the whey utilization
Biotechnological transformation of whey includes biochemical or enzymatic treatment to obtain oligosaccharides (e.g., galacto-oligosaccharides from lactose - prebiotics), hydrolysis (bioactive peptides), amino acids, and sugars (e.g., glucose, galactose), or microbial fermentation leading to various products. Recently, much attention has been paid to the synthesis of whey biopolymers because of the interest in bioplastics due to their biodegradability, thermoplasticity, biocompatibility, and nontoxicity. In addition, several other products can be considered as health-promoting, especially probiotics and postbiotics obtained by cultivation in whey-based media.

Examples of microbiological whey conversion of interest to industry
Table 1 provides some examples of whey conversion by microbial fermentation, such as the production of polyhydroxyalkanoates (PHA)-bioplastics (2021; Chang et al., 2021), postbiotics (Park et al., 2021), biosurfactants (Kachrimanou et al., 2021), ethanol (Carvalho et al., 2021), bacterial cellulose (Nguyen et al., 2022), and others will be presented. Among the newest products are postbiotics, byproducts of the whey fermentation process performed by probiotics (Park et al., 2021). Postbiotic products containing bioactive metabolites (bacteriocins, vitamins, organic acids, SCFA, exopolysaccharides EPS, etc.) alone or together with inanimate microorganisms or their components represent emerging products in the market of functional food, pharmaceuticals and veterinary products, although legislation in the EU for this group of products has not yet been harmonized.

Table 1: Commercially interesting products obtained by microbiological conversion of whey.

Product	**Production organism**	**Use**
Bioethanol	*Kluyveromyces, Saccharomyces*	Beverage, biofuel, etc.
Polyhydroxyalkanoates (PHA)	*Hydorgenophaga pseudoflava* *Cupriavidus necator* recombinant *Escherichia coli* *Pseudomonas hydrogenovora*	Bioplastics
Organic acids (lactic, acetic, propionic, citric, succinic, lactobionic, gluconic..)	Lactobacilli, acetic acid bacteria, *Propionibacterium, Candida, Aspergillus niger, etc.*	Food, chemical industry
Single cell protein	*Kluyverimyces* sp., kefir grains	Animal feed, foods
Single cell oil	*Fusarium, Mucor* etc.	Foods, pharmaceutical industry
Enzymes (β-galactosidase, α-amylase, proteases, etc.)	Lactic acid bacteria, *Bacillus*, *Kluyveromyces, Aspergillus,* etc.	Food and chemical industry
Glycerol	*Kluyveromyces, Aspergillus*	Biofuel, chemical industry
Cellulose	Acetic acid bacteria	Biocomposite materials
Biosurfactants	Lactobacilli, *Pseudomonas*, etc.	Food, pharmaceuticals, cosmetics
Probiotics	Lactic acid bacteria, bifidobacteria, etc.	Functional food, dietary supplements
Postbiotics (bacteriocins, EPS, CLA, etc.)	Lactic acid bacteria, bifidobacteria, *Bacillus*	Dietary supplements
Vitamins (B6, B12, etc.)	Propionibacteria	Food, pharmaceutical industry

Whey has also been shown to be a suitable and cost-effective medium for the production of postbiotics, byproducts of the fermentation process carried out by probiotics (Park et al., 2021). Postbiotic products containing bioactive metabolites (bacteriocins, vitamins, organic acids, SCFA, exopolysaccharides EPS, etc.) alone or metabolites together with inanimate microorganisms or their components represent an emerging group of products in the functional food, pharmaceutical and veterinary preparation markets, although legislation is not yet harmonised in the EU.

Conclusions

There is a great interest in the bioconversion of whey into different products, as it helps to reduce the environmental impact and produce different value-added products. Producers face obstacles such as high production costs (transportation, low concentrations of some ingredients, by-products, legal issues). The development of new products from whey can also contribute to the environmental sustainability of the dairy industry and improve public health.

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Izkoriščanje kisle sirotke za gojenje mlečnokislinskih bakterij in pridobivanje koristnih metabolitov

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Povzetek
Kislo sirotko, ki nastane pri proizvodnji skute, smo uporabili za produkcijo biomase mlečnokislinskih bakterij (MKB) kot starterskih in probiotičnih kultur ter bakteriocinov in vitamina B12. Sirotki je bil predhodno odstranjen del proteinov, ob optimizaciji pogojev gojenja pa smo jo obogatili še s kvasnim ekstraktom ter mineralnimi solmi. Vzpostavili smo pogoje rasti za uspešno produkcijo več MKB ter uspeli gojiti tudi različne seve, ki proizvajajo bakteriocine. Vzpostavili smo laboratorijsko produkcijo v kisli sirotki za enega izmed bolj široko uporabljenih bakteriocinov – nizina, ki je zelo robusten in močno protimikrobno aktiven ter zato zanimiv s stališča industrijske proizvodnje. Pri pridobivanju nizina smo uporabili več lastnih sevov laktokokov ter po koncu fermentacije nizin še delno prečistili in skoncentrirali z različnimi kromatografskimi tehnikami, ultrafiltracijo, precipitacijo s solmi in penjenjem. Izhodno gojišče za produkcijo vitamina B12 je imelo podobno sestavo kot gojišče za pridobivanje biomase MKB, smo pa tu tekom produkcije dodali še prekurzorje.

Ključne besede: mlečnokislinske bakterije, probiotiki, bakteriocini, postbiotiki, vitamin B12, kisla sirotka

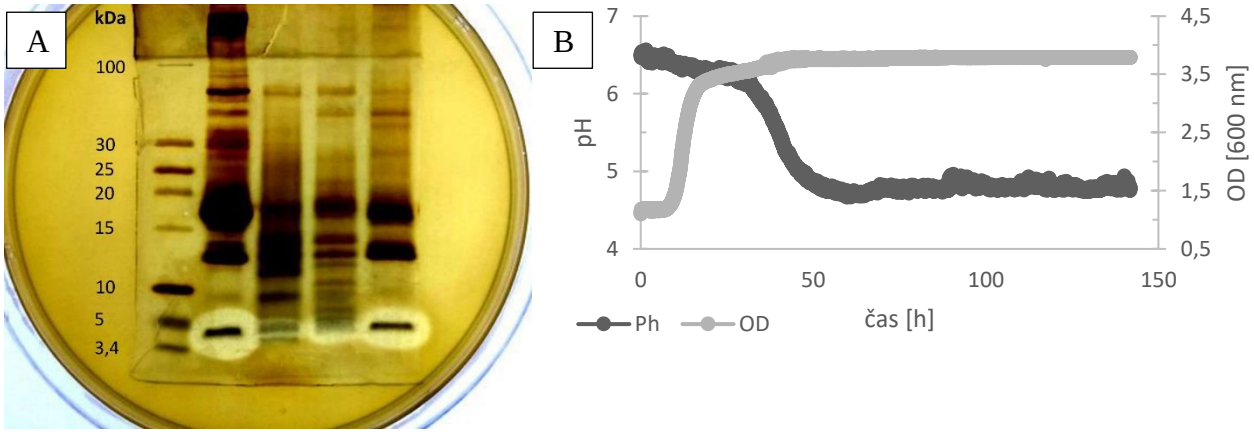
Uvod
Mlečnokislinske bakterije (MKB) so zelo razširjena skupina mikroorganizmov, ki jih najdemo tako v fermentiranih mlečnih, mesnih in rastlinskih izdelkih, kot tudi v prebavilih ter uro-genitalnem traktu ljudi in živali, pa tudi v tleh in vodi. MKB proizvajajo mlečno kislino kot glavni končni produkt njihovega anaerobnega metabolizma. Znane pa so tudi po sposobnosti sinteze širokega spektra metabolitov, ki ugodno vplivajo na prehranske, senzorične in tehnološke lastnosti fermentiranih živil. Zaradi teh razlogov se MKB v veliki meri uporabljajo kot starterske kulture, probiotiki in nutracevtiki (Wang in sod., 2021).

Priprava sirotke za gojenje MKB
Osiromašeno kislo sirotko iz proizvodnje skute, ki je bila mikrofiltrirana (0,5 µm) in ji je bil odvzet laktoferin in del drugih proteinov, smo uporabili za gojenje MKB ter proizvodnjo koristnih metabolitov. Sirotko smo dodatno obogatili s kvasnim ekstraktom (1-10 %), v nekaterih primerih tudi z različnimi mineralnimi solmi in pri produkciji vitamina B12 še s $CoCl_2$ in 5,6-dimetilbenzimidazolom (DMBZ). Pred pričetkom fermentacije smo pH uravnali na 6,5 in nato inokulirali z različnimi sevi vrste *Lactiplantibacillus* (*L.*) *plantarum*, s probiotičnim sevom *Lacticaseibacillus* (*L.*) *rhamnosus* GG, s proizvajalcem vitamina B12 *Propionibacterium* (*P.*) *freudenreichii* subsp. *freudenreichii* van Niel 1928 ali s sevi, ki proizvajajo bakteriocine: *Lactobacillus* (*L.*) *paragasseri* K7 (gasericini), različnimi sevi vrste *Lactococcus lactis* (nizin), *L. johnsonii* IM123 (laktacin F), *Pediococcus* (*Pd.*) *acidilactici* IM1 (pediocin AcH), *Pd. acidilactici* IM2 (pediocin PA-1) in *Enterococcus faecium* IM273 (enterocini A, B, P).

Produkcija biomase MKB v kisli sirotki
Optimizacijo gojenja smo pričeli na laboratorijskem nivoju v volumnu 10 mL nadaljevali pa na bioreaktorskem nivoju v 1 L in 2,5 L. Uspeli smo pridobiti biomaso različnih sevov MKB v koncentraciji 10^8-10^{10} KE/mL. Tekom bioreaktorskega procesa smo spremljali optično gostoto, število KE/mL, pH, obrate mešala, vsebnost kisika in temperaturo. Preskuse smo pri MKB vodili do stacionarne faze, ki je nastopila med 7-imi in 9-imi urami. Pri proizvodnji bakteriocinov smo čas fermentacije tudi podaljševali preko noči, fermentacija pri produkciji B12 pa je trajala 5-6 dni in smo jo zaustavili, ko je bila vsebnost B12 najvišja. Ugotovili smo, da uravnavanje pH tekom bioprocesa in dodatek mineralnih soli na samo produkcijo biomase MKB nista imela bistvenega vpliva. Smo pa z uravnavanjem pH na 6,4 tekom bioprocesa izboljšali produkcija nizina in vitamina B12.

Produkcija koristnih metabolitov v kisli sirotki

Brozgo iz bioprocesa smo centrifugirali in uporabili supernatant za nadaljnje analize protimikrobne aktivnosti proizvedenih metabolitov – bakteriocinov. Bakteriocine MKB predstavljajo ribosomsko sintetizirani protimikrobni peptidi, ki jih lahko v grobem razdelimo v dva razreda. V razred I spadajo peptidi, ki so podvrženi post-translacijskim modifikacijam, k peptidom razreda II pa tisti, ki se po sintezi več ne spremenijo. Po mehanizmu delovanja jih lahko nadalje razdelimo na tiste, ki delujejo na celično ovojnico in tiste, ki so dejavni predvsem znotraj celice in vplivajo na izražanje genov in sintezo proteinov (Cotter in sod., 2013). V nadaljnjih poskusih smo se osredotočili predvsem na produkcijo nizina s sevom *Lactococcus lactis* IM145. Nizin spada k skupini lantibiotikov (razred I), njegovo protimikrobno delovanje pa temelji na motnji sinteze celične stene sorodnih bakterijskih vrst ter posledično celične smrti (Małaczewska in Kaczorek-Łukowska, 2021). Nizin smo delno očistili s pomočjo ultrafiltracije, tekočinske kromatografije visoke ločljivosti z obrnjeno fazo in kationsko-izmenjevalne kromatografije. Čistost in funkcionalnost frakcij smo ugotavljali z NaDS-PAGE in preskusi protimikrobne aktivnosti (Slika 1A). Usmerili smo se tudi v produkcijo B12 s sevom *P. freudenreichii* subsp. *freudenreichii* van Niel 1928 (Slika 1B). Povpraševanje po vitaminu B12 je namreč veliko, saj se velik del prebivalstva, zlasti tisti, ki ne uživajo živil živalskega izvora, sooča z nezadostnim vnosom tega vitamina. Pri produkciji smo vsebnost vitamina B12 vrednotili z analizno metodo LC-MS/MS. Vzorec smo prehodno obdelali s KCN, da smo pridobili stabilnejšo ciano obliko vitamina B12.



Slika 1: Spremljanje proizvodnje biomase MKB in produkcije koristnih metabolitov. (A) Spremljanje protimikrobne aktivnosti nizina na NaDS-PAGE gelu; (B) spremljanje bioprocesa pri produkciji B12.

Zaključek

Proizvodnja biomase MKB in njenih metabolitov odpira številne aplikacije v farmacevtski in živilski industriji (Moradi in sod., 2021). Ker so ti mikroorganizmi dokaj zahtevni glede svojih prehranskih potreb, predvsem virov dušika, smo njihovo rast spodbudili z dodatkom kvasnega ekstrakta k deproteinizirani kisli sirotki ter tako dosegli nadaljnjo izrabo sirotke z zadovoljivim prirastom MKB in pridobivanjem različnih bakteriocinov ter vitamina B12.

Zahvala

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Utilization of acid whey for the cultivation of lactic acid bacteria and extraction of beneficial metabolites

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Abstract
Acid whey, produced during the manufacture of fresh curd cheese, was used for the production of biomass of lactic acid bacteria (LAB) as starter and probiotic cultures, and for the production of bacteriocins and vitamin B12. Some of the whey proteins were previously removed, and during optimization of the growth conditions, the whey was enriched with yeast extract and mineral salts. We have established the growth conditions for the successful production of several LAB and also managed to cultivate various strains that produce bacteriocins. We set up laboratory production in acid whey for one of the more widely used bacteriocins – nisin, which is very robust and highly antimicrobially active and therefore interesting for industrial production. We used our own strains of lactococci to obtain nisin, and after completion of fermentation, the nisin was also partially purified and concentrated by various chromatographic techniques, ultrafiltration, salt precipitation and foaming. The medium for vitamin B12 production was based on the same source as for the other LABs, but here we also added precursors during production process.

Keywords: lactic acid bacteria, probiotics, bacteriocins, postbiotics, vitamin B12, acid whey

Introduction
Lactic acid bacteria (LAB) are a widespread group of microorganisms found in fermented dairy, meat, and plant products, as well as in the gastrointestinal and genitourinary tracts of humans and animals, and also in soil and water. LABs produce lactic acid as the major end product of their anaerobic metabolism. They are also known for their ability to synthesize a wide range of metabolites that have beneficial effects on the nutritional, sensory and technological properties of fermented foods. For these reasons, LABs are widely used as starter cultures, probiotics, and nutraceuticals (Wang et al., 2021).

Preparation of whey for LAB cultivation
Depleted acid whey from fresh curd cheese production, microfiltered (0.5 µm) and with previously removed lactoferrin and some other proteins, was used as a base for cultivation of LAB and production of beneficial metabolites. The whey was additionally enriched with yeast extract (1-10 %), in some cases with various mineral salts, and in the production of vitamin B12 with $CoCl_2$ and 5,6-dimethylbenzimidazole (DMBZ). Before fermentation, the pH was adjusted to 6.5 and then inoculated with various strains of *Lactiplantibacillus* (*L.*) *plantarum*, with the probiotic strain *Lacticaseibacillus* (*L.*) *rhamnosus* GG, with vitamin B12 producer *Propionibacterium* (P.) *freudenreichii* subsp. *freudenreichii* van Niel 1928 and with the strains producing bacteriocins: *Lactobacillus* (*L.*) *paragasseri* K7 (gassericins), different strains of *Lactococcus lactis* (nisin), *L. johnsonii* IM123 (lactacin F), *Pediococcus* (*Pd.*) *acidilactici* IM1 (pediocin AcH), *Pd. acidilactici* IM2 (pediocin PA -1), and *Enterococcus faecium* IM273 (enterocins A, B, P).

Production of LAB biomass in acid whey
The optimization of the cultivation started in a volume of 10 mL and was then continued in bioreactors of 1 L and 2.5 L. We were able to obtain biomass of different LAB strains at a concentration of 10^8-10^{10} CFU/mL. Optical density, CFU/mL, pH, stirrer speed, oxygen content and temperature were monitored during the bioreactor process. Experiments with the LAB strains were performed until stationary phase, which occurred at 7 to 9 hours after inoculation. For bacteriocin production, the fermentation time was additionally extended overnight, while for B12 production, the fermentation lasted 5-6 days and was stopped when the B12 content was at its highest. We found that regulating pH during the bioprocess and adding mineral salts had no significant effect on the production of LAB biomass itself. However, by adjusting the pH to 6.4 during the bioprocess, we improved the production of nisin and vitamin B12.

Production of beneficial metabolites in acid whey

The broth from the bioprocess was centrifuged and the supernatant was used for further analysis of antimicrobial activity of the produced metabolites – bacteriocins. Bacteriocins from LAB are ribosomally synthesized antimicrobial peptides that can be broadly divided into two classes. Class I includes peptides that undergo post-translational modifications, and class II includes peptides that are not modified after synthesis. Depending on the mechanism of action, they can be further divided into those that act on the cell envelope and those that are mainly active inside the cell and affect gene expression and protein synthesis (Cotter et al., 2013). In further experiments, we focused mainly on the production of nisin using the *Lactococcus lactis* IM145 strain. Nisin belongs to the group of lantibiotics (class I), and its antimicrobial activity is based on the disruption of cell wall synthesis of related bacterial species and the resulting cell death (Małaczewska and Kaczorek-Łukowska, 2021). The nisin was partially purified by ultrafiltration, reversed-phase high-performance liquid chromatography, and cation-exchange chromatography. The purity and functionality of the fractions were determined by SDS-PAGE and antimicrobial activity assays (Figure 1A). We also focused on B12 production using *P. freudenreichii* subsp. *freudenreichii* van Niel 1928 (Figure 1B). The need for vitamin B12 is actually quite high, since a large part of the population, especially those who do not eat foods of animal origin, faces an insufficient intake of this vitamin. During production, the vitamin B12 content was determined using the analytical method LC-MS/MS. The sample was also pre-treated with KCN to obtain a more stable cyano form of vitamin B12.

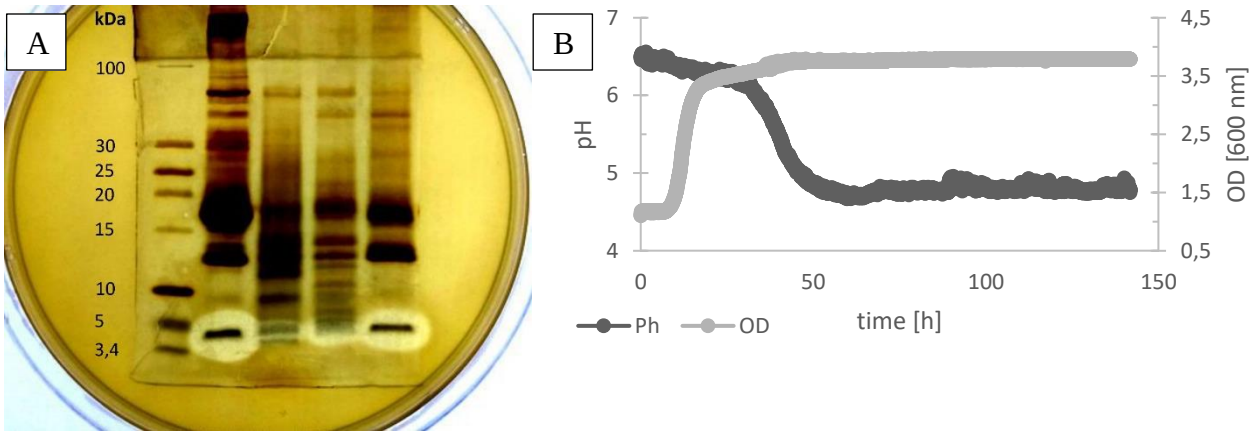

Figure 1: Monitoring LAB biomass production and production of beneficial metabolites. (A) Monitoring the antimicrobial activity of nisin on SDS- PAGE gel; (B) monitoring the bioprocess in B12 production.

Conclusion

The production of LAB biomass and its metabolites opens many applications in the pharmaceutical and food industries (Moradi et al., 2021). Since these microorganisms are quite demanding in terms of their nutrient requirements, especially nitrogen sources, we stimulated their growth by addition of the yeast extract to deproteinized acid whey, thus achieving subsequent use of whey with satisfactory growth of LAB and production of various bacteriocins and vitamin B12.

Acknowledgements

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Pridobivanje bioplina iz sirotke

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Povzetek
Pregledni prispevek obravnava možnosti za proizvodnjo bioplina iz odpadne kisle sirotke. Čeprav je ta energetsko bogata, je potrebno bioplinsko tehnologijo za maksimalni izplen energije ustrezno prilagoditi zaradi njene nizke pufrske kapacitete. Tu pride v poštev predvsem kodigestija sirotke z drugimi organskimi odpadki, kot so odpadno biološko blato iz čistilnih naprav, goveja in prašičja gnojevka in gnoj ter odpadki iz živilske industrije. Ustrezna tehnologija omogoča velik izplen toplotne ali/in električne energije iz sirotke, kar podpira sonaravni razvoj in krožno gospodarstvo v agroživilskem sektorju.

Ključne besede: sirotka, bioplin, obnovljiv vir energije, krožno gospodarstvo

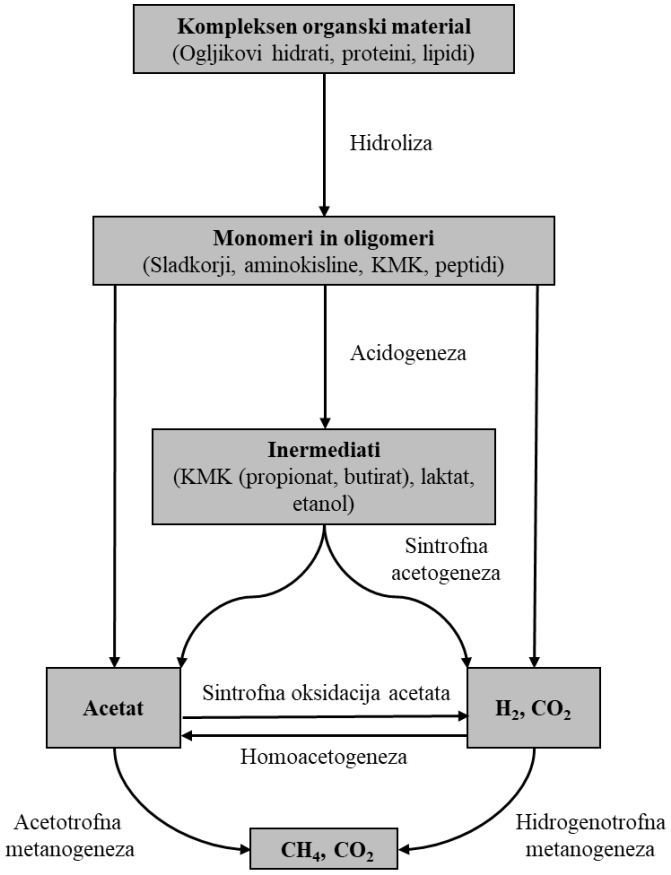
Uvod
Sladka sirotka v mlečni industriji večinoma ne predstavlja odpadka, ker se predela v različne prehranske proizvode, kisla sirotka pa je težavnejša za predelavo, jo je pa možno z anaerobno razgradnjo predelati v bioplin, ki predstavlja obnovljiv vir energije. Kisla sirotka je sicer organsko bogat stranski produkt (KPK je večinoma večji od 70 g/L) z veliko vsebnostjo ogljikovih hidratov (predvsem laktoze, do 50g/L), proteinov, lipidov in mineralnih snovi, ima pa nizko pH vrednost in majhno pufrsko kapaciteto (Miller in sod., 2007; Dufton in sod., 2018), zato tudi v procesu anaerobne razgradnje lahko povzroča težave. Težavam pri proizvodnji bioplina iz sirotke se je najlažje izogniti s kodigestijo z drugimi organskimi odpadki (Escalante in sod., 2018). Iz sirotke pa je možno poleg metana (bioplina) proizvesti tudi biovodik (Kozlowski in sod., 2016).

Anaerobna razgradnja sirotke v bioplin
Sirotko anaerobni mikroorganizmi v bioreaktorjih razgradijo v 4 zaporednih stopnjah do mešanice CH_4 in CO_2, ki jo imenujemo bioplin in predstavlja obnovljiv vir energije (Slika 1). Anaerobne hidrolitske bakterije v tem procesu hidrolizirajo polimerne snovi (npr. proteine sirotke) do monomernih sestavin, te pa dalje fermentirajo acidogene bakterije, ki proizvedejo kratkoverižne maščobne kisline (KMK), pri čemer je pri sirotki pomembno, da laktozo acidogene bakterije zelo hitro fermentirajo do KMK. KMK dalje pretvorijo acetogene bakterije v acetat, H_2 in CO_2. To pa so neposredni substrati za metanogenezo, ki jo opravijo metanogene arheje (Li in Khanal, 2017). Uspešnost proizvodnje bioplina je odvisna od narave substrata (snovi, ki jih uvajamo v bioreaktor) in njegovega C:N razmerja, temperature, pH, inhibitornih snovi, obremenitve bioreaktorja z organsko snovjo… Bioplin lahko direktno uporabimo kot toplotni vir v industrijskih obratih, za ogrevanje sosesk ali za proizvodnjo električne energije, ki jo oddajamo v električno omrežje, kar pomeni, da se ta tehnologija odlično vklaplja v krožno gospodarstvo na agroživilskem področju (Antonelli in sod., 2016).

Energetski in drugi potenciali sirotke
Sirotka ima velik energetski potencial, če jo z ustrezno vodenim tehnološkim postopkom predelamo v bioplin. Ustrezna bioplinska tehnologija lahko zagotovi izplen okoli 10.000 MWh toplotne energije iz 1000 ton sirotke. Iz kg suhe snovi pri kodigestiji sirotke in prašičje gnojevke lahko pridobimo od 149 do 268 L metana (Antonelli, 2016). Predelava sirotke v bioplin omogoča več vidikov trajnostnega razvoja v agroživilskem sektorju: poleg recikliranja odpadne organske snovi v obnovljivo energijo nastaja kot stranski produkt t.i. bioplinska gnojevka (tekoči ostanek anaerobne razgradnje), ki je odlično tekoče gnojilo za rastlinsko pridelavo (Tirichine in sod., 2020).

Težave in rešitve pri proizvodnji bioplina iz sirotke
Kljub temu, da ima sirotka velik energetski potencial, predstavlja problematičen substrat za proizvodnjo bioplina predvsem zaradi svoje majhne pufrske kapacitete (nizke alkalinitete), ki lahko hitro povzroči zakisanje v bioreaktorju in s tem nestabilnost procesa, posledično se zmanjša izplen bioplina in vsebnost metana v bioplinu (Bijnea in sod., 2015). K hitremu znižanju pH vrednosti pomembno prispeva tudi zelo hitra fermentacija laktoze v KMK, ki se lahko zgodi že med skladiščenjem in transportom sirotke do bioplinske naprave. Rešitev za to procesno težavo je kofermentacija sirotke z drugimi substrati, ki imajo večjo pufrsko kapaciteto. Sirotko pogosto presnavljajo v bioplin skupaj z odvečnim aktivnim blatom iz komunalnih čistilnih naprav, govejo gnojevko ali gnojem, perutninskim gnojem in celo skupaj z odpadki iz ribogojnic, s čimer

povečamo pufersko kapaciteto in dosežemo optimalno C:N razmerje, ki je v območju 30 do 10. Pri čisti sirotki je namreč prenizko zaradi prebitka dušika (Vivekanad in sod., 2018). Take rešitve omogočajo uspešno in stabilno proizvodnjo bioplina iz sirotke (Petek, 2019).



Slika 1: Shema mikrobne razgradnje organskih snovi iz sirotke v bioplin (CH_4+CO_2)

Zahvala

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Biogas production from whey

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Abstract
The review article discusses the possibilities for biogas production from waste acid whey. Although it is energy-rich, biogas technology needs to be adapted to maximize energy yield due to its low buffering capacity. This includes, in particular, whey codigestion with other organic wastes, such as waste biological sludge from sewage treatment plants, bovine and pig slurry and manure and waste from the food industry. Appropriate technology enables high yields of heat and/or electricity from whey, which supports sustainable development and the circular economy in the agri-food sector.

Key words: whey, biogas, renewable energy source, circular economy

Introduction
Sweet whey in the dairy industry is mostly non-waste because it is processed into various food products, while acid whey poses greater processing problems, but can be processed by anaerobic digestion into biogas, which is a renewable energy source. Acid whey is an organically rich by-product (COD is mostly greater than 70 g/ L) with a high content of carbohydrates (especially lactose, up to 50 g/L), proteins, lipids and minerals, but has a low pH value and low buffer capacity (Miller et al., 2007; Dufton et al., 2018), therefore, can also cause problems in the process of anaerobic digestion. Problems with acid whey biogas production are best avoided by codigestion with other organic wastes (Escalante et al., 2018). In addition to methane (biogas), biohydrogen can also be produced from whey (Kozlowski et al., 2016).

Anaerobic degradation of whey into biogas
Whey is decomposed by anaerobic microorganisms in bioreactors in 4 successive stages to a mixture of CH4 and CO_2, which is called biogas and represents a renewable energy source (Figure 1). In this process, anaerobic hydrolytic bacteria hydrolyze polymeric substances (eg. whey proteins) to monomeric components, which are further fermented by acidogenic bacteria producing short-chain fatty acids (SCFAs). It is important that lactose from whey is fermented very quickly by acidogenic bacteria to SCFAs. SCFAs are further converted by acetogenic bacteria to acetate, H2 and CO2. These, in turn, are direct substrates for methanogenesis performed by methanogenic archaea (Li and Khanal, 2017). The efficiency of biogas production depends on the nature of the substrate (substances introduced into the bioreactor) and its C:N ratio, temperature, pH, inhibitory substances, bioreactor's organic load… Biogas can be used directly as a heat source in industrial plants, for district heating or for the production of electricity that is transmited to the electricity grid, which means that this technology fits perfectly into the circular economy in agri-food sector (Antonelli at al., 2016).

Energy and other potentials of whey
Whey has a great energy potential if it is processed into biogas by a properly managed technological process. Appropriate biogas technology can provide about 10,000 MWh of thermal energy from 1,000 tons of whey. 149 to 268 L of methane can be obtained from 1 kg of dry matter in whey and pig manure codigestion (Antonelli, 2016). The processing of whey into biogas supports several aspects of sustainable development in the agri-food sector: in addition to the recycling of waste organic matter into renewable energy, liquid biogas manure (liquid residue of anaerobic digestion) is formed as a by-product which is an excellent fertilizer for crop production (Tirichine et al., 2020).

Problems and solutions in biogas production from whey
Despite the fact that whey has a high energy potential, it is a problematic substrate for biogas production mainly due to its low buffering capacity (low alkalinity), which can quickly cause acidification in the bioreactor and therefore process instability resulting in reduced biogas yield and methane content in biogas (Bijnea et al., 2015). The very rapid fermentation of lactose into SCFAs additionally adds to the low pH. The solution of this problem is the cofermentation of whey with other substrates that have a higher buffer capacity. Whey is often digested to biogas together with addition of activated sludge from municipal wastewater treatment plants, cattle and pig slurry and manure, poultry manure and even together with waste from fish farms, thus increasing buffer capacity and achieving an optimal C:N ratio of 30 to 10, which is too low in pure whey due to excess nitrogen

(Vivekanad et al., 2018). Such solutions enable a successful and stable production of biogas from whey (Petek, 2019).

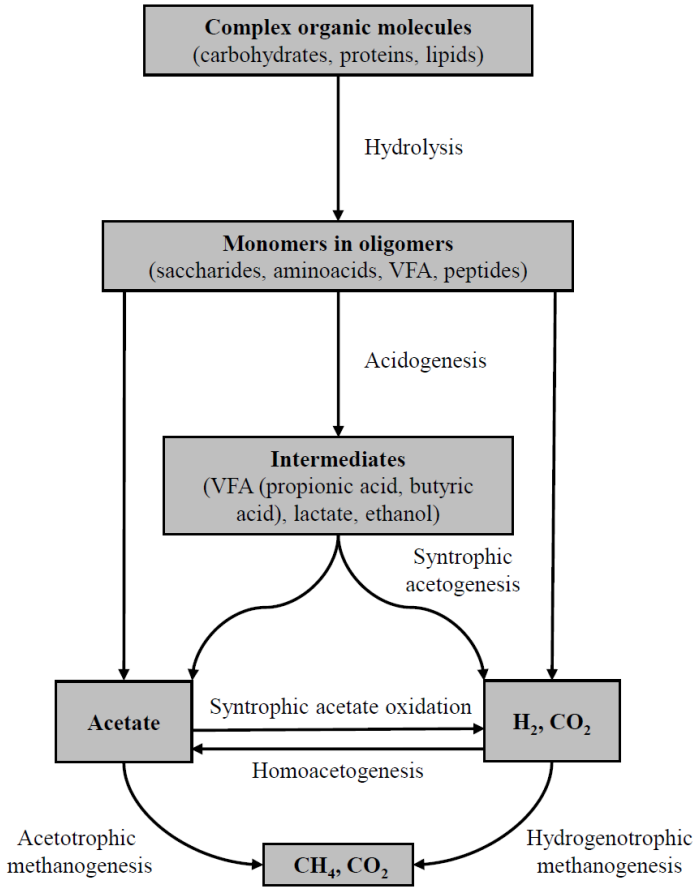

Figure 1: Scheme of microbial degradation of organic matter from whey to biogas (CH_4+CO_2)

Acknowledgements
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Sirotka kot dragocen vir funkcionalnih napitkov

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Izvleček
Sirotka je eden glavnih stranskih proizvodov mlečne industrije, ki izvira iz proizvodnje sira ali kazeina. Opisujejo jo kot rumenkasto-zelenkasto tekočino, ki ostane po koagulaciji kazeina z encimi in/ali kislinami. Proizvodnja in predelava mleka se nenehno povečujeta, pri čemer je sir najbolj razširjen mlečni izdelek. Posledično se povečujejo količine proizvedene sirotke. Sirotkine beljakovine veljajo za najpomembnejše sestavine, ki prispevajo k visoki hranilni vrednosti sirotke. Vse več je znanstvenih dokazov, ki potrjujejo bioaktivnost peptidov, ki nastanejo pri hidrolizi sirotkinih beljakovin s prebavnimi encimi, proteolitičnimi mikroorganizmi ali rastlinskimi proteazami. Med najbolj raziskanimi koristnimi učinki so zniževanje krvnega tlaka, antikancerogene, protimikrobne in antioksidativne lastnosti; imunomodulatorni učinki, uravnavanje sitosti in uravnavanje telesne teže, ohranjanje zdravja kosti in ohranjanje zdravja kože.
Raziskave so pokazale različne tehnološke rešitve za proizvodnjo živil na osnovi sirotke, pri čemer se je izkazalo, da je proizvodnja napitkov z ekonomskega in tehnološkega vidika najbolj sprejemljiva. Njihove hranilne lastnosti in potencialni učinki na zdravje izpolnjujejo zahteve sodobnih potrošnikov, ki se vse bolj zavedajo pomembnega vpliva hrane na človeka. Največ pozornosti so bile do zdaj deležni napitki z dodanim sadjem ali gazirani napitki, ki so bili ocenjeni kot sprejemljivi za potrošnika. Poleg tega je veliko zanimanja tudi za fermentirane napitke, zlasti če vsebujejo probiotične seve.
Tako je cilj te predstavitve podati pregled aktualnega znanja glede možnosti kreiranja napitkov iz sirotke. Zdi se, da je glavni problem v pomanjkanju informacij o prednostih teh izdelkov, ki jih mlekarska industrija posreduje potrošnikom. Poslecično ni dovolj zanimanja za takšne napitke. V prihodnosti je torej potrebno vlagati več truda v marketinške in aktivnosti in informiranje o izjemnih hranilnih in funkcionalnih lastnostih sirotke in napitkov iz sirotke, da bi pritegnili več pozornosti potrošnikov.

Ključne besede: sirotka, metode predelave, funkcionalne lastnosti, napitki, zdravstvene koristi

Uvod
Sirotka – vrste in hranilna vrednost
V odvisnosti od vrste koagulacije kazeina je sirotka lahko kisla ali sladka. V povprečju sirotka vsebuje približno 93 % vode in 6-7 % suhe snovi. Razen v kislosti se razlikujeta tudi v deležu nekaterih sestavin, kot so kalcij in sirotkine beljakovine. Dušikove sestavine sirotke veljajo za biološko najbolj dragocene sestavine in so odgovorne zato, da sirotko prištevajo med funkcionalna živila. Beljakovine sirotke vključujejo intaktne globularne frakcije, kot so termoobčutljivi ß-laktoglobulin, α-laktalbumin, goveji serumski albumin in imunoglobulini ter termostabilno frakcijo proteoznih peptonov. V manjših količinah so prisotni tudi laktoferin, laktolin, transferin in drugi glikolizirani proteini. Za beljakovine sirotke je značilna odlična aminokislinska sestava, ki vsebuje vse esencialne in ne-esencialne aminokisline. Razmerje med žveplovimi aminokislinami (Cys, Met) vpliva na njihovo biološko uporabnost in je v beljakovinah sirotke veliko višje kot v drugih beljakovinah živalskega ali rastlinskega izvora. Tako je biološka vrednost sirotkinih beljakovin za približno 15 % višja v primerjavi z jajčnimi beljakovinami, ki so glede na koncentracije esencialnih aminokislin veljale za referenčne. V skladu s tem je sirotka odličen vir funkcionalnih beljakovin in peptidov, vitaminov, mineralov in laktoze – sladkorja z nizkim glikemičnim indeksom. Zato jo smatramo za funkcionalno živilo za različne skupine potrošnikov, v prvi vrsti za športnike, pa tudi za otroke ali starejše.

Predelava sirotke – glavne težave in rešitve
Glavna težava, povezana s sirotko, je njena močna onesnaževalna moč zaradi visokih vrednosti (40-60 g/L) biokemijske potrebe po kisiku (BPK) in kemijska potreba po kisiku (KPK; 50-80 g/L), ki je približno 175-krat višja od vrednosti za tipične odplake. Zato je strogo prepovedano odlagati sirotko v okolje kot odpadno vodo ali jo razsipavati kot gnojilo, kar je bila v preteklosti redna praksa. Zaradi specifične sestave je sirotka podvržena hitremu mikrobnemu kvarjenju in zahteva toplotno obdelavo, pri čemer pride do denaturacije sirotkinih beljakovin in nastanka usedlin. Takšni pojavi so s stališča potrošnikov nezaželeni. Tako se sirotka večinoma uporablja neposredno kot živalska krma za govedo, vendar se glede na trenutno proizvedene količine lahko pojavijo težave v zvezi z visokimi stroški prevoza, zaradi česar je tak način uporabe nepraktičen. Za izboljšanje ekonomske valorizacije so bile uvedenedruge možnosti izrabe, kot je proizvodnja dehidriranih izdelkov, kot so sirotka v prahu, koncentrati sirotkinih beljakovin in izolati. Zelo priljubljeno je tudi frakcioniranje in izolacija beljakovinskih komponent, kot sta α-laktalbumin (α-La) ali glikomakropeptid (GMP). Vendar se zdi, da je proizvodnja napitkov na osnovi sirotke najbolj ekonomična in najenostavnejša

rešitev za uporabo sirotke v prehrani ljudi. Tehnološki razvoj odpira možnosti uporabe alternativnih metod v proizvodnji živil, kot so membranski procesi, visoko intenzivna ultrazvočna obdelava ali tehnologija superkritičnega ogljikovega dioksida. Z uporabo metod, drugačnih od termične obdelave, bi lahko premagali ovire, kot so kratek rok uporabnosti zaradi mikrobiološkega kvarjenja, pojav neželenih usedlin ali denaturacija sirotkinih beljakovin, izboljšali pa bi tudi obstoječe izdelke.

Napitki na osnosi sirotke – vrste in prehranska vrednost

Potreba po razvoju napitkov na osnovi sirotke je tesno povezana s hranilnimi in funkcionalnimi lastnostmi sirotkinih beljakovin ter z izpolnjevanjem pričakovanj sodobnih potrošnikov, ki zahtevajo inovativne izdelke z izboljšano funkcionalnostjo. V zadnjih dveh desetletjih so prišli na trg številni napitki na osnovi sirotke in podobni izdelki, ki vsebujejo posamezne sestavine sirotke (predvsem sirotkine beljakovine), kot osvežilna živila z dodano vrednostjo in/ali funkcionalna živila. Na splošno obstajata dve veliki skupini obstoječih napitkov - nefermentirani in fermentirani. Čeprav se zdi, da je najlažja rešitev za razvoj funkcionalnega napitka iz sirotke uporaba naravne sladke ali kisle sirotke kot osnove, se v zadnjem času uveljavlja tudi uporaba deproteinizirane sirotke ali sirotkinega permeata, ki ostane po ultrafiltraciji, pogosto v kombinaciji s CO_2. Pri fermentaciji sirotke se običajno uporabljajo starterske in/ali probiotične kulture, ki lahko presnavljajo laktozo. Napitki iz sirotke so priljubljene pri različnih skupinah potrošnikov bodisi kot nadomestki obrokov bodisi za druge namene, kot so rehidracija po vadbi, osvežitev in rehidracija med izpostavljenostjo visokim temperaturam okolja, kot lahko dostopni že pripravljeni zdravi prigrizki itd. Te večinoma uživajo športniki, zdravstveno ozaveščena populacija in potrošniki, ki želijo v vsakodnevno prehrano vključiti zdrave alternative. V primerjavi z nemlečnimi napitki sirotka predstavlja vir bioaktivnih beljakovin, peptidov in/ali lipidov s številnimi aktivnostmi, ki spodbujajo zdravje, kot so protimikrobno, antikancerogeno, antihipertenzično, antiulcerativno, protitumorsko ali antidiabetično delovanje, zniževanje holesterola in imunomodulatorno delovanje. Poleg tega je bogata tudi z vitamini skupine B, minerali, predvsem s kalcijem, natrijem in kalijem, ter laktozo – disaharidom z relativno nizkim glikemičnim indeksom..

Trenutni tržni trendi in prihodnost

Napitki na osnovi sirotke so namenjeni širokemu krogu potrošnikov – od majhnih otrok do starejših ljudi. Njihove hranilne lastnosti in potencialni učinki na zdravje zadovoljujejo zahteve sodobnih potrošnikov. Največ zanimanja je bilo do zdaj za napitke z določeno količino sadja ali za gazirani napitki, ki jih potrošniki dobro sprejemajo. Zelo veliko zanimanja je tudi za fermentirani napitki, zlasti tiste, ki vsebujejo probiotične seve. Vendar se zdi, da je glavni problem v pomanjkanju informacij, ki so na voljo potrošnikom glede prednosti teh izdelkov. Po drugi strani pa je zanimanje za tovrstne napitke še premajhno, zaradi česar je njihova proizvodnja z vidika mlečne industrije precej tvegana. Prihodnost je torej v pospešenih aktivnostih na področju trženja in informiranja potrošnikov, da bodo spoznali izjemne prehranske in funkcionalne lastnosti sirotke.

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Whey as a valuable source of functional beverages

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Abstract
Whey is one of the main by- products of the dairy industry originating from cheese or casein production. It can be defined as a yellowish-greenish liquid that remains after casein coagulation by enzymes and/or acids. Milk production and processing are increasing continuously, with cheese making being the most abundant dairy product. Consequently, there are growing amounts of the produced whey. Whey proteins are considered as the main contributors to the high nutritional value of whey. There is a growing number of scientific evidence confirming bioactivity of peptides arising from whey protein hydrolysis by digestive enzymes, proteolytic microorganisms or plant proteases. Some of the most examined beneficial effects are the blood pressure lowering action, anticancerogenic, antimicrobial and antioxidative properties; immunomodulatory effects, satiety regulation and weight management, bone health protection and dermoprotective action.
Various technological solutions have been studied in order to increase the production of foods based on whey whereby the production of beverages appeared to be most acceptable from the economic and technological point of view. Their nutritional properties as well as potential health promoting effects meet the requirements of modern consumers who are increasingly becoming aware of the great influence food has on humans. Beverages containing a certain amount of fruits or carbonated beverages have gained the most attention up to now, and were evaluated as acceptable to the consumer. Besides, large interest has also been detected for fermented beverages, especially if those contain probiotic strains.
Thus, the aim of this presentation is to give an overview of current acknowledgments considering the possibilities of creating whey beverages. However, the main problem seems to be in the lack of information that dairy industry has put on disposal to the consumers considering the advantages of these products. In turn, there is insufficient interest for such beverages. So, future perspective lies in putting more efforts into marketing and informing activities in order to draw the consumers' attention to the outstanding nutritional and functional properties of whey and whey beverages.

Keywords: whey, processing methods, functional properties, beverages, health benefits

Introduction / subtitles
Whey – types and nutritional value
Depending on the type of casein coagulation, whey can be acid or sweet. On average whey contains approximately 93 % of water and 6-7% solids. Besides the differences in acidity, the proportions of certain constituents such as calcium or the present whey protein fractions are also distinct. Nitrogen components of whey have been recognised as biologically most valuable constituents and are responsible for it's high potential to be regarded as functional food. Whey proteins include intact globular fractions such as thermo-sensitive ß-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins as well as thermostable fraction of proteose-peptones. In smaller quantities also lactoferrin, lactolin, transferrin and other glycolysated proteins are present. Whey proteins are characterised by an excellent amino-acid composition which contains all essential and non-essential amino acids. The ratio between sulphuric amino acids (Cys, Met) influences their bioavailability and is much higher in whey proteins than in other proteins of animal or vegetable origin. Thus, biological value of whey proteins is approximately 15 % higher in comparison to egg proteins which were regarded as referent considering essential amino acid concentrations. Accordingly whey is an excellent source of functional proteins and peptides, vitamins, minerals and lactose – a sugar with low glycemic index. It may be considered as functional food for various groups of consumers, targeting in the first line athletes, but also children or elderly individuals.

Whey processing – main difficulites and solutions
The main problem associated to whey is it's strong polluting power due to high values (40-60 g/L) of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD; 50 -80 g/L) which is approximately 175 times higher than typical sewage effluent. Consequently it is strictly forbidden to dispose whey in the environment as waste water or to spread it as a fertiliser, which was a regular practice in the past. Owing to specific composition, whey is subject to rapid microbial spoilage and requires thermal treatment whereby whey protein denaturation and sediment formation occur. Such phenomena are undesired from the consumers' perspective. Thus whey is mostly used as a direct animal feed for cattle but in relation to currently produced volumes, difficulties concerning high transportation costs might occur that make such type of utilization impractical. In order to improve economic valorisation, new opportunities have been introduced like the production of dehydrated products such as whey powder, whey protein concentrates and isolates. Very popular is also the fractionation and isolation of protein components like α-lactalbumin (α-La) or

glycomacropeptide (GMP). However, production of whey-based beverages appears to be the most economical and simplest solution for whey utilization in human nutrition. Along with technological development, the application of alternative food processing methods such as membrane processes, high intensity ultrasound or supercritical carbon dioxide technology is being investigated too. By applying nonthermal processing methods, hurdles like the low shelf life due to microbiological deterioration, occurrence of undesired sediments or whey protein denaturation might be overcome, but also the existing products could be improved.

Whey based beverages – types and nutritional value

The need for development of whey-based beverages is closely related to nutritional and functional properties of whey proteins, as well as to fulfilling the expectations of modern consumers who demand innovative products with enhanced functionality. Over the past two decades, numerous whey-based beverages and similar products containing isolated whey components (mainly whey proteins) have been placed on the market as refreshing, value-added and/or functional foods. In general, there are two large groups of existing beverages - the non fermented and fermented beverages. Although the easiest solution for designing a functional whey beverage seems to be using native sweet or acid whey as a basis, recently it has been suggested to use deproteinised whey or whey permeate remaining after ultrafiltration, often in combination with CO_2. Whey fermentation usually employs starter and/or probiotic cultures able to metabolise lactose. Whey beverages are popular among various consumer groups either as meal replacements or for purposes like rehydration after exercise, refreshment and rehydration during exposure to high environmental temperatures, easily available and ready-to-eat healthy snacks, etc. These are mostly consumed by athletes, health-conscious working population and consumers eager to include healthy alternatives into the everyday diet. Compared to non-diary beverages, whey is considered as a pool of bioactive proteins, peptides and/or lipids with numerous health-promoting activities such as antimicrobial, anticancerogenic, antihypertensive, antiulcerative, antitumour, antidiabetic, cholesterol-lowering and immunomodulatory. Besides, it is also rich in B-group vitamins, minerals, especially calcium, sodium and potassium, and lactose – a disaccharide with relatively low glycemic index.

Current market trends and future perspective

Whey-based beverages target a large scale of consumers – from little children to old people. Their nutritional properties and potential health-promoting effects meet the requirements of modern consumers. Beverages containing a certain amount of fruits or carbonated beverages have gained the most attention up to now, and were evaluated as acceptable to the consumer. Very large interest has also been noted for fermented beverages, especially those containing probiotic strains. However, the main problem seems to be in the lack of information available to the consumers considering the advantages of these products. In turn, there is insufficient interest for such beverages, which makes the reviewing of their production rather risky from the dairy industry point of view. So, future perspective lies in putting more efforts into marketing and informing activities in order to draw the consumer attention to the outstanding nutritional and functional properties of whey.

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Uporaba sirotke v kozmetologiji: primeri kozmetičnih izdelkov

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Povzetek
Kozmetologija je eno najhitreje razvijajočih se znanstvenih področij, kar se odraža v široki paleti kozmetično aktivnih sestavin in inovativnih dostavnih sistemih. Kozmetični izdelki z vgrajenimi biološkimi makromolekulami kot so proteini in peptidi, dandanes pridobivajo na pomenu. Podobno kot druga področja se tudi kozmetologija sooča z vizijo "zero waste" (op. nič odpadkov) in se povezuje z drugimi industrijskimi panogami, med katerimi je ena bolj zanimivih povezava z mlečno industrijo. Sirotka je stranski produkt pri proizvodnji mlečnih izdelkov in predstavlja veliko obremenitev za okolje. Po drugi strani se v kozmetični industriji proteini živalskega izvora kot so mlečni proteini, uporabljajo kot kozmetično aktivne sestavine, ki dokazano izboljšajo stanje zrele kože. Sirotkini proteini v tekoči ali sušeni obliki delujejo tudi kot površinsko aktivne snovi, sredstva za geliranje in stabilizatorji pene. Uporaba kozmetičnih izdelkov za nego kože z vgrajenimi peptidnimi in proteinskimi molekulami je povezana z njihovo sposobnostjo, da vežejo in zadržijo vodo v zgornjih plasteh kože ter tako zagotavljajo mehko in gladko kožo. Namen pričujočega prispevka je bil razviti kozmetične izdelke s sirotko in tako prispevati k zmanjšanju odpadne sirotke. Za izdelavo šampona za lase in hidrogela za čiščenje obraza smo uporabili tako tekočo kot sušeno obliko sirotke. Ugotovili smo, da koncentracija sirotke bistveno vpliva na lastnosti kozmetičnih izdelkov. Medtem ko je pri tekoči sirotki najvišja koncentracija za vgradnjo do 20 % (m/m), je optimalna koncentracija za sušeno sirotko 2 % (m/m). Na podlagi rezultatov lahko zaključimo, da smo razvili stabilna kozmetična izdelka, katerih dodano vrednost predstavljajo proteini sirotke.

Ključne besede: kozmetologija, peptidi in proteini, nič odpadkov, sirotka, šampon, hidrogel

Uvod
Kozmetologija se kot eno najhitreje rastočih znanstvenih področij vse bolj osredotoča na potrebe in želje posameznika. Tako se soočamo z novimi trendi, ki vključujejo kozmetične izdelke z dodano vrednostjo in implementacijo vizije brez odpadkov. V to področje lahko nedvomno uvrščamo kozmetične izdelke na osnovi bioloških makromolekul. Proteini oz. večinoma peptidi so ene izmed najbolj inovativnih sestavin, ki se uporabljajo v kozmetiki, predvsem zaradi njihove biokompatibilnosti in nizke stopnje alergenosti. Začetki uporabe proteinov v kozmetične namene segajo v antiko, ko je egipčanska vladarica Kleopatra za nego kože uporabljala mlečne kopeli. Dandanes postajajo še posebej pomembni proteini, pridobljeni iz naravnih virov, kot so mleko, rastline ali morski organizmi. Sirotka, stranski proizvod sirarstva, se običajno šteje za odpadni material, katerega količina na letni ravni znaša kar 190 milijonov ton. Sirotka je motna rumenkasta tekočina, sestavljena večinoma iz vode in laktoze ter z bogato proteinsko sestavo. Proteine sirotke pridobivamo z obarjanjem kazeinov in obsegajo: β-laktoglobulin (48-58 %), α-laktalbumin (13-19 %), imunoglobuline (8-12 %), serumski albumin (5–6 %), laktoferin (2 %), laktoperoksidazo (0,5 %), glikomakropeptid (12–20 %), lizocim, laktofericin in citokine (Prokopowicz et al., 2017). Na področju kozmetologije so sirotka oz. proteini sirotke cenjeni tako zaradi doprinosa k formulaciji kot vpliva na stanje kože. Proteini sirotke učinkovito zavirajo draženje in zmanjšujejo rdečico kože, kar pospešuje regeneracijo kože po izpostavljenosti neugodnim zunanjim dejavnikom, na primer soncu in v primeru kožnih opeklin (Gorouhi et al., 2009).

Materiali in metode
Uporabljena sirotka je bila pridobljena iz domače proizvodnje mleka v tekoči obliki. V šampon (preglednica 1) in hidrogel za čiščenje obraza (preglednica 2) sta bili vključeni dve različni frakciji sirotke, in sicer kisla sirotka in filtrat sirotke (tako v tekoči kot sušeni obliki). Za pridobivanje sušene sirotke je bila na novo razvita in optimirana metoda sušenja z razprševanjem (BÜCHI Mini Spray Dryer B-290, Švica).

1.) ***Šampon:*** najprej smo pripravili 30 % raztopino natrijevega lavrilsulfata (del I) ter sestavine dela II segreli na vodni kopeli pri 60 °C. Dela I in II smo združili in mešali do ohladitve. Na koncu smo dodali sirotko in uravnali pH (4,5-5,5). V okviru vrednotenja stabilnosti (25 ºC in 40 ºC) smo izvedli meritve pH (Mettler Toledo GmbH, Švica) in viskoznosti (Anton Paar Austria GmbH) ter izdelke organoleptično ovrednotili.

2.) ***Čistilni hidrogel za obraz***: najprej smo natehtali sestavine iz dela Ia in jih postopoma dodali v del Ib. Nato smo pripravili del II in mu počasi dodajali del I. Na koncu smo dodali še konzervans in sirotko. V okviru vrednotenja stabilnosti (ciklični testi na 5 ºC, 25 ºC in 40 ºC) smo določili pH, viskoznost in organoleptične lastnosti.

Preglednica 1. Sestava šamponov s sirotko.

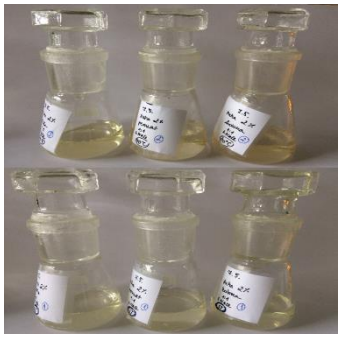
	tekoča sirotka % [m/m]	sušena sirotka % [m/m]
Del I		
30 % raztopina natrijevega lavrilsulfata	46,60	46,60
Del II		
kokamidopropil betain	1,50	1,50
kokamid DEA	1,50	1,50
propilenglikol	1,00	1,00
natrijev klorid	2,00	2,00
prečiščena voda	ad 100	ad 100
sirotka	**20,0**	**2,0**

Preglednica 2. Sestava hidrogelov s sirotko.

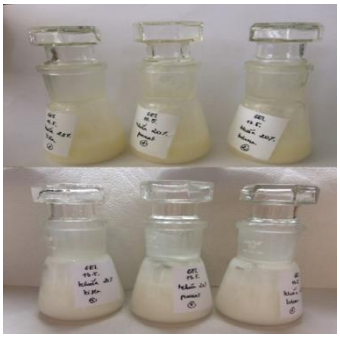
		tekoča sirotka % [m/m]	sušena sirotka % [m/m]
Del I			
Ia	prečiščena voda	29,00	47,00
	rožna voda	30,00	30,00
Ib	glicerol	7,50	7,50
	gumi ksantan	1,00	1,00
Del II			
decil glukozid		9,00	9,00
mandljevo olje		2,00	2,00
Del III			
Cosgard		1,50	1,50
sirotka		**20,00**	**2,00**

Rezultati

V prvem delu študije smo določili optimalno koncentracijo sirotke za vgradnjo v ***šampon***, pri čemer so vse proučevane koncentracije, z izjemama 2 % (m/m) in 20 % (m/m), vodile do vidnih nestabilnosti končnega izdelka. Nadalje smo ugotovili, da višanje koncentracije NaCl nad 2 % (m/m), ob stalni koncentraciji sirotke (t.j. sušeni 2 % (m/m); tekoči 20 % (m/m)), prav tako vodi k nestabilnemu izdelku. V naslednji stopnji smo v formulacijo dodali kokamid DEA (2% (m/m) NaCl) in s tem zagotovili stabilen produkt s sprejemljivo viskoznostjo. Med študijo stabilnosti pri 40 °C smo opazili povišanje pH vrednosti šamponov s sušeno sirotko, medtem ko pri šamponih s tekočo sirotko sprememb nismo zaznali. Prav tako smo ugotovili, da sirotka povzroči povišanje vrednosti pH v primerjavi s šamponi brez sirotke. Vrednosti viskoznosti se med stabilnostjo študijo niso bistveno razlikovale, opazna pa je bila porumenelost šamponov s specifičnim vonjem po mleku. V primeru ***hidrogela*** sta bili na podlagi predhodnih rezultatov za šampon kot končni koncentraciji izbrani 2 % (sušena sirotka) in 20 % (tekoča) (m/m) sirotke. Med študijo stabilnosti se viskoznost in pH hidrogelov nista bistveno spremenili, opazili pa smo spremembo v obarvanosti (slika 2).



Slika 1. Šamponi s sušeno (zgornja vrstica) in tekočo obliko sirotke (spodnja vrstica).



Slika 2. Rumenkasto obarvanje hidrogelov z vgrajeno sirotko med stabilnostno študijo (zgornja vrstica) v primerjavi s kontrolnimi vzorci (spodnja vrstica).

Zaključek

V okviru raziskovalnega dela smo razvili tehnološki postopek za pripravo šampona in hidrogela s sirotko ter nadalje optimirali metodo sušenja z razprševanjem za pridobivanje sušene frakcije, pri čemur smo upoštevali naravo sirotke. S stresno študijo smo dokazali, da pogoji shranjevanja ne vplivajo na stabilnost hidrogelov, nasprotno pa so šamponi z vgrajeno sirotko izkazovali znake nestabilnosti pri povišani temperaturi. Če povzamemo, smo razvili stabilen šampon in hidrogel s sušeno in tekočo (kisla in permeat) sirotko kar smatramo kot prispevek k viziji "nič odpadkov" in h krožnemu gospodarstvu.

Zahvala

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Use of whey in cosmetology: examples of cosmetic products

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Abstract
Cosmetology is one of the fastest-growing scientific areas, embracing wide range of cosmetically active ingredient and formulating novel delivery systems. Specifically, cosmetic products with incorporated biological macromolecules, i.e. proteins and peptides, that contribute to improved skin features, especially of aged skin, are gaining in importance. Similar to other fields, cosmetology is also facing with zero waste paradigm, and strives to a collaboration with other industries, amongst the one with milk industry is of great interest. Whey is a co-product in milk processing, and as such represents a high environmental burden. On the other hand, within the cosmetic industry proteins of animal origin, e.g. milk proteins, are used as cosmetically active ingredients. Whey proteins either in liquid or dry form act as emulsifiers, gelling agents, foam stabilizers and water bindings. The use of skin care cosmetic products with incorporated peptide and protein molecules is related to their ability to bind and retain water in the upper layers of the skin, and additionally providing emollient effect seen as soft and smooth skin The aim of the present research work was to develop cosmetic products with whey and thus ensure zero waste from milk processing. Liquid and dried form of whey in two different cosmetic products, i.e. hair shampoo and hydrogel for face cleaning, were under investigation. The results show that addition of all tested whey fractions leads to production of shampoo and gel in a concentration dependent manner. While for liquid whey the highest amount is up to 20 % (w/w), the optimal concentration for dried whey is 2 % (w/w). According to obtained results, stable whey-based cosmetic products with added value were formulated.

Keywords: cosmetology, peptides and proteins, zero waste, whey, shampoo, cleansing hydrogel

Introduction
Cosmetology as one of the fastest-growing scientific areas is increasingly focused towards individuals' needs and preferences. Thus, we are facing with new trends that include cosmetic products with added value plus a zero-waste mentality which is changing current consumers' habits. Cosmetic products based on biological macromolecules can indisputably be enclosed within this field. Proteins, or for the most part, peptides, are one of the most innovative ingredients used in cosmetics, valued in particular for their high biocompatibility and low potential to induce allergies. The beginnings of proteins' use for cosmetic purposes date back to antiquity when the Egyptian ruler Cleopatra used milk baths as luxury skin care treatment. Nowadays, proteins derived from natural sources such as milk, plants or marine organisms become especially recognised. Whey, a coproduct of cheese-making, is usually considered as waste material, and it is reported that 190 million tonnes of whey are produced annually. It is a turbid yellowish liquid composed mostly of water, lactose, fats minerals, but also having a superior protein composition. Whey proteins are a group of soluble milk proteins obtained by pH-dependent caseins precipitation and comprising: β-lactoglobulin (48-58 %), α-lactalbumin (13–19 %), immunoglobulins (8–12 %), serum albumin (5–6 %), lactoferrin (2 %), lactoperoxidase (0.5 %), glycomacropeptide (12–20 %), lysozyme, lactoferricin and cytokines (Prokopowicz et al., 2017). The advantage of whey and whey proteins in cosmetics is their double performance as they can affect the formulation properties as well as skin features. Used as cosmetically active ingredient, whey proteins effectively inhibit skin irritation and reduce skin redness through the promotion of inflammatory mediators, which accelerates skin regeneration after exposure to stressors, such as sun exposure and skin-burn (Gorouhi et al., 2009).

Materials and methods
The whey was obtained from domestic milk industry in a liquid form. Two different whey fractions, acid whey and permeate whey (both in liquid and dried form) were incorporated in the composition of shampoo *(Table 1)* and cleansing gel *(Table 2*). To obtain dried form of whey, spray drying method (BÜCHI Mini Spray Dryer B-290, Switzerland) was newly developed and optimized.
1.) ***Shampoo:*** First, 30 % solution of sodium lauryl sulfate was prepared. In addition, part II was weighed and heated in a water bath at 60 °C. Parts I and II were combined and stirred until cooled. At the end, whey was added and pH was adjusted to 4.5-5.5. As a part of stability test, pH (Mettler Toledo GmbH, Switzerland), viscosity (Anton Paar Austria GmbH) and organoleptic evaluation were performed after exposure to 25 and 40 ºC.
2.) ***Cleansing hydrogel:*** First, part Ia was weighed and gradually added into part Ib to provide gelation of xanthan gum. Second, part II was prepared and part I was slowly added. At the end, preservative and whey

were added. As a part of stability test, pH, viscosity, and organoleptic evaluation were performed after cyclic exposure to 5, 25 and 40 ºC.

Table 1. Composition of shampoos with liquid and dried whey.

	Product with liquid whey % [w/w]	Product with dried whey % [w/w]
Part I		
30 % solution of soduim lauril sulfate	46.60	46.60
Part II		
cocamidopropyl betaine	1.50	1.50
cocamide DEA	1.50	1.50
propylene glycol	1.00	1.00
sodium chloride	2.00	2.00
purified water	ad 100	ad 100
whey	**20**	**2**

Table 2. Composition of cleansing hydrogel with liquid and dried whey.

		Product with liquid whey % [w/w]	Product with dried whey % [w/w]
Part I			
Ia	purified water	29.00	47.00
	flower water	30.00	30.00
Ib	gycerol	7.50	7.50
	xanthan gum	1.00	1.00
Part II			
decyl glucoside		9.00	9.00
almond oil		2.00	2.00
Part III			
Cosgard		1.50	1.50
whey		**20.00**	**2.00**

Results

In the first part of the study, the optimal concentration of whey ***in shampoo*** was assessed. It was found out that all concentrations, apart from 2 % (w/w) and 20 % (w/w), led to visible instability of the final product. At constant rate of whey (i.e., dried 2 % (w/w); liquid: 20 % (w/w)), the addition of NaCl above 2 % (w/w) and cocamidopropyl betaine resulted in unstable shampoo with sedimentation observed. Addition of cocamide DEA at 2 % (w/w) of NaCl led to stable formulation with accepted viscosity (Figure 1). During stability study performed at 40 °C, the pH of shampoos with dried whey increased noticeably, while there were no significant changes in case of shampoos with other whey forms incorporated. It was also observed that addition of whey resulted in higher pH values comparing to shampoo with no whey. Viscosity was not altered, while yellowing of shampoos with specific milk odor were noticeable. Regarding ***hydrogels***, based on the previous results obtained for shampoos, 2 % (dried whey) and 20 % (liquid) (w/w) of whey were chosen as leading concentrations also for hydrogel. Within stability study, the viscosity and pH were not significantly altered, while minor colour alternations were observed (Figure 2).

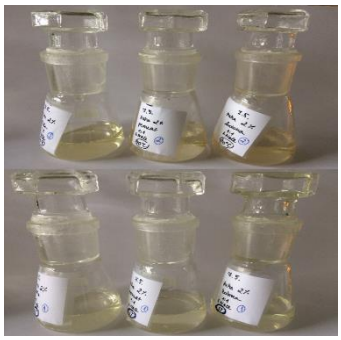

Figure 1. Shampoos containing dried (upper row) and liquid form of whey (lower row).

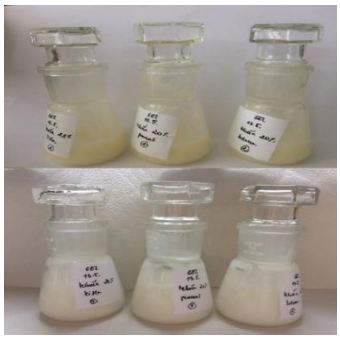

Figure 2. Yellowing of hydogels (upper row) during stability study comparing to hydrogels at the beginning (lower row).

Conclusion

During the study unique technological procedures for shampoo and hydrogel were developed, moreover spray drying method was prior optimized considering the nature of whey to obtain dried fraction of whey. Stability issues were successfully addressed, i.e. hydrogels expressed enhanced stability independently of stressed conditions, while the shampoos were unstable at elevated temperature. Finally, stable cosmetic products with incorporated liquid and dried whey were formed (both in acid and permeat form), thus contributing toward zero waste and circular economy.

Acknowledgements

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Posterji

Posters

Analizni pristop za vrednotenje stabilnosti laktoferina

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Povzetek
Laktoferin (Lf) je multifunkcionalen glikoprotein, ki veže železo in ima številne ugodne učinke na zdravje ljudi. Kot protein je Lf nagnjen k razgradnji, kar lahko kritično vpliva na kakovost izdelkov. Zato je bil glavni namen raziskovalnega dela razviti analizno metodologijo, ki omogoča vrednotenje stabilnosti Lf. Osredotočili smo se na reverznofazno in izključitveno kromatografijo, ki sta komplementarni metodi. Obe metodi smo uspešno validirali in potrdili njuno stabilnostno indikativnost. Pristop smo dodatno razširili s tremi spektroskopskimi tehnikami, s katerimi lahko v nekaterih primerih pridobimo koristne komplementarne informacije o stabilnosti Lf.

Ključne besede: laktoferin, stabilnost, HPLC-UV, spektroskopske tehnike, stabilnostno-indikativni pristop

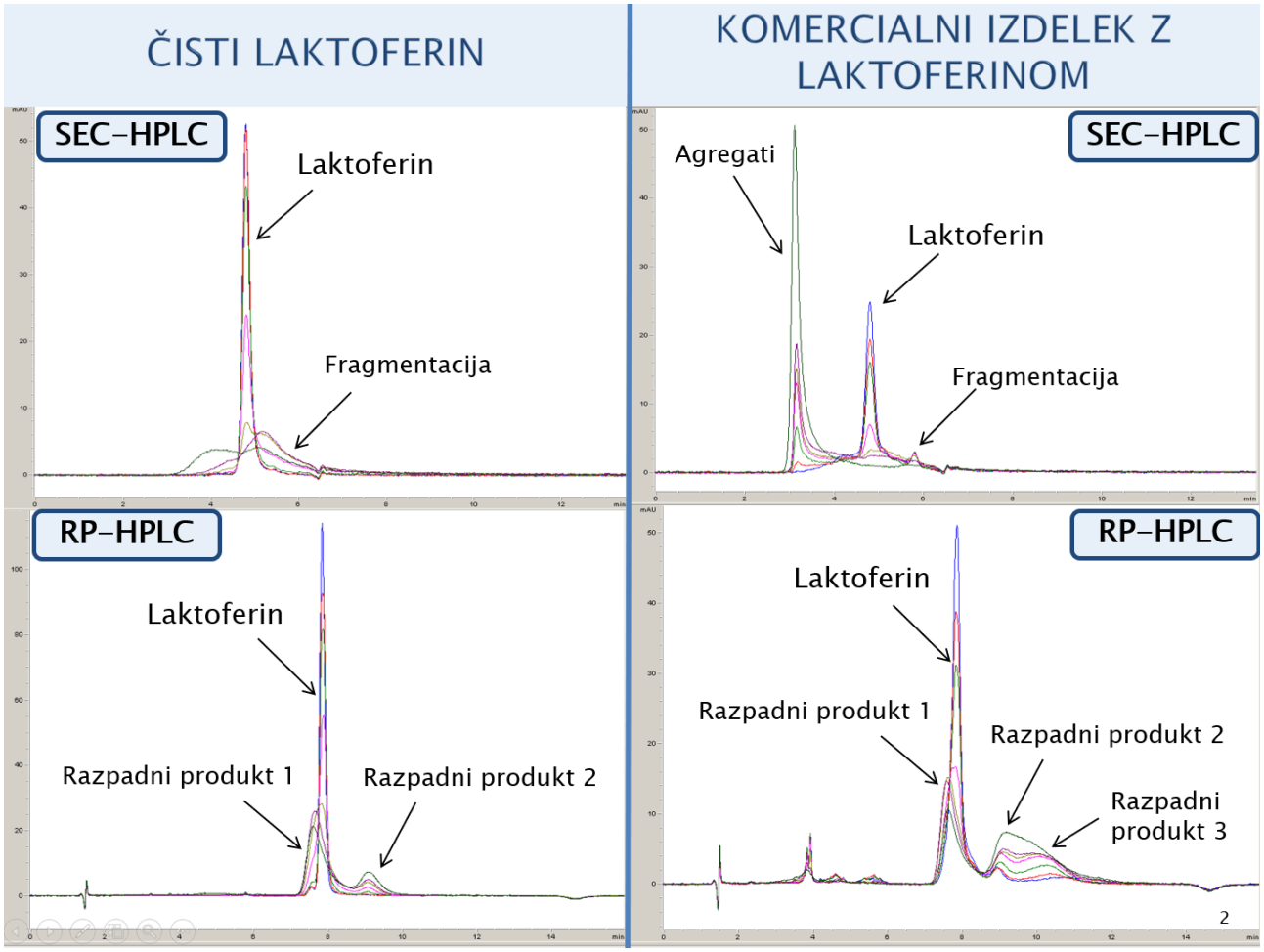
Uvod
Laktoferin (Lf) je približno 80 kDa velik globularni glikoprotein, ki veže železo v človeškem mleku. Najdemo ga v izločkih eksokrinih žlez in telesnih tekočinah. Deluje protimikrobno, protivnetno, imunomodulatorno, protirakavo in ima še številne druge fiziološke vloge (García-Montoya in sod., 2012). Lf je kot protein nestabilen, kar lahko kritično vpliva na kakovost izdelkov. Pri proteinskih učinkovinah moramo zagotoviti kakovost, varnost in učinkovitost med proizvodnjo, shranjevanjem in transportom, kar predstavlja velik izziv. Poleg tega je vrednotenje stabilnosti proteinov kompleksen in večplasten analizni izziv. Proteini se namreč od nizkomolekularnih spojin razlikujejo po večji strukturni kompleksnosti (ICH Q5C, 1995).

Namen
Primarni namen raziskovalnega dela je bil razviti analizno metodologijo, ki omogoča vrednotenje stabilnosti Lf v predformulacijskih študijah in v končnih izdelkih.

Materiali in metode
Standard govejega Lf smo kupili pri Sigmi-Aldrich. Analizirali smo tudi komercialno dostopna prehranska dopolnila z Lf. Preizkusili smo reverznofazne (RP) in izključitvene (SEC) kromatografske kolone petih različnih proizvajalcev (Agilent, Phenomenex, Sigma-Aldrich, Vydac in Waters). Uporabili smo Agilentov 1100/1200 HPLC sistem z detektorjem z nizom diod.

Rezultati in razprava
Osredotočili smo se na dve komplementarni kromatografski metodi. Med preizkušenimi RP kolonami (C3, C4, C8 in C18) se je najbolje izkazala kolona BioZen™ Intact XB-C8 150×4,6 mm, 3,6 µm (Phenomenex). Pri RP metodi smo optimalno ločbo dosegli s počasnim gradientnim programom in mobilno fazo, ki jo sestavljata 0,1 % TFA v vodi in 0,1 % TFA v acetonitrilu. Pri optimizirani SEC metodi uporabljamo kolono XBridge Protein BEH SEC 150×7,8 mm, 3,5 µm (Waters) in fosfatni pufer z dodatkom NaCl kot mobilno fazo. S stresnimi testi smo potrdili stabilnostno indikativnost kromatografskega analiznega pristopa in ugotovili, da je Lf najbolj občutljiv pri bazičnih pogojih in povišani temperaturi. V različnih vzorcih smo z RP metodo zaznali do 3 razpadne produkte Lf, s SEC metodo pa smo zaznali agregate in fragmente Lf (Slika 1). Razvite kromatografske metode smo uspešno validirali v skladu s smernico ICH in jih uporabili v stabilnostnih študijah (Osel in sod., 2021). Obe metodi smo uporabili tudi za vrednotenje vsebnosti Lf v komercialnih izdelkih, pri čemer smo ugotovili določeno stopnjo neujemanja med rezultati. RP metoda je primernejša za kvantitativno določanje Lf, saj lahko medij vzorca bistveno vpliva na kromatografski odziv pri SEC metodi.

Analizni pristop smo dodatno razširili s spektroskopskimi tehnikami (fluorimetrija, UV spektroskopija, določanje koncentracije celokupnih proteinov). Ugotovili smo, da so za kvalitativno in kvantitativno vrednotenje stabilnosti Lf primernejše kromatografske metode, vendar pa lahko z enostavnejšimi, hitrejšimi in dostopnejšimi spektroskopskimi tehnikami v nekaterih primerih pridobimo koristne komplementarne informacije o stabilnosti Lf.



Slika 1: Kromatogrami vzorcev čistega Lf in komercialnega izdelka z Lf, ki so bili shranjeni 0 min, 15 min, 30 min, 60 min, 120 min, 240 min in 24 h pri 60 °C.

Zaključek

Razvili smo stabilnostno-indikativni analizni pristop za vrednotenje stabilnosti Lf, ki temelji na komplementarnih RP in SEC kromatografskih metodah. Pristop omogoča ovrednotenje stabilnosti Lf v različnih vzorcih, pri čemer lahko zaznamo tudi razlike v mehanizmih razpada. Poleg tega je analizni pristop uporaben tudi pri kontroli kakovosti izdelkov z Lf.

Zahvala

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Analytical Approach for Stability Evaluation of Lactoferrin

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Abstract
Lactoferrin (Lf) is a multifunctional iron-binding glycoprotein that expresses several beneficial effects on human health. As a protein, Lf is prone to degradation which critically affects the quality of products. Therefore, the main purpose of our work was to develop a stability-indicating analytical approach for the evaluation of Lf stability. The approach was based on complementary reversed-phase and size-exclusion chromatographic methods which were successfully validated, also confirming their stability-indicating nature. The approach was additionally extended with three spectroscopic techniques which may provide valuable complementary information on Lf stability in some cases.

Keywords: lactoferrin, stability, HPLC-UV, spectroscopic techniques, stability-indicating approach

Introduction
Lactoferrin (Lf) is a globular glycoprotein with a molecular mass of about 80 kDa that is widely represented in various secretory fluids. As the main iron-binding in human milk it has anti-microbial, anti-inflammatory, immunomodulatory, anticancer, and many other biological activities (García-Montoya et al., 2012). As a protein, it is prone to degradation which critically affects the quality of products. There is a special challenge for proteins to ensure their quality, safety, and efficiency during processing, storage, and transportation; they differ from small molecules in their level of complexity. Therefore, protein characteristics demand a complex analytical approach and special handling in stability testing (ICH Q5C, 1995).

Objective
The aim of our work was to develop an analytical methodology for the stability evaluation of Lf in preformulation studies as well as in final products.

Experimental
Reference standard of bovine Lf was obtained from Sigma-Aldrich. Commercially available dietary supplements containing Lf were also used. Various reversed-phase (RP) and size exclusion (SEC) stationary phases were obtained from five manufacturers (Agilent, Phenomenex, Sigma-Aldrich, Vydac, and Waters). An Agilent 1100/1200 HPLC system, equipped with a diode array detector was used.

Results and Discussion
Our analytical methodology was focused on two complementary chromatographic methods. Among various types of RP columns (C3, C4, C8, and C18) tested, BioZen™ Intact XB-C8 150×4.6 mm, 3.6 µm column (Phenomenex) showed the best performance. The optimised RP method utilised a shallow gradient by using 0.1% TFA in water and 0.1% TFA in acetonitrile. The optimised SEC method was comprised of an XBridge Protein BEH SEC 150×7.8 mm, 3.5 µm column (Waters) and phosphate buffer with the addition of NaCl as a mobile phase. The stability-indicating nature of the chromatographic analytical approach was proven by stress testing, where it was shown that Lf is most prone to degradation under thermal and alkaline conditions. Up to three degradation products, as well as aggregates and fragments of Lf, were detected in various samples using RP and SEC chromatographic methods (Figure 1). The developed methods were successfully validated according to ICH guideline and applied to stability studies (Osel et al., 2021). Both methods were also used for the determination of Lf in commercial products and showed a certain degree of disagreement between the obtained results. In addition to other advantages, RP chromatographic method is more appropriate for quantification of Lf because it was noticed that the sample media may substantially affect the SEC chromatographic response.

The analytical approach was additionally extended with three spectroscopic techniques (fluorimetry, UV spectroscopy, determination of total protein concentration). Chromatographic methods proved to be more appropriate for qualitative and quantitative evaluation of Lf stability. However, the complementary information from simpler, faster, and more accessible spectroscopic techniques may be valuable in some cases.

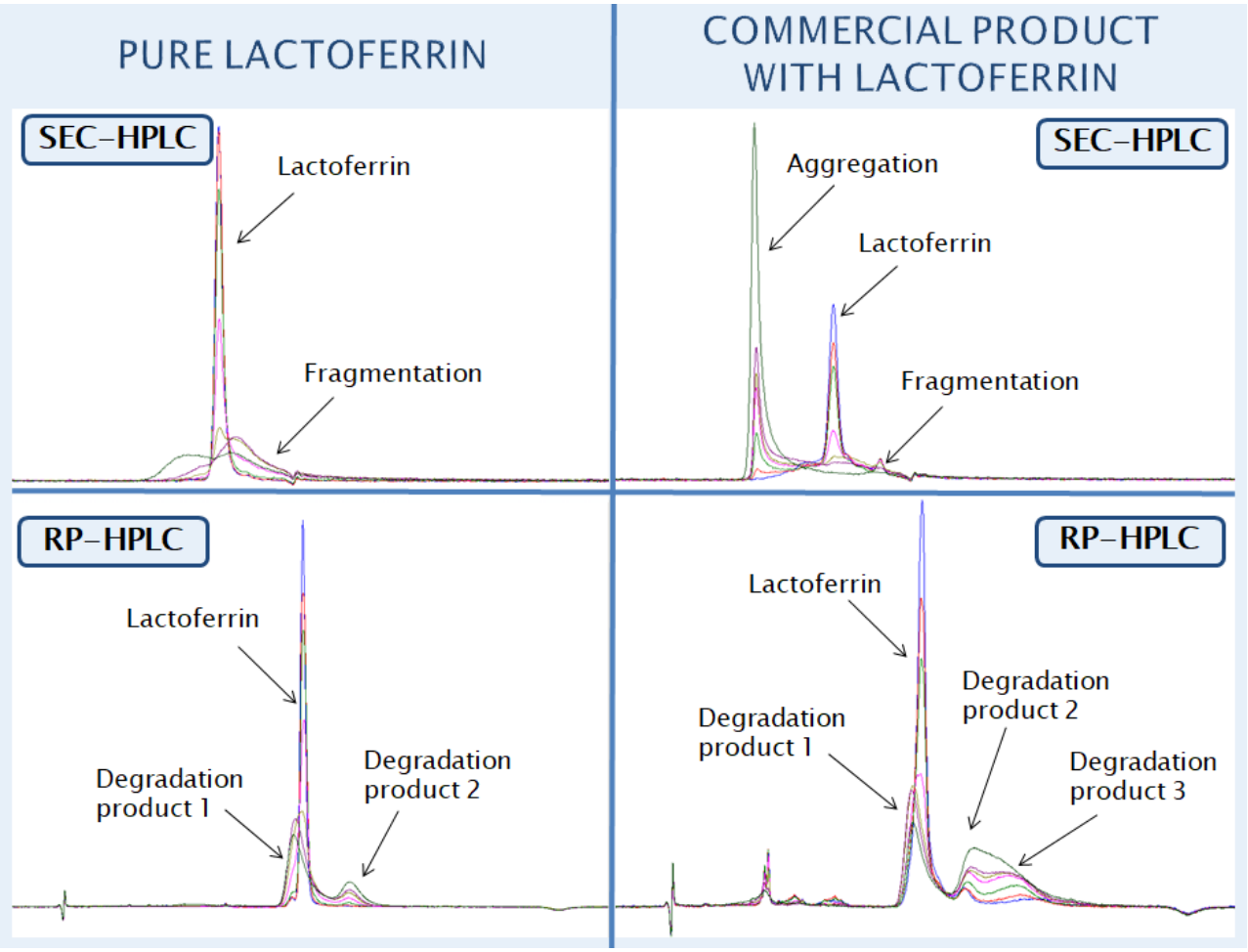

Figure 1: Chromatograms of pure Lf and commercial product with Lf stored for 0 min, 15 min, 30 min, 60 min, 120 min, 240 min, and 24 h at 60 °C.

Conclusions

A stability-indicating analytical approach for the evaluation of Lf stability based on complementary RP and SEC chromatographic methods was established. It allows the evaluation of Lf stability in various samples, including the ability to detect differences in degradation mechanisms. Moreover, it can be used for the quality control of products containing Lf.

Acknowledgment

This research was funded by Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020, Support of Research and development projects (TRL 3–6), S4—Networks for the transition to a circular economy, Biomass and alternative raw materials in the frame of the project LAKTIKA (OP20.03521), Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements, http://laktika.arhel.si/en.

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Ovrednotenje stabilnosti laktoferina v vodnih raztopinah

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Povzetek
Laktoferin (Lf) je približno 80 kDa velik multifunkcionalen globularen glikoprotein, ki veže železo in ima številne pozitivne učinke na zdravje ljudi. Ker je Lf podvržen razgradnji, je bil glavni namen raziskovalnega dela ovrednotiti njegovo stabilnost v različnih tekočih vzorcih pri različnih pogojih shranjevanja. Vzorce smo analizirali z validirano stabilnostno-indikativno reverznofazno HPLC metodo. Stresni testi so pokazali, da je temperatura eden od glavnih dejavnikov, ki vplivajo na stabilnost Lf. Pri različnih temperaturah shranjevanja je prišlo do sprememb v hitrosti in mehanizmu razpada. Na stabilnost Lf vpliva tudi prisotnost (proteinskih) nečistot ali različnih koncentracij soli v vzorcih, medtem ko koncentracija Lf v vzorcu ne vpliva na njegovo stabilnost.

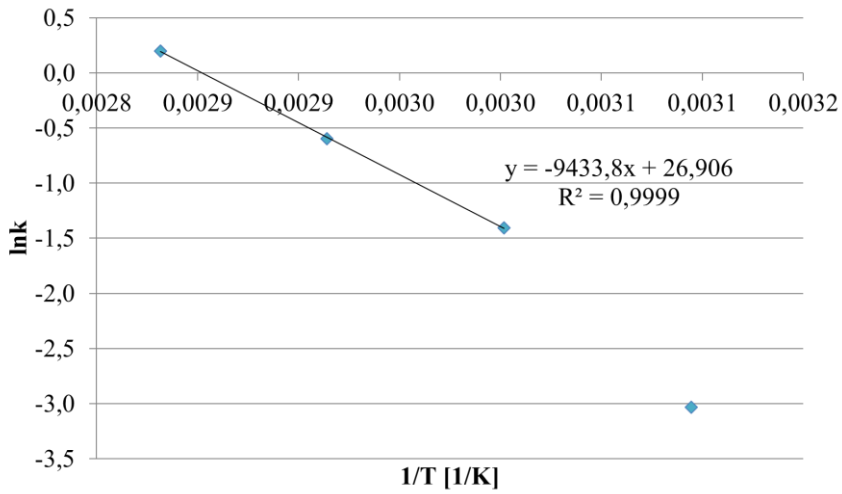
Ključne besede: laktoferin, stabilnost, kinetika, temperatura, vodne raztopine

Uvod
Laktoferin (Lf) je približno 80 kDa velik multifunkcionalen globularen glikoprotein, ki veže železo. Najdemo ga v izločkih eksokrinih žlez in telesnih tekočinah. Zaradi njegovega potenciala za zdravljenje različnih bolezni se zanimanje za izdelke z Lf povečuje. Vendar pa je Lf kot protein podvržen razgradnji, kar lahko kritično vpliva na kakovost izdelkov (García-Montoya in sod., 2012).

Namen
Namen raziskave je bil ovrednotiti stabilnost Lf v različnih vzorcih pri različnih pogojih shranjevanja.

Materiali in metode
Vzorce smo analizirali z reverznofazno HPLC metodo na koloni BioZen™ Intact XB C8 (150×4,6 mm, 3,6 µm; Phenomenex), termostatirani na 30 °C. Elucija je potekala po gradientnem programu (0,1 % TFA v vodi in 0,1 % TFA v acetonitrilu) pri pretoku 1,0 mL/min (Osel in sod., 2021). Analize smo izvajali na HPLC sistemu Agilent 1100/1200 z detektorjem z nizom diod. Valovno dolžino detekcije smo nastavili na 280 nm. Analizirali smo tekoče vzorce z Lf (elucije E1 in E3 ter koncentrirana elucija E3) in vodne raztopine trdnih vzorcev (izolati) z Lf, ki je bil izoliran iz sirotke.

Rezultati in razprava
Stresni testi so pokazali, da je Lf najbolj občutljiv na bazični medij in povišano temperaturo, najmanj pa na izpostavitev svetlobi. V različnih vzorcih smo zaznali največ tri razpadne produkte. Ugotovili smo, da razpad Lf poteka po kinetiki ničtega reda. Arrheniusova enačba je linearna v območju 60–80 °C, med 50 in 60 °C pa se spremeni mehanizem razpada (Slika 1).



Slika 1: Arrheniusov graf za vzorec izolata Lf 1 (c = 1.0 mg/mL), ki temelji na konstantah hitrosti razpada 0. reda za razpad Lf med 50 in 80 °C.

Konstante hitrosti razpada so bile za več kot dva velikostna reda nižje pri realnih pogojih shranjevanja kot pri temperaturah nad 50 °C. Vzorci so bili predvidljivo bolj stabilni v hladilniku ali zamrzovalniku kot pri sobni temperaturi (Preglednica 1). Večkratni cikli zamrzovanja in odmrzovanja vzorcev niso vplivali na stabilnost Lf v primerjavi z istimi vzorci, ki niso bili ponovno zamrznjeni. Opazili smo znatne razlike v stabilnosti Lf med vzorci, ki so se razlikovali v koncentraciji in čistosti Lf. To je najverjetneje posledica prisotnosti drugih proteinskih nečistoč ali različnih koncentracij soli. Pri vzorcih z različnimi koncentracijami istega Lf namreč nismo opazili bistvenih razlik v konstantah hitrosti razpada (Preglednica 1).

Preglednica 1: Konstante hitrosti razpada za različne vodne vzorce Lf pri različnih temperaturah.

Vzorec	**k [% dan^{-1}] [a]**			
	50 °C	**Sobna T**	**4 °C**	**−20 °C**
Vzorec izolata Lf 1 (c = 1.0 mg/mL)	124,5	/	/	/
Vzorec izolata Lf 2 (c = 1.0 mg/mL)	105,0	/	/	/
Vzorec izolata Lf 3 (c = 1.0 mg/mL)	179,1	/	/	/
Vzorec E1 (c = 0.55 mg/mL)	/	5,585	/	/
Vzorec E3 (c = 1.9 mg/mL)	/	0,438	Stabilen [b]	Stabilen [b]
Vzorec koncentrirane E3 (c = 30.5 mg/mL)	/	1,302	0,529	0,814
Vzorec izolata Lf 3 (c = 0.55 mg/mL)	/	0,326	/	/
Vzorec izolata Lf 3 (c = 1.9 mg/mL)	/	0,353	/	/
Vzorec izolata Lf 3 (c = 10.0 mg/mL)	/	0,409	/	/
Vzorec izolata Lf 3 (c = 30.5 mg/mL)	/	0,426	/	/

[a] Konstante hitrosti razpada 0. reda. Vsebnost Lf [%] smo izračunali relativno glede na točko nič in jo uporabili za izračun konstant hitrosti razpada. Tako smo dobili primerljive rezultate, saj se je koncentracija Lf v različnih vzorcih zelo razlikovala.
[b] Po 4 tednih je razpadlo manj kot 5 % Lf
/ Nismo ovrednotili.

Zaključek

Temperatura je eden od glavnih dejavnikov, ki vplivajo na stabilnost Lf. Pri različnih temperaturah je prišlo do sprememb v hitrosti in mehanizmu razpada. Na stabilnost Lf vpliva tudi prisotnost (proteinskih) nečistot ali različnih koncentracij soli v vzorcih, medtem ko koncentracija Lf v vzorcu ne vpliva na njegovo stabilnost.

Zahvala

Raziskava je bila sofinancirana z Operativnim programom za izvajanje evropske kohezijske politike v obdobju 2014 – 2020, Spodbujanje izvajanja raziskovalno-razvojnih projektov (TRL 3-6), S4 - Mreže za prehod v krožno gospodarstvo, Biomasa in alternativne surovine, v okviru projekta LAKTIKA (OP20.03521), Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil, http://laktika.arhel.si.

Reference

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Osel N in sod. Pharmaceutics. 13(7): 1065 (2021).

Overview of Lactoferrin Stability in Aqueous Solutions

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Abstract
Lactoferrin (Lf) is an 80 kDa multifunctional iron-binding globular glycoprotein that exerts several beneficial effects on human health. Since it is prone to degradation, the main purpose of our work was to evaluate the stability of Lf in various liquid samples at various storage conditions using the validated stability-indicating reversed-phase HPLC method. The forced degradation studies showed that temperature is one of the main factors affecting the Lf stability. Changes in degradation mechanisms and rate constants at different temperatures were observed. Lf stability in various samples was also affected by the presence of other (protein) impurities or different salt concentrations but not by Lf concentration.

Keywords: lactoferrin, stability, kinetics, temperature, aqueous solutions

Introduction
Lactoferrin (Lf) is an 80 kDa multifunctional iron-binding globular glycoprotein that is widely represented in various secretory fluids. The interest in products containing Lf is increasing due to its potential for the treatment of various diseases. However, as a protein, Lf is prone to degradation which critically affects the quality of products (García-Montoya et al., 2012).

Objective
The aim of our work was to evaluate the stability of Lf in various samples at various storage conditions.

Materials and Methods
The validated reversed-phase HPLC method utilized a BioZen™ Intact XB C8 column (150×4.6 mm, 3.6 µm; Phenomenex) at 30 °C and a gradient elution (0.1% TFA in water and 0.1 % TFA in acetonitrile) at a flow rate of 1.0 mL/min (Osel et al., 2021). Analysis was performed on an Agilent 1100/1200 series HPLC system, equipped with a diode array detector. Detection was carried out at 280 nm. Liquid samples (elutions E1 and E3 as well as concentrated elution E3) and aqueous solutions of solid samples (isolate samples) with Lf isolated from whey were tested.

Results and Discussion
The forced degradation study showed that Lf was most prone to degradation under alkaline and thermal conditions and at least affected by exposure to light. Up to three degradation products were detected in various samples. Lf degradation followed the zero-order kinetic model and the Arrhenius equation was linear between 60 and 80 °C. Changes in the degradation mechanism of Lf occurred between 50 and 60 °C (Figure 1).

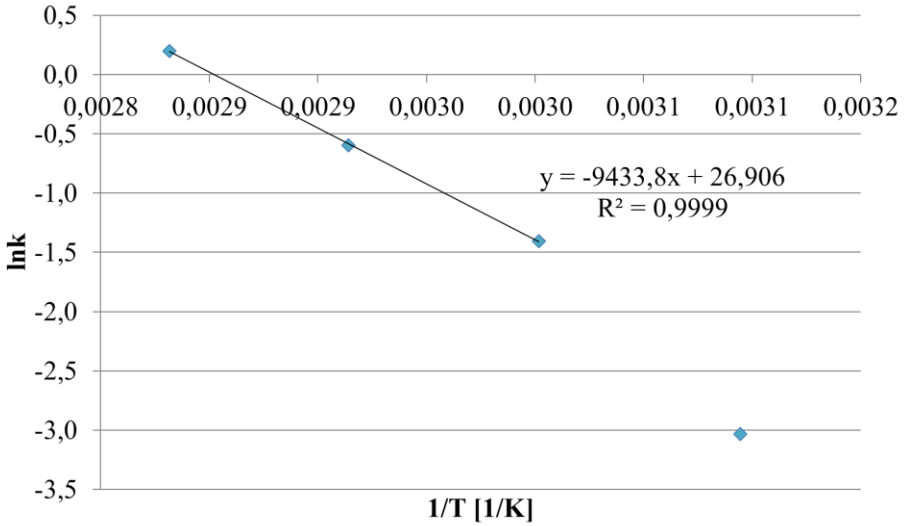

Figure 1: Arrhenius plot for Lf isolate sample 1 (c = 1.0 mg/mL) based on zero-order rate constants for the degradation of Lf at 50–80 °C.

The rate constants were more than two orders of magnitude lower at real storage conditions compared to temperatures above 50 °C. The samples were predictably less stable at room temperature than in the

refrigerator or in the freezer (Table 1). It was observed that multiple cycles freezing and thawing of Lf samples compared to the same samples that were not refrozen did not affect the stability of Lf. Significant differences in Lf stability among various samples, which differed in Lf concentration and purity, were observed. This is most likely due to the presence of other protein impurities or different salt concentrations since samples with various concentrations of the same Lf did not affect Lf rate constants (Table 1).

Table 1: Degradation rate constants for various aqueous Lf samples at different temperatures.

Sample	k [% Day^{-1}] [a]			
	50 °C	Room T	4 °C	−20 °C
Lf isolate sample 1 (c = 1.0 mg/mL)	124.5	/	/	/
Lf isolate sample 2 (c = 1.0 mg/mL)	105.0	/	/	/
Lf isolate sample 3 (c = 1.0 mg/mL)	179.1	/	/	/
E1 sample (c = 0.55 mg/mL)	/	5.585	/	/
E3 sample (c = 1.9 mg/mL)	/	0.438	Stable [b]	Stable [b]
Concentrated E3 sample (c = 30.5 mg/mL)	/	1.302	0.529	0.814
Lf isolate sample 3 (c = 0.55 mg/mL)	/	0.326	/	/
Lf isolate sample 3 (c = 1.9 mg/mL)	/	0.353	/	/
Lf isolate sample 3 (c = 10.0 mg/mL)	/	0.409	/	/
Lf isolate sample 3 (c = 30.5 mg/mL)	/	0.426	/	/

[a] Zero-order degradation rate constant. Content of Lf [%] was calculated relatively to the zero-time point and used for the calculation of rate constants to obtain comparable results due to the high concentration difference.
[b] <5% Lf degradation after 4 weeks.
/ not evaluated

Conclusions

Temperature is one of the main factors affecting Lf stability. Changes in degradation mechanisms and rate constants at different temperatures were observed. Lf stability in various samples was also affected by the presence of other (protein) impurities or different salt concentrations but not by Lf concentration.

Acknowledgement

This research was funded by Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020, Support of Research and development projects (TRL 3–6), S4—Networks for the transition to a circular economy, Biomass and alternative raw materials in the frame of the project LAKTIKA (OP20.03521), Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements, http://laktika.arhel.si/en.

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Osel N et al. Pharmaceutics. 13(7): 1065 (2021).

Analizna metoda za določanje vitamina B_{12} v vzorcih fermentirane kisle sirotke

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Povzetek
Cianokobalamin (CNCbl) je najstabilnejša in najpogosteje uporabljena oblika vitamina B_{12}. Zaradi kompleksne strukture se ga pridobiva s fermentacijo z bakterijami, kjer se lahko kot gojišče uporablja tudi kisla sirotka. Za namen ugotavljanja uspešnosti fermentacije smo razvili analizno metodo za določanje vitamina B_{12} v vzorcih, pridobljenih s fermentacijo kisle sirotke. Ugotovili smo, da je za sprostitev vitamina B_{12} iz bakterijskih celic ključno vsaj 10 minutno avtoklaviranje pri 121 °C. Za pretvorbo kobalaminov naravnega izvora v CNCbl pa je optimalen dodatek 0,1 % KCN v 0,2 M acetatnem pufru s pH vrednostjo 4,5 v razmerju z vzorcem 1:1. Vzorce smo analizirali s predhodno razvito in validirano instrumentalno LC-MS/MS metodo.

Ključne besede: vitamin B_{12}, LC-MS/MS, *Propionibacterium freudenrechii*, avtoklaviranje, gojišče fermentirane kisle sirotke

Uvod
Vitamin B_{12} predstavlja skupino spojin kobalaminov, med katerimi so adenozilkobalamin (ACbl), metilkobalamin (MeCbl) in hidroksokobalamin (OHCbl) naravnega izvora. Cianokobalamin (CNCbl) je sinteznega izvora in je tudi najstabilnejša oblika, ki se najpogosteje uporablja v prehranskih dopolnilih in zdravilih. Pridobiva se s procesom biosintezne fermentacije z bakterijami *P. denitrificans* in *P. shermanii*. Po fermentaciji sledi ekstrakcija vitamina B_{12}, kjer se bakterijske celice običajno segreva 10–30 minut pri 80–120 °C in pri pH vrednosti 6,5–8,5. Nato se z obdelavo vzorca s cianidom oziroma tiocianatom izvede pretvorba v CNCbl (Martens in sod., 2002).

Namen
Namen raziskovalnega dela je bil razvoj analizne metode za določanje vsebnosti vitamina B_{12} v vzorcih, pridobljenih s fermentacijo kisle sirotke s sevom *Propionibacterium freudenrechii* subsp. *freudenrechii* van Niel 1928.

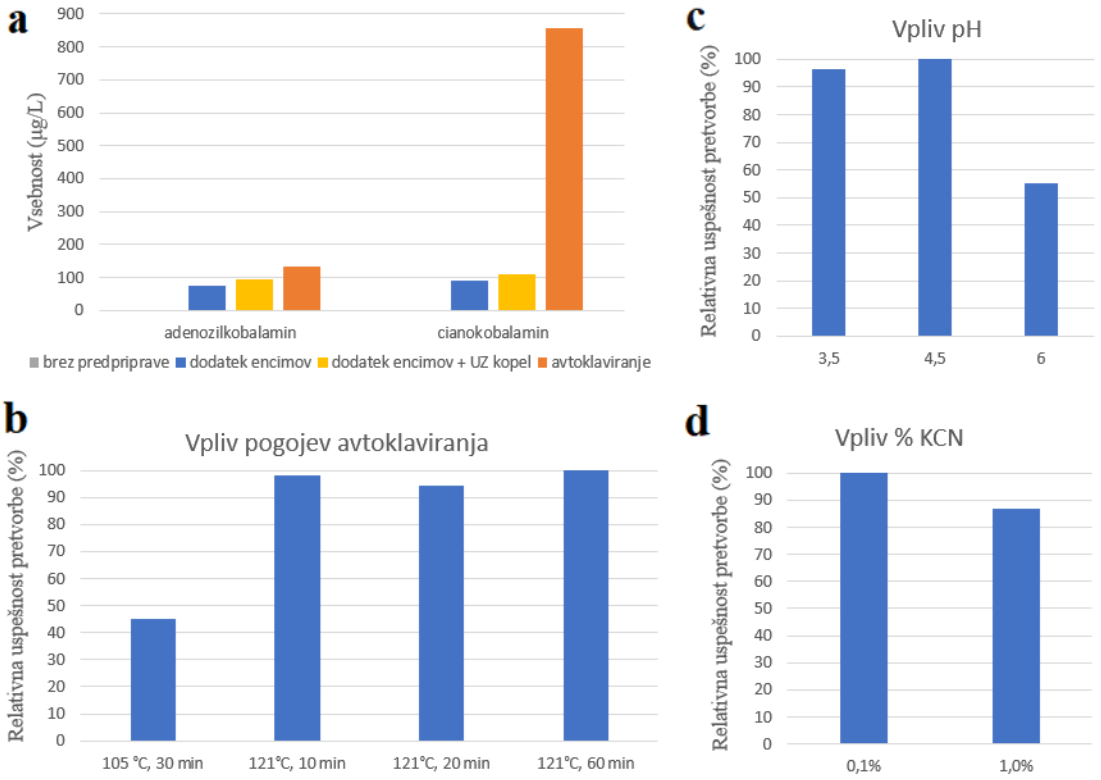
Materiali in metode
Razvoj analizne metode za določanje vitamina B_{12} v vzorcih iz gojišč je potekal s standardi CNCbl, ACbl, OHCbl (vsi Sigma-Aldrich) in MeCbl (Carbosynth). Vzorce, ki so bili pridobljeni s fermentacijo obogatene kisle sirotke s sevom *Propionibacterium freudenrechii* subsp. *freudenrechii* van Niel 1928, smo pridobili od projektnih partnerjev (UL BF). Vzorce smo analizirali s predhodno razvito in validirano instrumentalno LC-MS/MS metodo. Ločba analitov je potekala na koloni Kinetex C18 50 × 2,1 mm, 2,6 µm delci (Phenomenex, USA) pri 40 °C z gradientnim programom in mobilno fazo, ki jo sestavljata 0,05 % mravljinčna kislina v MilliQ vodi ter metanol s pretokom 0,5 mL/min. Uporabili smo UHPLC Infinity 1290 sistem, sklopljen s 6460 QQQ masnim spektrometrom in ESI ionskim izvorom (Agilent Technologies, USA) za kvantifikacijo pa smo uporabili multirezidualno analizo. Pri razvoju postopka priprave vzorcev smo za ekstrakcijo vitamina B_{12} uporabili več načinov razbitja bakterijskih celic (avtoklaviranje, uporaba encimov, ultrazvočna kopel) ter različne pogoje avtoklaviranja in dodatek KCN za pretvorbo kobalaminov naravnega izvora v stabilnejšo obliko – CNCbl. Po optimizaciji smo analizno metodo tudi ovrednotili ter jo uporabili pri vrednotenju vsebnosti vitamina B_{12} v realnih vzorcih.

Rezultati in razprava
Vitamin B_{12} se ne izloča iz bakterijskih celic, zato jih je treba najprej uničiti. Za sprostitev vitamina B_{12} iz bakterijskih celic smo uporabili več metod, in sicer dodatek encimov, ki razgradijo bakterijsko celično steno (lizocim in mutanolizin), ekstrakcijo v ultrazvočni kopeli in postopek avtoklaviranja, kjer celice uničimo s povišano temperaturo. Ugotovili smo, da se z avtoklaviranjem sprosti največ vitamina B_{12} iz celic (Slika 1a).

Avtoklaviranje je dolgotrajen postopek, zato je bilo treba pogoje čim bolje optimizirati. Preverili smo različne temperature in čas avtoklaviranja. Ugotovili smo, da je avtoklaviranje pri 105 °C manj učinkovito, saj je bila

količina nastalega CNCbl iz MeCbl najnižja. Najvišjo pretvorbo smo dosegli z avtoklaviranjem pri 121 °C (Slika 1b). Za končni postopek priprave vzorcev smo izbrali najkrajši čas (10 minut) avtoklaviranja z zadovoljivo pretvorbo.

Pri fermentaciji z bakterijami nastaja vitamin B_{12} v obliki ACbl ali MeCbl. Ti obliki sta pod vplivom svetlobe nestabilni in se pretvarjata v obliko OHCbl. Da bi dobili stabilnejšo obliko vitamina B_{12}, smo uporabili dodatek KCN, ki omogoča pretvorbo teh oblik v CNCbl. Pripravili smo 0,2 M acetatni pufer z različnimi pH vrednostmi (3,5–6,0) in različnim deležem KCN (0,1 in 1,0 %). Pripravljen pufer smo dodali k vzorcu v razmerju 1:1 ter avtoklavirali pri izbranih pogojih. Ugotovili smo, da je bila pretvorba najbolj uspešna pri uporabi 0,1 % KCN v 0,2 M acetatnem pufru s pH vrednostjo 4,5 (Sliki 1c in 1d). Prav tako smo ugotovili, da se ACbl v večjem obsegu pretvarja v CNCbl kot MeCbl. V okviru validacije analizne metode smo preverjali linearnost, ponovljivost, točnost, selektivnost, stabilnost in učinek matrice, s čimer smo glede na predhodno postavljene kriterije potrdili ustreznost metode. Analizno metodo smo v nadaljevanju aplicirali na realne vzorce in ugotovili, da je dodatek KCN potreben, saj smo v vzorcu brez dodanega KCN skupno določili 40 % manj vitamina B_{12}. Ker ACbl s postopkom avtoklaviranja in dodatka KCN nismo popolnoma pretvorili v CNCbl, iščemo alternativne poti za optimizacijo, npr. dodatna izpostavitev vzorca sončni svetlobi.



Slika 1: Vpliv dejavnikov na nastanek CNCbl; a – vpliv metod za razbitje bakterijskih celic na sprostitev vitamina B_{12}, b – vpliv pogojev avtoklaviranja, c – vpliv pH raztopine s KCN, d – vpliv % KCN. Relativno uspešnost pretvorbe (%) smo podali glede na najboljši rezultat.

Zaključek

Razvili smo analizno metodo za določanje vsebnosti vitamina B_{12} v vzorcih, pridobljenih s fermentacijo kisle sirotke. Ugotovili smo, da sta pri pripravi vzorcev zelo pomembna dva koraka. S postopkom avtoklaviranja uničimo bakterijsko celično steno in povzročimo sprostitev vitamina B_{12}, z dodatkom KCN pa ACbl oziroma MeCbl pretvorimo v stabilnejšo obliko CNCbl. Validirana analizna metoda nam omogoča določanje vsebnosti vitamina B_{12} v gojiščih s kislo sirotko, prav tako bi jo lahko prenesli tudi na druga gojišča.

Zahvala

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Analytical method for vitamin B_{12} determination in fermented acid whey

Timeja Planinšek Parfant[1], Nika Osel[1], Albin Kristl[1], Diana Paveljšek[2], Bojana Bogovič Matijašić[2], Robert Roškar[1]
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Abstract
Cyanocobalamin (CNCbl) is the most stable and most commonly used form of vitamin B_{12}. Due to its complex structure, it is produced by a biosynthetic fermentation process, where acid whey-based media can also be used. In order to evaluate the efficiency of fermentation, an analytical method for vitamin B_{12} determination in samples obtained by fermentation of acid whey-based media was developed. We showed that autoclaving at 121 °C for at least 10 minutes is essential to extract vitamin B_{12} from bacterial cells. The optimal conversion of cobalamins of natural origin to CNCbl was obtained by mixing the fermented acid whey-based media with 0.1% KCN in 0.2 M acetate buffer (pH 4.5) in the ratio of 1:1. Previously developed and validated instrumental LC-MS/MS method was used for the sample analysis.

Keywords: Vitamin B_{12}, LC-MS/MS, *Propionibacterium freudenrechii*, autoclaving, fermented acid whey-based media

Introduction
Vitamin B_{12} is a group of cobalamin compounds, among which adenosylcobalamin (ACbl), methylcobalamin (MeCbl), and hydroxocobalamin (OHCbl) are of natural origin. Cyanocobalamin (CNCbl), a synthetic form of vitamin B_{12}, is the most stable form and most commonly used in nutritional supplements and medicines. It is produced by a biosynthetic fermentation process using *P. denitrificans* and *P. shermanii* bacterial species. Fermentation is followed by the extraction of vitamin B_{12}, where the whole broth is usually heated at 80–120 °C for 10–30 min at pH 6.5–8.5. The conversion to CNCbl is obtained by treating the sample with cyanide or thiocyanate (Martens et al., 2002).

Objective
The aim of this work was to develop an analytical method for vitamin B_{12} determination in acid whey-based media fermented by *Propionibacterium freudenrechii* subsp. *Freudenrechii* van Niel 1928.

Materials and methods
For the method development of vitamin B_{12} in acid whey-based media, the CNCbl, ACbl, OHCbl (all Sigma-Aldrich), and MeCbl (Carbosynth) standards were used. Acid whey-based media samples fermented by the *Propionibacterium freudenrechii* subsp. *Freudenrechii* van Niel 1928 were provided by a project partner (UL BF). Previously developed and validated instrumental LC-MS/MS method was used for sample analysis. A Kinetex C18 50 × 2.1 mm, 2.6 µm particle size column (Phenomenex, USA) at 40 °C with gradient elution at a flow rate 0.5 mL/min using 0.05% formic acid in Milli Q water and methanol was used for the separation on a UHPLC Infinity 1290 coupled with 6460 QQQ mass spectrometer equipped with Jetstream ESI source (Agilent Technologies, USA) operating in MRM mode. For the vitamin B_{12} extraction, several methods (autoclaving, addition of enzymes, ultrasonic bath) for bacterial membrane lysis were used. For the conversion of cobalamins of natural origin into a more stable form – CNCbl, different autoclaving conditions and the addition of KCN were used. The developed analytical method was validated and subsequently used for vitamin B_{12} determination in real samples.

Results and discussion
Bacterial membrane lysis is necessary, due to the intracellular production of vitamin B_{12}. Several methods have been used to extract the vitamin B_{12}, namely the addition of enzymes that lyse bacterial cell membranes (lysozyme and mutanolysin), ultrasonic bath extraction, and autoclaving, during which cell membranes are disrupted by elevated temperature. The most efficient method for the extraction of vitamin B_{12} was autoclaving (Figure 1a).
Autoclaving is a time-consuming process, so the conditions had to be optimized. Different temperatures and time variations of autoclaving were studied. Autoclaving at 105 °C was found to be less efficient since the amount CNCbl converted from MeCbl was the lowest. The highest conversion rate was achieved by autoclaving at 121 °C (Figure 1b). Even the shortest tested autoclaving time (10 minutes) was sufficient and was chosen for the final sample preparation.

The bio-produced vitamin B_{12} forms are ACbl or MeCb, which are known to be sensitive to light and are easily converted to OHCbl. In order to obtain a more stable form of vitamin B_{12}, the addition of KCN was used, which caused the conversion of these forms into CNCbl. 0.2 M acetate buffer with different pH values (3.5–6.0) and different KCN additions (0.1 and 1.0%) were tested. The prepared buffer was added to the sample in the ratio of 1:1 and autoclaved under selected conditions. The conversion was the most effective by using 0.1% KCN in 0.2 M acetate buffer with a pH of 4.5 (Figures 1c and 1d). It was also observed that ACbl is converted to CNCbl to a greater extent than MeCbl. The developed analytical method was then validated in terms of linearity, accuracy, precision, selectivity, stability and matrix effect, which complied with the set acceptance criteria. The analytical method was afterward applied to real sample analyses and confirmed that the KCN addition was necessary, as in total less than 40% of vitamin B_{12} was detected in the samples without treatment with KCN. Since the conversion of ACbl to CNCbl was not completed, alternative conversion methods have been explored to obtain the optimal conversion to CNCbl (e.g. additional exposure to the sunlight).

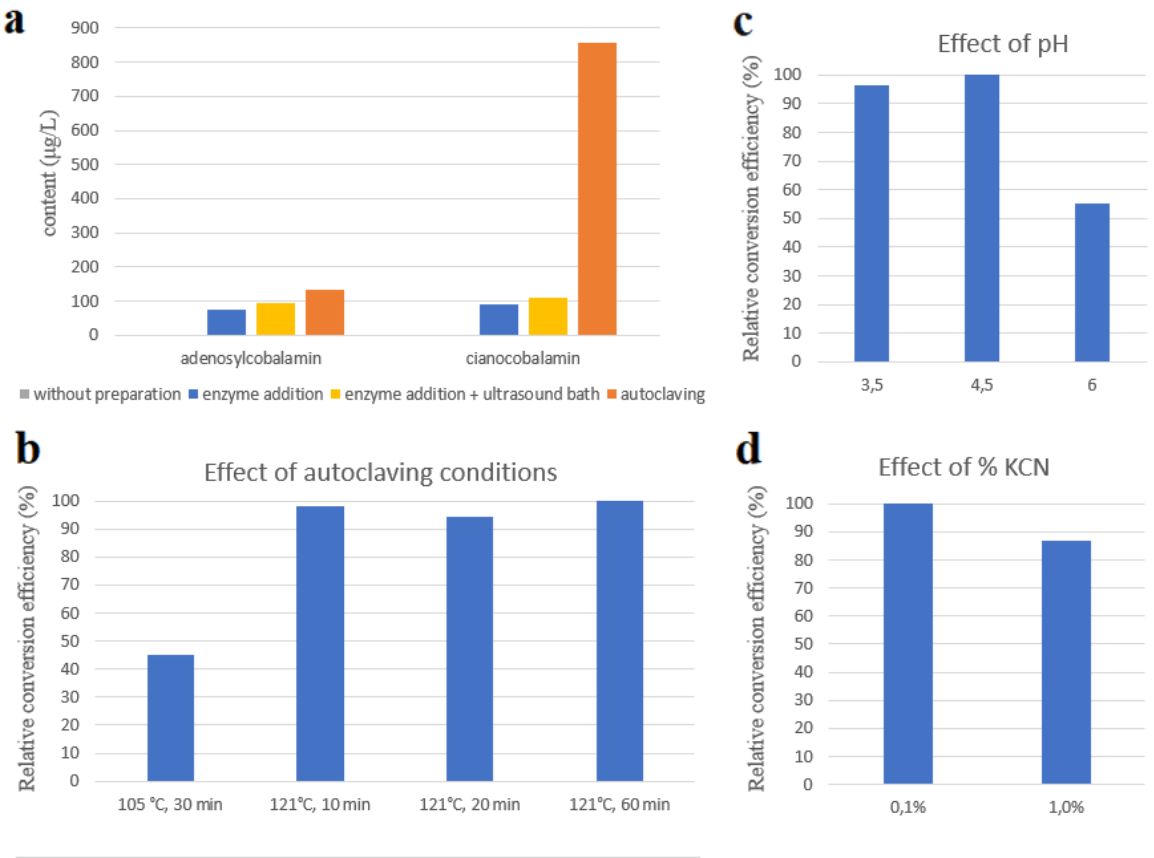

Figure 1: Influence of factors on CNCbl formation; a – influence of methods for bacterial cell membrane lysis on vitamin B_{12} extraction, b – influence of autoclaving conditions, c – influence of KCN solution pH value, d – influence of KCN content. The relative conversion efficiency (%) has been presented considering the best result.

Conclusion

An analytical method for vitamin B_{12} determination in samples obtained by acid whey-based media fermentation was developed. Two steps are very important in the sample preparation procedure. Extraction of vitamin B_{12} is achieved by bacterial membrane lysis with autoclaving, while the conversion of ACbl or MeCbl into a more stable CNCbl form is achieved with the addition of KCN. A validated analytical method allows the vitamin B_{12} determination in acid whey-based media and could also apply to other growth media.

Acknowledgements

This research was co-funded by the project LAKTIKA (contract No. OP20.03521) in the frame of the Operational Programme ECP 2014 – 2020 and the project LIFE for Acid Whey (contract No. LIFE16 ENV/SI/000335) of the European financial instrument LIFE.

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Stabilnostna študija gastrorezistentnih pelet z laktoferinom

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Povzetek
Laktoferin (Lf) je multifunkcionalen glikoprotein, ki veže železo in ima številne ugodne učinke na zdravje ljudi, zato se zanimanje za izdelke z Lf povečuje. Namen raziskovalnega dela je bil ovrednotiti kakovost in stabilnost gastrorezistentnih pelet z Lf, ki smo jih pripravili v Wursterjevi komori z oblaganjem nevtralnih peletnih jeder z Lf in gastrorezistentno oblogo. Vsebnost Lf v formulaciji je bila 200 mg. S testi sproščanja smo ugotovili, da gastrorezistentna obloga zadostno zaščiti Lf, saj se je po dveh urah v kislem mediju sprostilo manj kot 10 % Lf. Rezultati stabilnostne študije pri dolgoročnih in pospešenih pogojih shranjevanja so pokazali, da gastrorezistentna obloga, želatinska kapsula in Alu-Alu ovojnina stabilizirajo Lf. Raziskava je pokazala, da imata izbira ustrezne ovojnine in pogojev shranjevanja pomembno vlogo pri zagotavljanju kakovosti in stabilnosti izdelka z Lf.

Ključne besede: laktoferin, gastrorezistentne pelete, stabilnostna študija, testi sproščanja, kontrola kakovosti

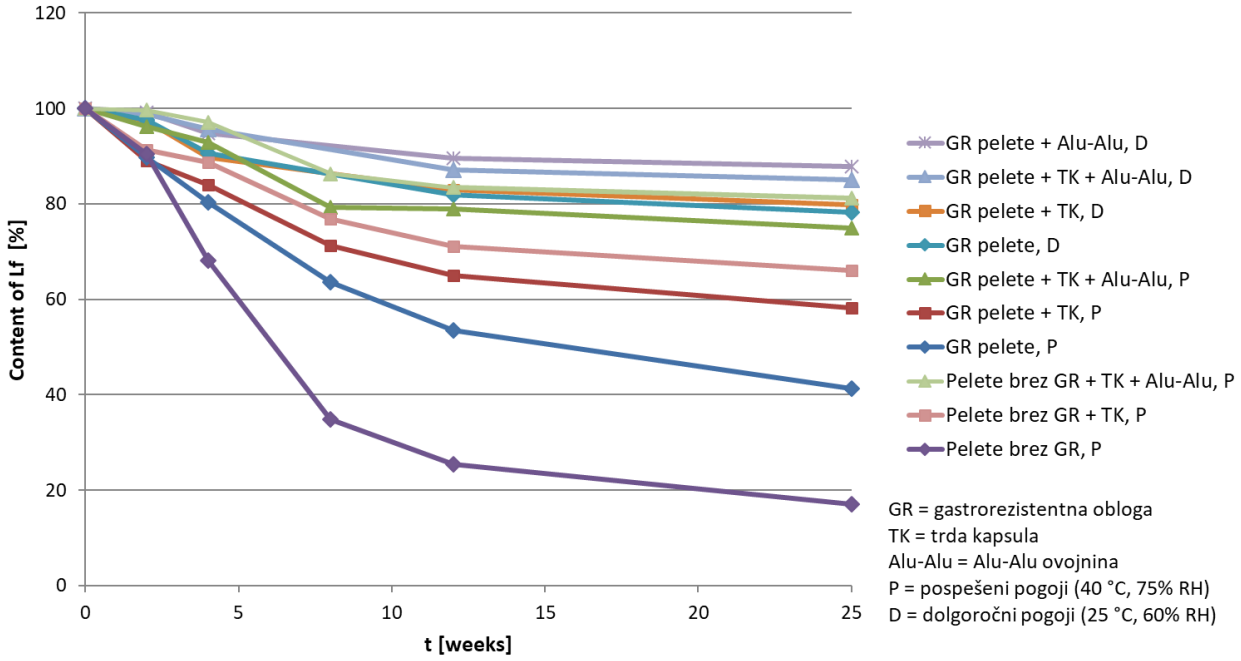
Uvod
Laktoferin (Lf) je multifunkcionalen glikoprotein, ki veže železo in ima številne ugodne učinke na zdravje ljudi, zato se zanimanje za izdelke z Lf povečuje. Lf pa je nestabilen, kar kritično vpliva na kakovost izdelkov (García-Montoya in sod., 2012).

Namen
Namen raziskovalnega dela je bil ovrednotiti kakovost in stabilnost gastrorezistentnih pelet z Lf.

Materiali in metode
Lf 90-odstotne čistosti smo prejeli od projektnega partnerja Arhel d.o.o. (Slovenija). Gastrorezistentne pelete z Lf smo pripravili v Wursterjevi komori z oblaganjem nevtralnih peletnih jeder (Cellets 200) z dvema oblogama. Prva obloga je vsebovala Lf, HPMC in PEG 6000. Druga obloga pa je bila gastrorezistentna obloga, sestavljena iz Eudragit-a L30-D55, Tween-a 80, trietil citrata in glicerol monostearata. Trde kapsule velikosti 0 smo napolnili s peletami in jih zavarili v Alu-Alu ovojnino. Vzorce smo analizirali z validirano stabilnostno-indikativno reverznofazno HPLC metodo (Osel in sod., 2021). Ovrednotili smo vsebnost, profil sproščanja in stabilnost Lf v izdelku. Vzorce za stabilnostno študijo smo shranjevali v klimatskih komorah pri pospešenih (40 °C in 75 % RV) in dolgoročnih pogojih shranjevanja (25 °C in 60 % RV). V vnaprej definiranih časovnih točkah (0, 2, 4, 8 (slednja samo pri 40 °C in 75 % RV), 12 in 25 tednov) smo analizirali po tri paralele vsakega vzorca.

Rezultati
Vsebnost Lf v formulaciji je bila 200 mg. Da bi ovrednotili kakovost gastrorezistentne obloge, smo po standardnem farmkopejskem postopku (Ph. Eur. 2.9.3) izvedli teste sproščanja. Uporabili smo aparat z vesli (USP 2) in teste izvedli v šestih paralelah. Ugotovili smo, da gastrorezistentna obloga zadostno zaščiti Lf, saj se je po dveh urah v kislem mediju sprostilo manj kot 10 % Lf. Na koncu testa pa se je sprostilo več kot 90 % odmerka.

Nadalje smo ovrednotili tudi stabilnost Lf v formulaciji. V stabilnostno študijo smo vključili različne vzorce, da bi ovrednotili vpliv gastrorezistentne obloge, trde kapsule in Alu-Alu ovojnine na stabilnost Lf. Rezultati stabilnostne študije pri pospešenih pogojih shranjevanja (40 °C in 75 % RV) so pokazali, da gastrorezistentna obloga, želatinska kapsula in Alu-Alu ovojnina stabilizirajo Lf (Slika 1). Podoben učinek smo opazili tudi pri dolgoročnih pogojih shranjevanja (25 °C in 60 % RV), le da je bil razpad Lf hitrejši pri pospešenih pogojih shranjevanja. Po šestih mesecih shranjevanja pri dolgoročnih pogojih je v končni formulaciji (gastrorezistentne pelete z Lf v trdi kapsuli v Alu-Alu ovojnini) ostalo več kot 85 % začetne vsebnosti Lf (Slika 1).



Slika 1: Stabilnost Lf v peletah pri pospešenih in dolgoročnih pogojih shranjevanja.

Razprava

V primerjavi s komercialno dostopnimi izdelki razvita formulacija vsebuje relativno visok odmerek Lf. Gastrorezistentna obloga ščiti Lf pred želodčnimi encimi in kislim okoljem, kar je ključnega pomena za dostavo aktivnega proteina na mesto delovanja. Izdelano formulacijo bi lahko uporabili kot prehransko dopolnilo pri tako imenovani popotniški diareji, saj preprečuje gastrointestinalne težave in predstavlja obetavno alternativo antibiotikom ali aktivnemu oglju.

Rezultati stabilnostne študije so pokazali, da ima izbira ustrezne ovojnine in pogojev shranjevanja pomembno vlogo pri zagotavljanju kakovosti in stabilnosti izdelka z Lf. Gastrorezistentna obloga do neke mere stabilizira Lf. Vendar pa je bil Lf v gastrorezistentnih peletah manj stabilen kot v neobloženih peletah, če so bile pelete dodatno zaščitene s trdo kapsulo ali z Alu-Alu ovojnino. Poleg tega je bil Lf v gastrorezistentnih peletah, ki so bile direktno zapakirane v Alu-Alu ovojnino, bolj stabilen, kot če so bile gastrorezistentne pelete napolnjene v trde kapsule in šele nato zapakirane v Alu-Alu ovojnino (Slika 1). Oba vpliva bi bila lahko posledica interakcij med Lf in gastrorezistentno oblogo oziroma trdo kapsulo.

Zaključek

Raziskava je pokazala, da ima izbira ustrezne ovojnine in pogojev shranjevanja pomembno vlogo pri zagotavljanju kakovosti in stabilnosti izdelka z Lf.

Zahvala

Raziskava je bila sofinancirana z Operativnim programom za izvajanje evropske kohezijske politike v obdobju 2014 – 2020, Spodbujanje izvajanja raziskovalno-razvojnih projektov (TRL 3-6), S4 - Mreže za prehod v krožno gospodarstvo, Biomasa in alternativne surovine, v okviru projekta LAKTIKA (OP20.03521), Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil, http://laktika.arhel.si.

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Stability Study of Enteric-Coated Pellets Containing Lactoferrin

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Abstract
Lactoferrin (Lf) is a multifunctional iron-binding glycoprotein that expresses several beneficial effects on human health; therefore, the interest in products containing Lf is increasing. Our work aimed to evaluate the quality and stability of the enteric-coated pellets with Lf which were prepared in a Wurster chamber by coating the Cellets 200 with Lf layer and enteric coating. The formulation contained 200 mg of Lf. The dissolution tests proved that enteric coating provides sufficient protection of Lf since less than 10% of Lf were released in acidic media in two hours. The stability study at accelerated and long-term storage conditions showed that the enteric coating, gelatinous capsule, and Alu-Alu packing provide a stabilizing effect on Lf. The present study demonstrated that the selection of appropriate packaging and storage conditions play a significant role in ensuring the quality and stability of the product containing Lf.

Keywords: lactoferrin, enteric-coated pellets, stability study, dissolution tests, quality control

Introduction
Lactoferrin (Lf) is a multifunctional iron-binding glycoprotein that expresses several beneficial effects on human health; therefore, the interest in products containing Lf is increasing. However, Lf is prone to degradation which critically affects the quality of products (García-Montoya et al., 2012).

Objective
The aim of this work was to evaluate the quality and stability of the enteric-coated pellets containing Lf.

Materials and Methods
Lf at 90% purity was provided by industry partner Arhel d.o.o (Slovenia). The enteric-coated pellets with Lf were prepared in a Wurster chamber by coating the Cellets 200 with two layers. The first layer was prepared from the aqueous solution of Lf, HPMC, and PEG 6000. The second layer was enteric coating, composed of Eudragit L30-D55, Tween 80, triethyl citrate, and glycerol monostearate. The pellets were encapsulated in a size 0 hard capsule and packed in an Alu-Alu packing. The validated stability-indicating reversed-phase HPLC method was used for the sample analysis (Osel et al., 2021). The assay, dissolution profile, and stability of the product were evaluated. Samples for the stability study were stored in climatic chambers at accelerated (40 °C and 75% RH) and at long-term storage conditions (25 °C and 60% RH). Three replicates of each sample were analysed at predefined time points (0, 2, 4, 8 (the latter only at 40 °C and 75% RH), 12, and 25 weeks).

Results
The formulation contained 200 mg of Lf. In order to test the quality of the enteric coating, the dissolution tests were carried out in six replicates by the standard pharmacopoeia procedure (Ph. Eur. 2.9.3) using a USP Apparatus 2. The dissolution tests proved that enteric coating provides sufficient protection of Lf since less than 10% of Lf was released in acidic media. At the end of the dissolution test, more than 90% of the dose was released.

The stability of Lf in the formulation was further evaluated. Various samples were prepared to evaluate the effect of enteric coating, hard capsule, and Alu-Alu packing on the stability of Lf. The stability study at accelerated storage conditions showed that the enteric coating, gelatinous capsule, and Alu-Alu packing provide a stabilizing effect on Lf (Figure 1). A similar effect was observed at long-term storage conditions with Lf degradation being faster at accelerated storage conditions. After 6 months at long-term conditions, more than 85% of the initial Lf content remained in the final formulation (enteric-coated Lf pellets in a hard capsule in an Alu-Alu packing) (Figure 1).

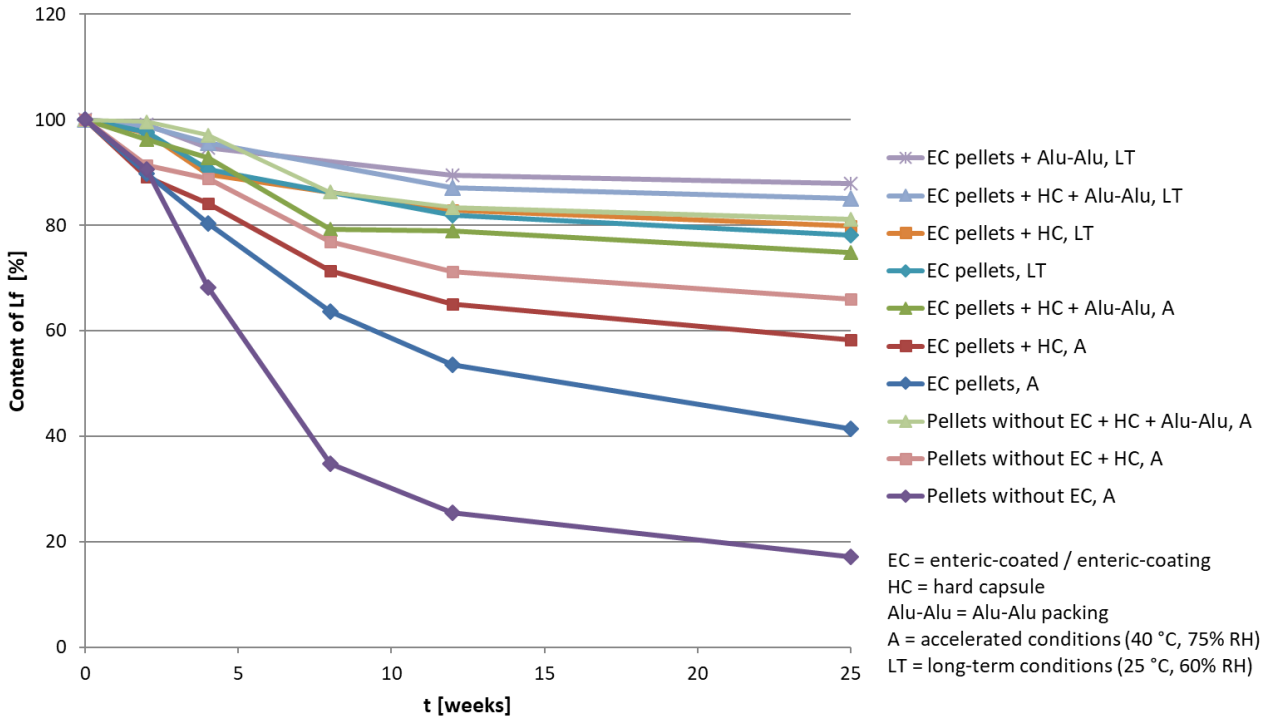

Figure 1: Stability of Lf in pellets at accelerated and long-term storage conditions.

Discussion

The formulation contains a relatively high dose of Lf compared to commercially available products. The enteric coating protects Lf from gastric enzymes and a low pH environment which is crucial for the delivery of the active protein to the site of action. This formulation could be used as a dietary supplement for so-called Traveller's disease since it prevents gastrointestinal distress and presents a promising alternative to antibiotics or activated carbon.

The stability study showed that storage conditions and packaging significantly affect the stability of Lf. The enteric coating provided some stabilizing effect compared to the uncoated pellets. However, Lf in the enteric-coated pellets was less stable than in the uncoated pellets when the pellets were additionally protected by a hard capsule or Alu-Alu packing. Moreover, Lf in enteric-coated pellets in Alu-Alu packing without a hard capsule was found to be more stable than when both a hard capsule and Alu-Alu packing were used (Figure 1). Both effects could be a consequence of some interactions between Lf and enteric coating / hard capsule.

Conclusions

The present study demonstrated that the selection of appropriate packaging and storage conditions play a significant role in ensuring the quality and stability of the product containing Lf.

Acknowledgement

This research was funded by Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020, Support of Research and development projects (TRL 3–6), S4—Networks for the transition to a circular economy, Biomass and alternative raw materials in the frame of the project LAKTIKA (OP20.03521), Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements, http://laktika.arhel.si/en.

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Osel N et al. Pharmaceutics. 13(7): 1065 (2021).

Čiščenje imunoglobulina G iz sirotke in njegova stabilizacija

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Povzetek
S pomočjo monolitne protein G-afinitetne kromatografije smo izolirali imunoglobulin G (IgG) iz osiromašene kisle in sladke sirotke. Določili smo karakteristike nalaganja IgG na kolono. Z izoliranimi frakcijami smo izvedli presejalni test različnih pogojev skladiščenja (dodatek/odsotnost stabilizatorjev, liofilizacija/tekoče stanje/odmrzovanje in zamrzovanje) na agregacijo in koncentracijo IgG v raztopinah. Dodatek manitola, saharoze in NaCl ali le acetatnega pufra je v primerjavi z dodatkom samega PBS bolje zaščitil IgG pred agregacijo pri vseh preskušenih načinih skladiščenja. Za pripravo formulacij aktivnega IgG je smiselno dodati stabilizatorje.

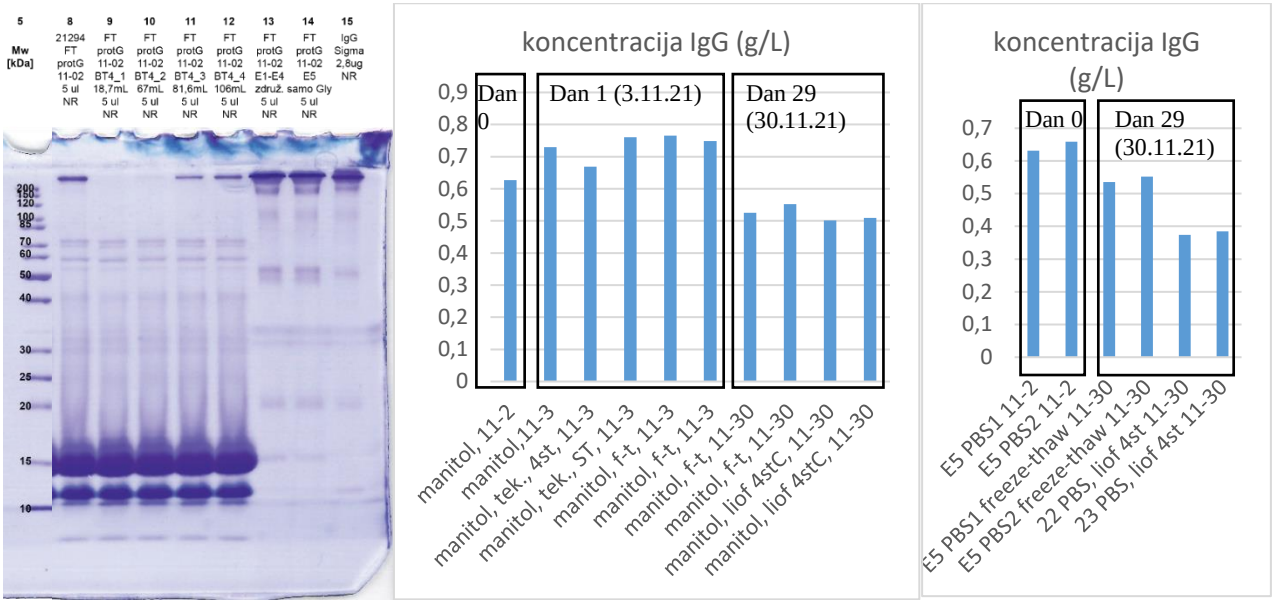
Ključne besede: sirotka, IgG, čiščenje, afinitetna kromatografija, stabilizacija.

Uvod
IgG je glavni imunoglobulin v kravjem mleku. V zrelem mleku doseže koncentracije do 0,7 g/L, v kolostromu pa celo do 32 – 212 g/L. Drugi dve varianti imunoglobulinov v mleku, IgA in IgM, sta v zrelem kravjem mleku zastopani v precej nižjih koncentracijah - do 0,1 g/L (Gapper et al., 2017). Poznamo 4 razrede IgG, v kravjem mleku prevladuje IgG1. Mlečni imunoglobulini ščitijo tako mlečno žlezo kot tudi tele pred okoljskimi patogeni in toksini, ko še nima razvitega imunskega sistema. Imunoglobulini med dozorevanjem imunskih celic s selekcijo pridobijo zelo visoko afiniteto do tarčnega antigena ($K_D = 10^{-15}$). Na koncu težkih (57 kDa) in lahkih (26 kDa) verig se nahajajo zanke, ki so podvržene mutacijam in ki oblikujejo hipervariabilno regijo za vezavo antigena. Imunoglobulini z izjemo sekrecijskega IgA niso odporni proti proteolizi s prebavnimi encimi. IgG vsebujejo več disulfidnih vezi, ki povezujejo težke verige z lahkimi verigami in težke verige med sabo v monomer z molekulsko maso 150 kDa. Pri koagulaciji kazeinov se del IgG obori ali veže na kazeine. V sirotki so koncentracije zato nižje kot v mleku.

Molekule IgG so zaradi občutljive in razvejane 3D-strukture podvržene agregaciji in so zaradi tega nestabilne pri daljšem hranjenju ali zamrzovanju. V komercialnih proizvodih proizvajalci v produkte z deklarirano aktivnostjo dodajajo stabilizatorje, npr. glicerol, NaCl, saharozo in BSA. Problem je predvsem pomemben v farmaciji pri proizvodnji bioloških zdravil, ki morajo zaradi intrevenoznega vnašanja biti brez agregatov. Park et al. (2013) so ugotovili, da dodatek 4% manitola in 2% saharoze pri pH 5 preprečuje agregiranje IgG in podaljša stabilnost formulacij monoklonskih IgG.

Metode
Iz 100 do 200 mL osiromašene kisle (FT) in sladke sirotke (FTS) smo s protein G-afinitetno kromatografijo z uporabo monolitne kolone CIMmultus™-r protein G-1 v več poskusih izolirali IgG. Interakcija IgG s proteinom G je odvisna od pH, zato smo eluirali z glicinom pri pH 2. Elucijskim frakcijam smo umerili pH na višjo vrednost in nekaterim dodali fosfatni pufer in NaCl (PBS) ali raztopino NaCl, manitola in saharoze. Frakcije smo nato skladiščili pri različnih pogojih (liofilizacija, v tekočem stanju pri različnih temperaturah in z več cikli zamrzovanja in odmrzovanja), nato pa izmerili koncentracijo z analitsko protein G-afinitetno HPLC in z vizualnim opazovanjem spremljali pojav agregatov v raztopinah.

Rezultati
Slika 1 (levo) prikazuje analizo FT, nevezanih frakcij, elucij in komercialnega IgG (Sigma-Aldrich) z SDS-PAGE. Slika 1 (sredina in desno) prikazuje koncentracijo IgG ob različnih dnevih v frakcijah z dodanim manitolom in brez manitola ter z različnimi načini skladiščenja: tekoče pri 4 °C, tekoče pri sobni temperaturi (ST), pri zamrzovanju in odmrzovanju (f-t, freeze-thaw) in liofilizaciji.



Slika 1: Levo: Analiza vhodne kisle sirotke (FT), nevezanih frakcij, elucij in komercialnega IgG (Sigma-Aldrich) z SDS-PAGE. Sredina: koncentracija IgG v frakcijah z manitolom. Desno: koncentracija IgG v frakcijah brez manitola.

Izmerjena koncentracija je bila bolj odvisna od dneva meritve kot od razlike med vzorci, kar nakazuje, da uporabljena metoda ni zanesljiva. Vseeno smo lahko med sabo primerjali koncentracije različnih vzorcev na isti dan merjenja. V FT in FTS smo izmerili podobno koncentracijo IgG (FT 0,089 in FTS 0,085 mg/mL). Pri nanašanju na kolono smo dosegli preboj IgG pred nanosom 105 mL FTS, predvidoma pri enakem volumnu kot v kisli sirotki (80 mL, naši predhodni rezultati) glede na podobno koncentracijo IgG. Kolona se je nasičila z IgG pri nanosu 157 do 186 mL FTS. Pri nanašanju večjega volumna se koncentracija v nevezani frakciji več ni povečevala in ni presegla koncentracije v nanešenem FTS, kot smo opazili z nekaterimi drugimi proteini in drugimi kolonami CIMmultus™. Dodatek manitola, saharoze in NaCl (in v primeru izolacije iz FTS tudi dodatek le acetatnega pufra) je stabiliziral mlečni IgG pri liofilizaciji in odmrzovanju/zamrzovanju do te mere, da ni bilo jasne razlike v koncentraciji med različnimi obdelavami vzorcev. Le glicin ali kombinacija glicina in PBS nista stabilizirala IgG pri liofilizaciji ali zamrzovanju/odmrzovanju: IgG se je pri tem delno oboril.

Zaključki

Kolona CIMmultus™-r protein G-1 je zaradi mašenja (predvidoma s hidrofobnimi nečistotami) manj primerna za izolacijo snovi iz sladke sirotke. Pri izolaciji iz kisle sirotke takšnega mašenja nismo opazili. Dodatek manitola in saharoze ali le acetatnega pufra zavaruje mlečni IgG pred agregacijo in je torej smiseln, če želimo proizvajati formulacijo z aktivnim IgG.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey (št. pogodbe LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (št. pogodbe OP20.03521) Operativnega programom EKP 2014 – 2020.

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Purification of immunoglobulin G from whey and its stabilization

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Abstract
Immunoglobulin G (IgG) was isolated from depleted acid and sweet whey by monolithic protein G-affinity chromatography. We determined the characteristics of IgG loading on the column. Using the isolated fractions, we performed a screening test of different storage conditions (addition / absence of stabilizers, lyophilization / liquid state / thawing and freezing cycles) on the aggregation and concentration of IgG in solutions. The addition of mannitol, sucrose and NaCl or acetate buffer alone compared with the addition of PBS alone protected IgG from aggregation under all tested storage conditions. Stabilizers should therefore be added to prepare active IgG formulations.

Keywords: whey, IgG, purification, affinity chromatography, stabilization.

Introduction
IgG is the major immunoglobulin in bovine milk. It reaches concentrations of up to 0.7 g/L in mature milk and up to 32-212 g/L in colostrum. The other two variants of immunoglobulins in milk, IgA and IgM, are present in mature bovine milk at much lower concentrations – up to 0.1 g/L (Gapper et al., 2017). There are 4 classes of IgG, with IgG1 being the predominant form in bovine milk. Immunoglobulins in milk protect both the mammary gland and the calf from environmental pathogens and toxins when the calf's immune system is not yet developed. During the maturation of immune cell immunoglobulins acquire a very high affinity for the target antigen ($K_D = 10^{-15}$). At the end of the heavy (57 kDa) and light (26 kDa) chains are mutation-prone loops that form a hypervariable region for antigen binding. Immunoglobulins, with the exception of secretory IgA, are not resistant to proteolysis by digestive enzymes. IgGs contain multiple disulfide bonds that link the heavy chains to the light chains and the heavy chains to each other, forming a monomer with a molecular weight of 150 kDa. In the process of casein coagulation, part of the IgG seems to precipitate or bind to caseins. IgG concentrations in whey are thus lower than in milk.

IgG molecules are subject to aggregation due to their delicate and branched 3D structure and are therefore unstable during prolonged storage or freezing. In commercial products, manufacturers add stabilizers (e.g., glycerol, NaCl, sucrose, and BSA) to products with declared IgG activity. The problem of aggregation is particularly prominent in the production of biologic drugs, which must be free of aggregates due to intravenous administration. Park et al. (2013) found that the addition of 4% mannitol and 2% sucrose at pH 5 prevented IgG aggregation and prolonged the stability of monoclonal IgG formulations.

Methods
In several experiments, IgG was isolated from 100 to 200 mL of depleted acid (FT) and sweet whey (FTS) by protein G-affinity chromatography using a monolithic CIMmultus™-r protein G-1 column. The interaction of IgG with protein G is pH-dependent, therefore, the bound IgG was eluted with glycine at pH 2. The elution fractions were adjusted to a higher pH value. Phosphate buffer and NaCl (PBS), or NaCl, mannitol and sucrose solution were added to some of the fractions. The fractions were then stored under different conditions (lyophilization, liquid at different temperatures, or with multiple freeze-thaw cycles) and then the concentration was measured by analytical protein G-affinity HPLC and the appearance of aggregates in solutions was monitored by observation.

Results
Figure 1 (left) shows analysis of FT, unbound fractions, elutions, and commercial IgG (Sigma-Aldrich) by SDS-PAGE. Figure 1 (middle and right) shows IgG concentration on different days in fractions with added mannitol and without mannitol and with different storage methods: liquid at 4 °C, liquid at room temperature (ST), freezing and thawing (f-t) and lyophilization.

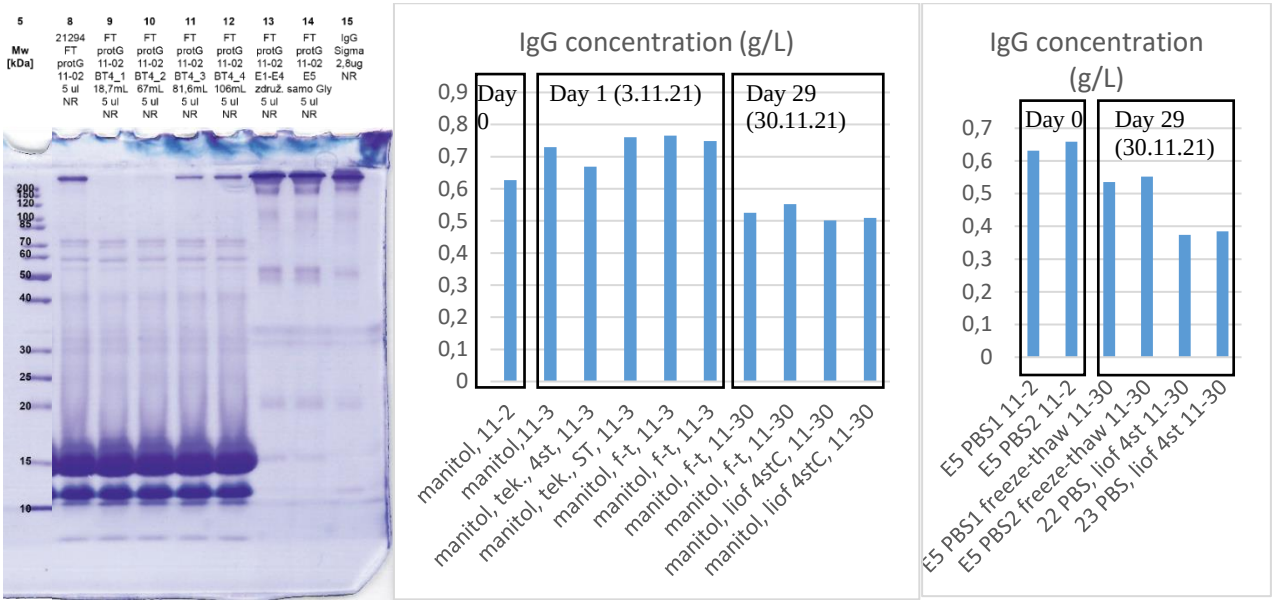

Figure 1: Left: Analysis of input acid whey (FT), unbound fractions, elutions, and commercial IgG (Sigma-Aldrich) by SDS-PAGE. Middle: IgG concentration in mannitol fractions. Right: IgG concentration in mannitol-free fractions.

The measured concentration was more dependent on the day of measurement than on the difference between samples, indicating that the method used was not reliable. However, we were able to compare the concentrations of different samples on the same day of measurement. Similar IgG concentrations (FT 0.089 and FTS 0.085 g/L) were measured in FT and FTS. When applied to the column, IgG breakthrough was achieved before application of 105 mL of FTS, presumably at the same volume as in acid whey (80 mL, our previous results) due to a similar IgG concentration. The column was saturated with IgG at 157 to 186 mL of loaded FTS. When a larger volume was applied, the concentration in the unbound fraction no longer increased and did not exceed the concentration in the loaded FTS, as observed with some other proteins and other CIMmultus™ columns. The addition of mannitol, sucrose, and NaCl (and in the case of isolation from FTS, the addition of acetate buffer alone) stabilized the milk IgG during lyophilization and thawing / freezing to such an extent that there were no clear differences in concentration between the different sample treatments. Only glycine or the combination of glycine and PBS did not stabilize the IgG during lyophilization or freezing / thawing: in this case, the IgG partially precipitated.

Conclusions

The CIMmultus™-r protein G-1 column is less suitable for isolation of substances from sweet whey due to clogging (presumably by hydrophobic impurities). No such clogging was observed when isolating from acid whey. The addition of mannitol and sucrose or only acetate buffer protects milk IgG from aggregation and is therefore useful if we want to prepare a formulation with active IgG.

Acknowledgements

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Pridobivanje nizina in vitamina B12 z biokonverzijo kisle sirotke

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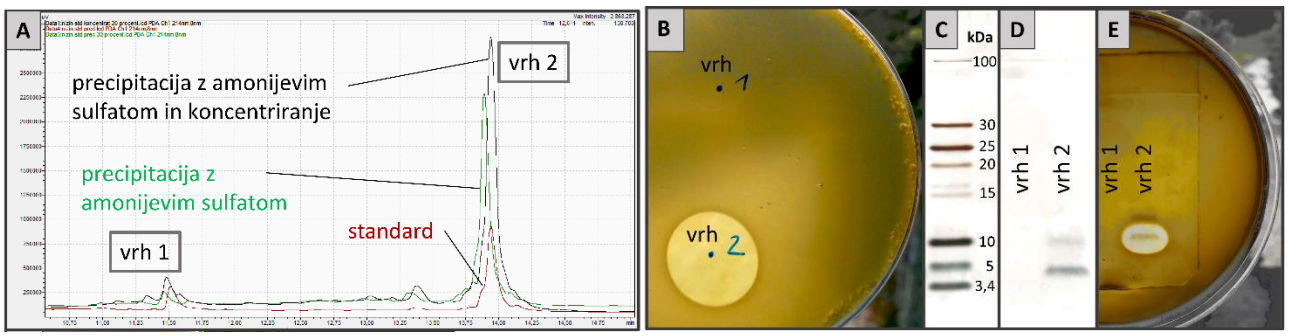
Povzetek
Optimizirali smo produkcijo nizina in vitamina B12 v bioreaktorjih različnega volumna. Preizkusili smo več metod koncentriranja, čiščenja ter analitike ugotavljanja nizina. Produkcija nizina je bila boljša, kadar smo med fermentacijo uravnavali pH nad 6, se je pa v tem primeru večji del nizina vezal na bakterije (pelet) (skoraj 50 %). Tako pridobljeni nizin lahko z nižanjem pH pod 3 sprostimo s celic v gojišče. Za koncentriranje nizina na večjem nivoju je tako najbolj primeren način ultrafiltracija, v kombinaciji z uravnavanjem pH. Nizin smo poskušali izolirati tudi s penjenjem z detergentom in nadaljnjo izolacijo z mešanico metanola in acetona. S penjenjem ob dodatku detergenta lahko nizin dodatno skoncentriramo, sama izolacija z metanolom in acetonom pa je primerna za pridobivanje bolj čiste oblike nizina. Vitamin B12 smo proizvajali v dveh stopnjah in na koncu fermentacije poželi bakterijske celice s centrifugiranjem ter iz njih sprostili vitamin B12, ki smo ga merili z LC-MS/MS.

Ključne besede: nizin, protimikrobna aktivnost, vitamin B12, kisla sirotka, bioproces

Uvod
Stranski proizvodi iz živilske industrije imajo lahko velik bremenilni vpliv na okolje. Eden izmed takih stranskih proizvodov je tudi sirotka, ki jo, odvisno od vrste izdelka s katero jo pridobimo, delimo na sladko in kislo sirotko. Ker je zelo hranilna, jo lahko uporabimo v različne namene – od napitkov ali kot dodatka živilom do izdelkov z višjo dodano vrednostjo, ki vsebujejo posamezne proteine, izolirane iz sirotke. Nadaljnja predelava sirotke se razlikuje predvsem glede na njen izvor. Predelava kisle sirotke, ki nastane pri fermentaciji oz. kislinski koagulaciji mleka, je veliko zahtevnejša od predelave sladke sirotke, ki je stranski produkt pri izdelavi sira. Predelava je zahtevnejša predvsem zaradi večje vsebnosti mlečne kisline, ki moti nadaljnje procese, zato so novi postopki in končni izdelki zelo zaželeni (Zandona in sod., 2021). Raziskali smo možnosti nadaljnje uporabe kisle sirotke, ki je bila predtem mikrofiltrirana in ji je bil s kromatografijo na monolitnih kolonah odvzet del proteinov. Tako obdelana sirotka vsebuje nespremenjeno koncentracijo laktoze, mlečne kisline, mineralov, posameznih sirotkinih proteinov (ki jih nismo odvzeli na kolonah), peptidov in drugih rastnih faktorjev, ki jih lahko mikroorganizmi izkoriščajo za rast in metabolno aktivnost. Sirotko smo dodatno obogatili s kvasnim ekstraktom in jo uporabili za proizvodnjo nizina in vitamina B12.

Produkcija nizina
Nizin smo pridobivali v bioreaktorjih volumna 1 L in 2,5 L z gojenjem seva *Lactococcus lactis* IM145. V predposkusih smo določili optimalne pogoje priprave gojišča, sestavljenega iz mikrofiltrirane in deproteinizirane kisle sirotke, ki smo ji dodali 2,5 % kvasnega ekstrakta ter uravnali pH na 6,3-6,5. Fermentacija je potekala pri 30 °C, 350 rpm, dokler nismo dosegli stacionarne faze rasti (7,5-8 h). Med fermentacijo smo spremljali pH, optično gostoto, v 1-urnih intervalih nacepljali na petrijeve plošče in ugotavljali protimikrobno aktivnost. Za namene sledenja proizvodnje bakteriocinov smo razvili metode za detekcijo nizina in njegove protimikrobne aktivnosti: kvalitativno na trdnem gojišču, semi-kvantitativno z mikrodilucijo, kvalitativno z identifikacijo lis z nizinsko aktivnostjo na poliakrilamidnih gelih NaDS-PAGE in kationsko izmenjevalno kromatografijo CIMac-COOH ter kromatografijo z obrnjenima fazama – RP-HPLC C-8 (Slika 1). Optimizirali smo tudi protokole za koncentriranje in delno čiščenje nizina iz gojišča na osnovi kisle sirotke, s pomočjo ultrafiltracije, precipitacije z amonijevim sulfatom ter preparativne kationsko-izmenjevalne kromatografije ob uporabi različnih mobilnih faz. Kromatografija z obrnjenima fazama se je izkazala kot najprimernejša za analitiko nizina med postopkom čiščenja, za detekcijo protimikrobne aktivnosti pa je bila najbolj primerna mikrodilucijska metoda. Težavo pri analitiki namreč predstavljajo številni sirotkini proteini, ki so v gojišču prisotni v višjih koncentracijah kot nizin in zato pogosto prekrijejo nizinske vrhove. V nadaljnjih poskusih smo opazili, da je produkcija nizina večja, če se tekom bioprocesa uravnava pH na 6,4, je pa v tem primeru tudi več nizina vezanega na bakterijske celice, ki ga lahko ob koncu fermentacije sprostimo v gojišče z nižanjem pH pod 3. Nizin smo poskušali izolirati tudi s penjenjem ob dodatku detergenta (0,1 % Tween 80) ter z mešanico metanola in acetona. S penjenjem lahko ob nizkem pH nizin skoncentriramo. Z izolacijo z metanolom in acetonom pa imamo sicer nekaj izgub, pridobimo pa na končni čistosti nizina.



Slika 1: Protimikrobna aktivnost frakcij, pridobljenih med analizo standarda nizina z RP-HPLC, ki odgovarjata dvema vrhovoma. (A) Analiza standardnega vzorca nizina, pred in po precipitaciji ter koncentriranju; (B) protimikrobna aktivnost odvzetih frakcij na RP-HPLC sistemu, pri vrhu 1 in 2; (C) proteinska lestvica pri elektroforezi NaDS-PAGE; (D) elektroforeza NaDS-PAGE za odvzete frakcije na RP-HPLC sistemu pri vrhu 1 in 2; (E) protimikrobna aktivnost gela pod točko D.

Produkcija vitamina B12

Vitamin B12 smo proizvajali s fermentacijo obogatene deproteinizirane kisle sirotke s sevom *Propionibacterium freudenreichii* subsp. *freudenreichii* van Niel 1928. Ugotovili smo, da je za produkcijo B12 potreben dodatek kvasnega ekstrakta, dodatek mineralov ni imel večjega vpliva na količino nastalega B12, dodatek prekurzorjev pa je produkcijo rahlo izboljšal. Fermentacija je potekala v dveh stopnjah. V prvi smo sev namnožili, druga pa je bila usmerjena v produkcijo B12. Preizkusili smo različne pogoje gojenja in ugotovili, da je celokupna vsebnost vitamina B12 največja, kadar v prvi stopnji 4 % prekonočne kulture inokuliramo v deproteinizirano kislo sirotko, obogateno s kvasnim ekstraktom (2,5 %) ter inkubiramo anaerobno 2 dni pri 30 °C. Po končani inkubaciji pričnemo z 2. stopnjo in vcepimo 4 % seva iz 1. stopnje v gojišče na osnovi deproteinizirane kisle sirotke, obogatene s kvasnim ekstraktom in $CoCl_2$ (40 mg/L) ter 5,6-dimetilbenzimidazolom – DMBMZ (70 mg/L). Drugo fermentacijo vodimo aerobno 6 dni pri 30 °C. Vsebnost vitamina B12 smo ovrednotili z analizno metodo LC-MS/MS, kjer smo zaradi stabilnosti vzorce predhodno obdelali s KCN.

Zaključek

Po koncu gojenja MKB v fermentacijski brozgi ostane še 2-3 g laktoze ter večja količina mlečne kisline (lahko tudi več kot 2 g / 100 g) kar je še vedno zelo obremenilno za okolje. Kislo sirotko sicer nadalje predelamo z anaerobno razgradnjo, kjer najprej skušamo doseči razgradnjo kompleksnih organskih molekul v enostavnejše in bolj topne komponente ali hlapne spojine, kot sta ocetna in propionska kislina. Te potem lažje pretvorimo v pline, med katerimi prevladujeta metan in ogljikov dioksid (Rivas et al., 2010). Ker propionibakterije med produkcijo B12 pretvorijo skoraj vso laktozo in mlečno kislino v ocetno in propionsko kislino, skušamo nadalje vzpostaviti tudi zaporedno fermentacijo laktokokov in nato še propionibakterij, ki bi pripomogla k še boljšemu izkoristku in uporabi kisle sirotke.

Zahvala

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Production of nisin and vitamin B12 by acid whey bioconversion

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Abstract
We optimized the production of nisin and vitamin B12 in bioreactors with different volumes. We tested several methods of concentration, purification and analytics for nisin determination. Nisin production was better when pH was adjusted above 6 during fermentation, but in this case most nisin was bound to the bacteria (pellet) (almost 50%). The nisin recovered this way can be released into medium by lowering the pH below 3. Ultrafiltration, in combination with pH adjustment, is the most appropriate way to concentrate nisin when production is scaled up. We also attempted to isolate the nisin by foaming with detergent and further isolating it with a combination of methanol and acetone. Foaming with the addition of detergent can further concentrate the nisin, and isolation with methanol and acetone itself is suitable for obtaining a purer form of nisin. Vitamin B12 was produced in two steps. At the end of fermentation, bacterial cells were harvested by centrifugation, vitamin B12 was then released from bacterial cells and determined by LC-MS/MS.

Keywords: nisin, antimicrobial activity, vitamin B12, acid whey, bioprocess

Introduction
By-products from the food industry can have a major impact on the environment. One of these by-products is also whey, which is divided into sweet and acid whey, depending on the type of origin product. Being highly nutritious, it can be used for a variety of purposes – from dairy beverages or as an additive to other food products, to high-added value products containing individual proteins isolated from whey. Further treatment of whey depends primarily on its origin. The processing of acid whey, which is produced during the fermentation or acid coagulation of milk, is much more demanding than the processing of sweet whey, which is a by-product of cheese production. Treatment is more difficult mainly because of the higher content of lactic acid, which hinders further processing techniques, making new approaches and final products highly desirable (Zandona et al., 2021). We have introduced the reuse of acid whey after microfiltration and partial extraction of proteins on the monolithic chromatography columns. Whey treated this way contains unchanged concentrations of lactose, lactic acid, minerals, individual whey proteins (which were not removed on the columns), peptides and other growth factors that microorganisms can use for their growth and metabolic activity. The whey was further enriched with yeast extract and used for the production of nisin and vitamin B12.

Nisin production
Nisin was obtained in 1-liter and 2.5-liter bioreactors by culturing the *Lactococcus lactis* IM145 strain. In preliminary experiments, we determined the optimal conditions for the media preparations, which consisted of deproteinized acid whey with the addition of 2.5 % yeast extract, with pH adjusted to 6.3-6.5. Fermentation was carried out at 30 °C and 350 rpm until and the stationary phase was reached (7.5-8 h). During fermentation, pH and optical density were monitored, Petri dishes were inoculated at 1 h intervals, and antimicrobial activity was also determined. To follow bacteriocin production, we developed methods to detect nisin and its antimicrobial activity: qualitatively on solid medium, semi-quantitatively using the microdilution technique, qualitatively by identification of nisin activity spots on polyacrylamide SDS-PAGE, cation exchange chromatography CIMac-COOH and reversed phase chromatography – RP-HPLC C-8 (Figure 1). We also optimized protocols for concentration and partial purification of nisin from a medium based on acid whey by ultrafiltration, ammonium sulfate precipitation, and preparative cation exchange chromatography using different mobile phases. Reverse phase chromatography was found to be most suitable for nisin analysis during the purification process, and the microdilution method was best for detection of antimicrobial activity. The problem with nisin analysis is that many whey proteins are present in the medium at higher concentrations than nisin and therefore often mask the nisin peaks. In further experiments, we observed that nisin production is higher when the pH is adjusted to 6.4 during the bioprocess. However, in this case, more nisin is bound to the bacterial cells and can be released from them at the end of the fermentation when the pH is lowered below 3. The nisin was also further isolated by foaming with the addition of detergent (0.1 % Tween 80) and a

combination of methanol and acetone. The nisin can be adequately concentrated by foaming at low pH. However, isolation with methanol and acetone results in some losses, but we gain in the final purity of the nisin.

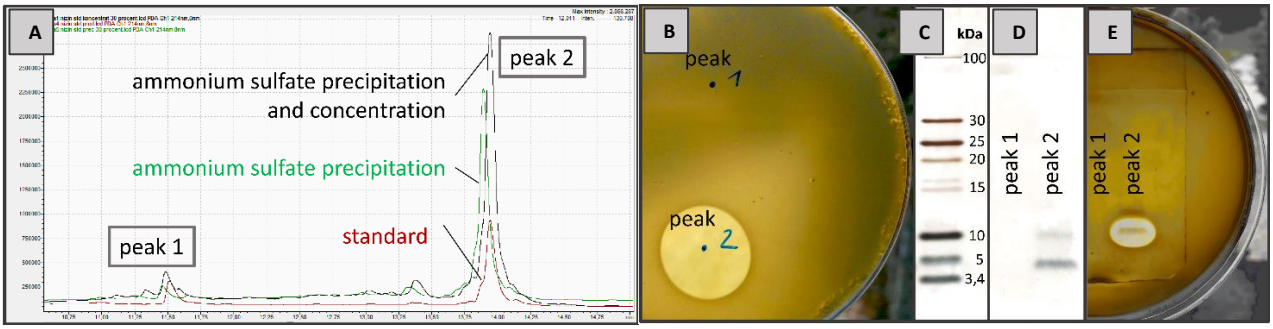

Figure 1: Antimicrobial activity of fractions obtained from analysis of nisin standard by RP-HPLC corresponding to two peaks. (A) Analysis of a standard nisin sample before and after precipitation and concentration; (B) antimicrobial activity of the obtained fractions from the RP-HPLC system, at peak 1 and 2; (C) protein scale in SDS- PAGE electrophoresis; (D) SDS- PAGE electrophoresis for fractions taken on the RP-HPLC system at peak 1 and 2; (E) antimicrobial activity of the gel at point D.

Production of vitamin B12

Vitamin B12 was produced by fermentation of enriched deproteinized acid whey with the strain *Propionibacterium freudenreichii* subsp. *freudenreichii* van Niel 1928. We found that the production of B12 required the addition of yeast extract. The addition of minerals had no significant effect on the amount of B12 produced, while the addition of precursors slightly improved production. Fermentation was performed in two stages. In the first step we promoted the growth of the strain, while the second step was focused on the production of B12. We tested different growth conditions and found that the highest total vitamin B12 content was obtained when 4 % of the overnight culture in the first processing step was inoculated into deproteinized acid whey enriched with yeast extract (2.5 %) and incubated anaerobically at 30 °C for 2 days. After incubation, the second production step begins, where we inoculate 4 % of the strain from step 1 into the medium based on deproteinized acid whey enriched with yeast extract, $CoCl_2$ (40 mg/L) and 5,6-dimethylbenzimidazole – DMBMZ (70 mg/L). The second fermentation was performed aerobically at 30 °C for 6 days. Vitamin B12 content was determined by the analytical method LC-MS/MS, with samples previously treated with KCN for better stability.

Conclusion

After the end of LAB cultivation, 2-3 g of lactose and a larger amount of lactic acid (often more than 2 g / 100 g) remain in the fermentation broth, which is still very burdensome for the environment. Acid whey can be further processed by anaerobic digestion, where first the complex organic molecules are broken down into simpler and more soluble components or volatile compounds such as acetic and propionic acids. These are then more readily converted to gases, of which methane and carbon dioxide predominate (Rivas et al., 2010). Since propionibacteria convert almost all lactose and lactic acid into acetic and propionic acid during B12 production, we are trying to further establish sequential fermentation of lactococci followed by fermentation with propionibacteria, which would contribute to even better utilisation and consumption of acid whey.

Acknowledgements

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Razvoj in optimizacija industrijskega postopka izolacije LPO iz sladke sirotke z uporabo CIM kromatografske kolone

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Povzetek
Za razvoj metode izolacije laktoperoksidaze (LPO) iz sirotke, smo opravili serijo eksperimentov, pri čemer smo LPO poskusili izolirati iz kisle (KS), nato pa še iz sladke sirotke (SS). Izkazalo se je, da je SS zaradi 20 - 50-krat višje vsebnosti LPO in 5 – 6-krat nižje vsebnosti LF bolj primerna za izolalcijo LPO. Eksperimente smo najprej opravili na laboratorijskem nivoju (CIMmultus SO3 1mL kolona). V začetni fazi smo elucijo LPO izvajali s solnim gradientom, kasneje pa razvili metodo s stopenjsko elucijo, ki omogoča enostavnejši prenos na večje kromatografske kolone. S ciljem izboljšanja stabilnosti eluirane LPO smo, s testiranji na pilotnem nivoju z uporabo CIMmultus SO3 8L kolone, dodatno prilagodili sestavo elucijskega pufra in še nekoliko optimizirali ostale elucijske pufre. Rezultat je ponovljiva metoda izolacije LPO iz SS, pri čemer se laktoperoksidazna aktivnost (237 – 305 U/mg) ohranja in je primerljiva z LPO standardom proizvajalca Sigma (261 – 283 U/mg).

Ključne besede: Laktoperoksidaza, CIM kromatografska kolona, izolacija, sirotka

Uvod
Sirotka, sladka ali kisla, vsebuje proteine, ki izkazujejo protimikrobne, protivirusne in antioksidativne lastnosti. Ti proteini lahko posledično nudijo zaščito pred rakavimi obolenji, boleznimi srca ter pomagajo pri krepitvi imunskega sistema (Gonzalez-Chavezin sod.,2009, Marwahain Kennedy, 1988, Macwan in sod., 2016). Proteini, ki predstavljajo večinski delež v sirotki so α-laktalbumin (α-LA), β-laktoglobulin (β-LG), goveji serumski albumin (BSA) in imunoglobulini (npr. IgG). Poleg omenjenih so prisotni tudi drugi proteini, kot sta laktoperoksidaza (LPO) in laktoferin (LF), a predstavljata občutno nižji delež celokupnih proteinov (<1%) (Hahn et al., 1998). Ionsko izmenjevalna kromatografija (IEX) na CIM kromatografskih kolonah se uporablja na širokem področju izolacije različnih bioloških molekul in virusov (Nesterenko, 2018). Prednosti CIM kromatografskih kolon (BiaSeparations, Sartorius) so predvsem v njeni visoki ločljivosti, hitrosti ločbe in enostavnem prenosu metod iz laboratorijske na industrijsko raven. Za izolacijo LPO/LF iz SS in KS smo uporabili kolono z močnim kationskim izmenjevalcem (SO_3^-). Površinski naboj obeh proteinovje pri pH omenjenih sirotk (pH SS 6,6 in pH KS 4,6) pozitiven, kar privede do njune vezave na kromatografski nosilec. Zaradi prisotnosti mikroorganizmov, proteinskih in maščobnih agregatov, drugih proteinov, sladkorjev in mlečne kisline, izolacija LPO ni enostavna. To zahteva ustrezno predpripravo sirotke na samo kromatografijo in uporabo ustrezne metode in pufernih raztopin.

Rezultati
Metodo za izolacijo LPO smo na laboratorijskem nivoju najprej razvili na KS, kar se zaradi nizke vsebnosti LPO (<5 mg/L) in visoke vsebnosti LF (80-150 mg/L) ni izkazalo kot učinkovito. Rezultati so pokazali, da zaradi preferenčne vezave LF, ki je v primerjavi z LPO bolj bazičen protein (Voswinkela in Kulozik, 2011), slednjega izrine. Uspešna izolacija je bila tako dosežena šele po predhodni odstranitvi LF iz KS, vendar smo se zaradi nizke vsebnosti LPO v KS nato preusmerili na SS, kjer je koncentracijsko razmerje med obema proteinoma obrnjeno. V primeru izolacije iz SS se je pristop pokazal kot uspešen. Zaradi slabše stabilnosti LPO v eluciji s fosfatnim pufrom (NaH2PO4), kjer se je le-ta oborila in hitro izgubljala peroksidazno aktivnost, smo uporabili in-house razvito metodo stabilizacije proteina, kar se je izkazalo v ohranitvi aktivnosti in nični precipitaciji. Metodo razvito na laboratorijskem nivoju smo nato preizkusili še na polindustrijskem nivoju z uporabo 8L kromatografske kolone in še nekoliko optimizirali ostale puferne raztopine. Rezultati testov ponovljivosti so pokazali dobro ponovljivost in stabilen produkt LPO, ki ohranja specifično aktivnost preko celotnega procesa kromatografije in zgoščevanja elucije (**Tabela 1**). Rezultati IEX HPLC analitike in SDS page so pokazali, da je čistost produkta med eksperimenti v rangu 49 – 71%. Opazili smo, da je stopnja čistosti proteina v pozitivni korelaciji s količino vezane LPO na kromatografski koloni. Pri tem smo količino vezane LPO grobo ocenili z encimskim testom peroksidazne aktivnosti koncentriranih LPO elucij. Rezultati so pokazali, da je bila pri večji masi vezane LPO na kromatografski koloni ,čistost končne elucije višja. Rezultatje tudi v skladu z dognanji spremljanja poteka izolacije LF iz kisle in sladke sirotke. Glavni razlog za ta pojav

lahko v obeh primerih pripišemo učinku izpodrivanja drugih beljakovin z nižjo afiniteto do vezave na kromatografski nosilec kot jo ima LPO oziroma LF.

Tabela 1. Laktoperoksidazna aktivnost zgoščin LPO izražena v U/mg. Za primerjavo je v tabeli predstavljena tudi aktivnost standarda LPO proizvajalca Sigma.

	Optimizacija pufrov			**Test ponovljivosti 3x**				
LPO Koncentrat	Lot 21231	Lot 21256	Lot 21263	Lot 21270	Lot 21312	Lot 21319	Lot 21319 (združen KLPO)	LPO (Sigma)
Skupni prot. (g/100g)	1.83	1.39	0.71	1.46	1.61	1.94	2.31	/
Koncentriran (K) LPO (IEX HPLC, purity %)	71.2	39.5	40.6	49.4	56.0	51.0	53	/
Skupni LPO prot. (g/100g)	1.303	0.549	0.288	0.721	0.902	0.990	1.2243	/
Volumen (L)	0.952	0.825	1.00	1.025	1.04	0.995	2.00	/
c LPO (mg/mL)	13.03	5.49	2.88	7.21	9.02	9.89	12.243	2.5
Specifična LPO aktivnost (U/mg)	**221.3**	**123.1**	**6.68**	**304.40**	**237.7**	**252.6**	**201.4**	**282.5/261.5**
Volumski delež (φ_i)	0.163	0.141	0.171	0.176	0.178	0.170	=1.00	/
Vsota LPO aktivnosti glede na volumski delež LPO elucije (21231 + 21256 + 21263 + 21270 + 21312 + 21319)							193.5	/

Zahvala
Raziskava je bila sofinancirana s projektom LAKTIKA - Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil (OP20.03521) Operativnega programom za izvajanje evropske kohezijske politike v obdobju 2014 – 2020 in projektom LIFE for Acid Whey - Ponovna uporaba odpadne sirotke za ekstrakcijo bioaktivnih beljakovin z visoko dodano vrednostjo (LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE.

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Development and optimization of an industrial process for the isolation of LPO from sweet whey using CIM chromatographic column

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Abstract
We performed a series of experiments to develop a method for lactoperoxidase (LPO) isolation from whey. First, we try to isolate LPO from acid (AW) and later from sweet whey (SW). It turned out that SW is more suitable due to 20-50-timeshigher LPO content and 5-6-timeslower LF content. The experiments were first performed at the laboratory level (CIMmultus SO3 1mL column). In the first stage, the LPO elution was performed with a conductivity gradient. Later, a step elution method was developed, which allowed upscaling to larger chromatographic columns with ease. Finally, to improve eluted LPO's stability, we adjusted the composition of the elution buffer and slightly optimized other elution buffers by testing on a pilot level using a CIMmultus SO3 8L column. The result is a repeatable method for LPO isolation from SW where by lactoperoxidase activity (237 - 305 U/mg) is maintained and is comparable to LPO standard from manufacturer Sigma (261 - 283 U/mg).

Keywords: Lactoperoxidase, CIM chromatographic column, isolation, whey

Introduction
Whey, sweet or acid, contains proteins that exhibit antimicrobial, antiviral and antioxidant properties. These proteins can therefore provide protection against cancer, heart disease and help strengthen the immune system (Gonzalez-Chavezin et al., 2009; Marwahain Kennedy, 1988; Macwan et al., 2016). The major proteins in whey are α-lactalbumin (α-LA), β-lactoglobulin (β-LG), bovine serum albumin (BSA) and immunoglobulins (e.g.IgG). In addition to those mentioned, other proteins are also present, likelactoperoxidase (LPO) and lactoferrin (LF). However, they represent a significantly lower proportion of total proteins (<1%) (Hahn et al., 1998). Ion exchange chromatography (IEX) on CIM chromatographic columns are used in the broad field of isolation of biological molecules and viruses (Nesterenko, 2018). The advantages of CIM chromatographic columns (Bia Separations, Sartorius) are mainly in theirhigh resolution, fast separation process, and simple upscalefrom laboratory to industrial level. A column with a strong cation exchanger (SO_3^-) was used to isolate the LPO / LF from SW and AW. The surface charge of both proteins is positive at the pH of whey (pH SW 6.6 and pH AW4.6), which leads to their binding to the chromatographic support. Due to the presence of microorganisms, protein and fat aggregates, other proteins, sugars and lactic acid, isolating LPO is not easy. It requires proper preparation (microfiltration) of the whey for chromatography and the use of an appropriate method and buffer solutions.

Results
The method for LPO isolation was first developed at the laboratory level for AW, which did not prove to be effective due to the low content of LPO (<5 mg/L) and high content of LF (80-150 mg/L). The results showed that, due to the preferential binding of LF, which is a more basic protein (Voswinkela and Kulozik, 2011) compared to LPO, the latter is displaced. Successful isolation was thus achieved only after removing LF from AW, but due to the low content of LPO in AW, we choose to use SW, where the concentration ratio between the two proteins is reversed. In the case of LPO isolation from SW, the approach proved to be successful. Due to the poorer stability of LPO in elution with phosphate buffer (NaH_2PO_4), where it precipitated and rapidly lost its peroxidase activity, we used an in-house developed protein stabilization method, which resulted in the preservation of LPO activity and zero precipitation. With some minor changes of the buffers, the method developed at the laboratory level was tested at the semi-industrial level using an 8L chromatographic column. The repeated isolation tests performed on a semi-industrial level give good repeatability results and a stable LPO product that retained specific activity throughout the chromatography and concentration processes (Table 1). The results of IEX HPLC analytics and SDS page showed that the purity of the product varies in the range of 49 to 71%. Furthermore, we observed that the degree of protein purity positively correlated with the amount of bound LPO on the chromatographic column. The amount of bound LPO was roughly estimated by the enzymatic test of peroxidase activity of concentrated LPO elutions. The results showed that the higher the mass of bound LPO on the chromatographic column, the higher the purity of the final elution. The result is

also in line with the monitoring findings of LF isolation from AW and SW. The main reason for this phenomenon lies in both cases in the displacement effect of other proteins with lower affinity for binding to the chromatographic ligand compared to LPO or LF.

Table2. Peroxidase activity of LPO concentrates in terms of U/mg of LPO. For the purpose of comparison, the activity of Sigma standard was also determined.

	Buffer optimization			**Repeatability experiments 3x**				
LPO Concentrate	Lot 21231	Lot 21256	Lot 21263	Lot 21270	Lot 21312	Lot 21319	Lot 21319 (gathered all KLPO)	LPO (Sigma)
Tot prot. (g/100g)	1.83	1.39	0.71	1.46	1.61	1.94	2.31	/
Concentrated LPO (IEX HPLC, purity %)	71.2	39.5	40.6	49.4	56.0	51.0	53	/
Tot. LPO prot. (g/100g)	1.303	0.549	0.288	0.721	0.902	0.990	1.2243	/
Volume (L)	0.952	0.825	1.00	1.025	1.04	0.995	2.00	/
c LPO (mg/mL)	13.03	5.49	2.88	7.21	9.02	9.89	12.243	2.5
Specific Lactoperoxidse activity (U/mg)	**221.3**	**123.1**	**6.68**	**304.40**	**237.7**	**252.6**	**201.4**	**282.5/261.5**
Volumefraction (φ_i)	0.163	0.141	0.171	0.176	0.178	0.170	=1.00	/
Sum of LPO activity in relation to concentrate volume fraction (21231 + 21256 + 21263 + 21270 + 21312 + 21319)							193.5	/

Acknowledgements

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Optimizacija diafiltracije in koncentriranja proteinskih elucij kot priprava na sušenje

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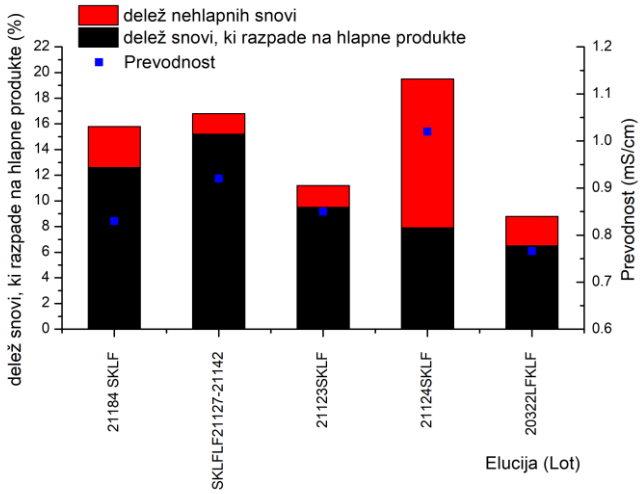
Povzetek
Za potrebe sušenja v vrtinčnoslojni komori (FBDS) in doseganja ciljne vsebnosti LF v končnem produktu (kapsuli) smo v navezavi s projektnim partnerjem FFA optimirali korak diafiltracije in zgoščevanja proteinskih elucij laktoferina (LF), laktoperoksidaze (LPO) in proteinske frakcije bogate z betalaktoglobulinom, alfalaktalbuminom in LF (BLG). Za diafiltracijo smo uporabljali tangencialno filtracijo z uporabo t.i. hollow fiber membrane. Za doseganje višjih izkoristkov sušenja v FBDS komori in ugoden potek samega sušenja je bilo potrebno v proteinski zgoščini doseči dovolj visoko vsebnost proteinskega produkta, medtem ko je bilo za doseganje končnega odmerka doseči tudi dovolj visoko čistost, kar velja predvsem za LF zgoščino, in nizko vsebnost soli v vseh omenjenih proteinskih zgoščinah namenjenih sušenju. Iz podatkov analiz o zgoščinah, ki zajemajo različne fizikalno kemijske lastnosti (vsebnost vode, suha snov, ki razpade v hlapne produkte, nehlapna suha snov, koncentracija LF, prevodnost zgoščine, pH in čistota LF) smo ugotovili, da na končni rezultat sušenja v FBDS komori najbolj vplivata delež nehlapne suhe snovi, suhe snovi, prevodnost proteinske zgoščine in čistost ter koncentracija LF v zgoščini. Zadovoljiv rezultat optimizirane diafiltracije so dosegli v primeru LF in BLG, medtem ko v primeru zgoščine LPO zaradi visoke viskoznosti koncentrata, kljub nizki vsebnosti proteinov nismo dosegli ustrezne sestave, ki bi omogočala dodatno zgoščevanje in kasneje stabilen potek sušenja v komori.

Ključne besede:laktoferin, laktoperoksidaza, diafiltracija, sušenje, FBDS komora

Uvod
Namen diafiltracije proteinskih elucijskih frakcij (LF/LPO/BLG) je odstranitev soli in morebitno prisotnih drugih sirotkinih proteinov iz raztopine s proteinskim produktom. Z uporabo membrane z ustrezno velikostjo por (50 kDa – LF/LPO in 10 kDa – BLG) in dodajanjem vode v raztopino omogočimo zadrževanje LF/LPO/BLG na retentatni strani, medtem ko voda z raztopljenimi solmi odteka preko permeatne strani membrane. Za diafiltracijo smo uporabljali tangencialno filtracijo s t.i. hollow fiber membranami proizvajalca Watersep (mini-Bioproducer24). Višje izkoristke sušenja v FBDS komori in ugoden potek samega sušenja je doseženo le ob dovolj visoki vsebnost proteinskega produkta, medtem ko je za doseganje končnega odmerka potrebno doseči tudi dovolj visoko čistost, kar velja predvsem za LF zgoščino, in nizko vsebnost soli v vseh omenjenih proteinskih zgoščinah namenjenih sušenju. S sledenjem različnim fizikalno kemijskim lastnostim kot so: vsebnost vode, suha snov, ki razpade v hlapne produkte, nehlapna suha snov, koncentracija in čistost LF, skupni proteini, prevodnost zgoščine in pH smo določili ključne parametre, ki morajo biti izpolnjeni, da postopek sušenja v FBDS komori dosega visoke izkoristke, je ponovljiv in je hkrati v suhem produktu dosežena ciljna vsebnost učinkovine (LF).

Rezultati
Rezultati, pridobljeni z merjenjem prevodnosti in termo gravimetričnim analizatorjem (TGA, **Slika 1**) so pokazali, da nekateri koncentrati LF po diafiltraciji še vedno vsebujejo večjo maso nehlapnih snovi, kar je predvsem posledica nezadovoljive odstranitve soli (NaCl) in se odraža tudi v višji prevodnosti raztopine. S podaljšanjem postopka diafiltracije smo poskusili dodatno znižati vsebnost soli in povišati vsebnost, ki razpade na hlapne snovi (proteini) ter to tudi dosegli. Poudariti je potrebno, da k prevodnosti zgoščine doprinesejo tudi prisotni proteini oz. proteinski izolat, zato se s podaljšanjem diafiltracije le ta ne zniža občutno, a kljub temu nakazuje na še dodatno zmanjšanje vsebnosti soli (**Slika 1**). Rezultati, ki v povzetku niso predstavljeni, so pokazali tudi, da vsebnost soli ter prisotnost neželenih proteinov (nižja čistost) v primeru LF koncentratov, negativno vplivata na odmerek v končnem produktu. Poleg tega je za doseganje visoke učinkovitosti sušenja potrebno v koncentratu dosegati dovolj visoko vsebnost proteinov (>10 wt%). Z uporabo omenjenih rezultatov smo kasneje določili parametre, ki naj bodo značilni za koncentriran LF izolat in mejne vrednosti, ki jih mora le ta dosegati po postopku diafiltracije in pred sušenjem v FBDS komori (**Tabela 1**).



Slika 1. Vsebnost snovi, ki med TGA analizo razpade na hlapne (proteini) in nehlapne produkte (soli), ter prevodnost analiziranega koncentrata.

Tabela 1: Parametri, ki so značilni za LF izolat in mejne vrednosti, ki jih mora dosegati zgoščina proteinskega izolata LF pred sušenjem v FBDS.

Proteinski izolat	**Zgoščina proteinskega izolata po diafiltraciji**			
EL3	KLF	Faktor zgostitve KLF	Prevodnost KLF	pH KLF
min. čistost elucije LF: 85% (IEX-HPLC); prevodnost: 45-75 mS/cm	min. čistost zgoščine LF: 95% (IEX-HPLC)	>50x oz. >10%wt vsebnost LF oz. celokupnih proteinov	0.3 – 0.5 mS/cm	6 -7

Po optimiranem postopku diafiltracije smo pripravili še dve zgoščini LF (**Tabela 2**), pripravljeni z združevanjem več predhodno diafiltriranih zgoščin LF, pri čemer smo dosegli dovolj nizko prevodnost koncentrata, visoko vsebnost LF ter visoko relativno čistost. Sušenje v FBDS komori je bilo v obeh primerih uspešno in dalo zadovoljiv rezultat.

Tabela 2. Zgoščini LF, ki smo ju pripravili po optimiranem postopku diafiltracije. Zgoščini sta bili pripravljeni po združenju več predhodno diafiltriranih LF izolatov, ki smo jih še dodatno diafiltrirali.

LF Lot	**Začetni volumen (L)**	**Končni volumen (L)**	**pH zgoščine**	**Končna prevodnost (mS/cm)**	**Masa LF (g) v zgoščini**	**Relativna čistota (area%)**
21228SKLF	9	1.224	6.55	0.279	239	99.1
21295SKLF	6.9	2.89	6.25	0.687	393.6	95.6

Glede BLG koncentrata, smo bili za doseganje zadovoljivega rezultata pri sušenju pozorni le na doseganje nizke prevodnosti in dovolj velike vsebnosti proteinov, medtem ko se je izkazalo, da LPO zaradi svojih značilnosti ne dovoljuje priprave ustreznega koncentrata visoke čistosti. Izkazalo se je namreč, da se viskoznost LPO koncentrata kljub nizki vsebnost skupnih proteinov (<5%) zelo poviša, kar pa onemogoča dodatno diafiltracijo in učinkovito sušenje v FBDS komori.

Zahvala
Raziskava je bila sofinancirana s projektom LIFE for Acid Whey (št. pogodbe LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (št. pogodbe OP20.03521) Operativnega programom EKP 2014 – 2020.

Optimization of diafiltration and concentration of protein elutions as preparation for drying

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Abstract
For the purpose of drying in fluidized bed drying chamber (FBDS) and achieving the target LF content in the final product (capsule), we optimized the step of diafiltration and concentration of protein elutions of lactoferrin (LF), lactoperoxidase (LPO) and protein fraction rich by betalactoglobulin, alphalactalbumin and LF (BLG). For diafiltration we used tangential filtration using hollow fiber membrane. In order to achieve higher drying efficiency in the FBDS chamber and a stable drying process, it was necessary to achieve a sufficiently high content of protein product in the protein concentrate, a sufficiently high purity, especially for LF, and low content of salts in all three protein concentrates used for drying tests. From the data obtained trough different physicochemical analysis (water content, dry matter that decomposes into volatile products, non-volatile dry matter, LF purity and concentration, conductivity of protein concentrate, and pH) we found that the major factors that influence drying result in FBDS chamber are content of non-volatile solids, dry matter, protein concentrate conductivity, and LF purity and its concentration in the concentrate. Satisfactory results of optimized diafiltration were achieved in the case of LF and BLG, while in the case of LPO concentrate, due to its high viscosity and despite low protein content, we did not achieve a suitable composition that would allow additional concentration and later stable drying in the chamber.

Keywords: lactoferrin, lactoperoxidase, diafiltration, drying, FBDS chamber

Introduction
The purpose of protein elution fractions (LF/LPO/BLG) diafiltration is to remove salts and other whey proteins present from the protein product concentrate. By using a membrane with an appropriate pore size (50 kDa - LF/LPO and 10 kDa - BLG), addition of water to the solution on retentate side, LF/LPO/BLG is retained on the retentate side, while water with dissolved salts flows through to permeate side of the membrane. For diafiltration we used tangential filtration using hollow fiber membranes from Watersep (mini-Bioproducer24). In order to achieve higher drying efficiency in the FBDS chamber and a stable drying process, it was necessary to achieve a sufficiently high content of protein product in the protein concentrate, a sufficiently high purity, especially for LF, and low content of salts in all three protein concentrates used for drying tests. We followed various physicochemical properties of protein concentrates, such as: water content, dry matter that decomposes into volatile products, non-volatile dry matter, concentration and purity of LF, total proteins, conductivity of concentrate and pH. After reviewing the data we determined the key parameters that must be met for the drying process in the FBDS chamber in order to achieve high efficiency, repeatability of the process and at the same time that the target content (LF) is achieved in the dry product.

Results
The results obtained by conductivity measurements and thermo gravimetric analyzer (TGA, **Figure 1**) showed that some LF concentrates after diafiltration still contain a significant amount of non-volatile substances, which is mainly due to unsatisfactory removal of salt (NaCl) and is also reflected in higher conductivity. By prolonging the diafiltration process, we tried and also achieved further reduction of salt content and increase the content of volatile substances (proteins). We need to emphasize that the proteins also contribute to the conductivity of the protein isolate concentrate, so prolonging diafiltration does not significantly reduce it, but it nevertheless indicates a further reduction in salt content (**Figure 1**). The results not presented in the summary also showed that the salt content and the presence of unwanted proteins (lower purity) in the case of LF concentrates negatively affected the dose in the final product. To achieve the targeted dose and high drying efficiency, a sufficiently high protein content (>10 wt%) and high LF purity (>95%) must be achieved. Using preliminary results, we determined the parameters that should be characteristic to concentrated LF isolate and the limit values that LF concentrate must reach after diafiltration process before drying in the FBDS chamber (**Table 1**).

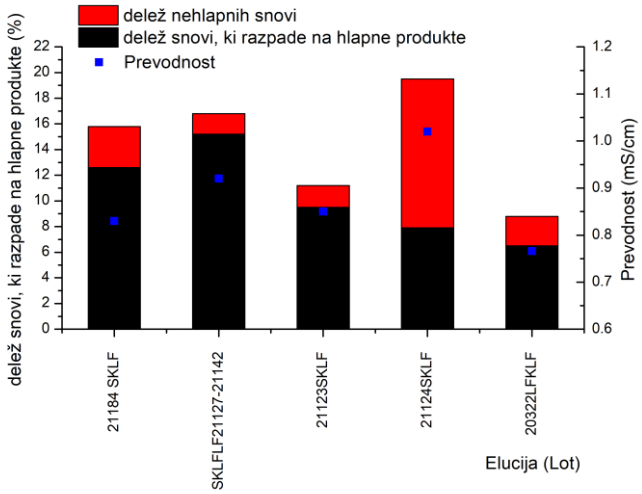

Figure 1. Content of volatile (proteins) and non-volatile products (salts) determined by TGA analysis and the conductivity of the analyzed LF concentrates.

Table 1: Parameters of LF isolate and determined limit values, which should be met for final LF concentrate before drying in FBDS.

Protein isolate	**Final protein concentrate after diafiltration**			
EL3	KLF	Concentration factor KLF	Conductivity of KLF concentrate	pH
Elution min. purity LF: 85% (IEX-HPLC); Conductivity: 45-75 mS/cm	min. purity of LF concentrate: 95% (IEX-HPLC)	>50x or >10%wt of LF or total protein	0.3 – 0.5 mS/cm	6 -7

After optimized diafiltration process, two more LF concentrates were prepared (**Table 2**). They were prepared by diafiltration of combined several pre-filtered LF concentrates, where a sufficiently low concentrate conductivity, high LF content and high relative purity were achieved. Drying of these two concentrates in the FBDS chamber was successful and gave a satisfactory result.

Table 2. LF concentrates prepared by an optimized diafiltration process. They were prepared by combining several pre-concentrated LF isolates, which were further diafiltered.

LF Lot	**Starting volume (L)**	**Final volume (L)**	**pH**	**Final conductivity (mS/cm)**	**LF Mass (g) in concentrate**	**Relative purity (area%)**
21228SKLF	9	1.224	6.55	0.279	239	99.1
21295SKLF	6.9	2.89	6.25	0.687	393.6	95.6

In order to achieve satisfactory results for BLG drying, we focused to concentrate low conductivity and sufficiently high protein content. For LPO it turned out that, due to its characteristics, it does not allow to prepare a suitable high purity concentrate. Namely, the viscosity of LPO increases significantly despite low content of total proteins (<5%), which prevents additional diafiltration and efficient drying in FBDS.

Acknowledgements
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Filtracija in diafiltracija sirotke - optimizacija masnega izkoristka za laktoferin in laktoperoksidazo

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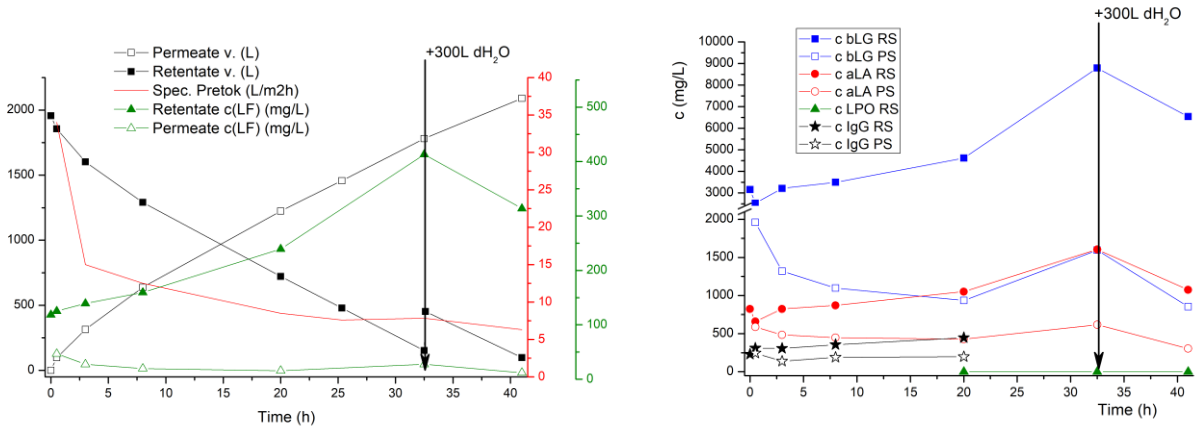
Povzetek
Pred kromatografsko izolacijo laktoferina (LF) in laktoperoksidaze (LPO) na kromatografski koloni je zaradi zaščite same kolone in njenega stabilnega delovanja potrebno sirotko prefiltrirati. Za izvedbo izolacij na pilotnem nivoju smo zato redno izvajali tudi filtracijo sirotke na pilotnem filtracijskem sistemu (JIUWU HI-TECH) in spremljali masno prehodnost sirotkinih proteinov (LF, LPO, IgG, aLA in bLG). Izkazalo se je, da na retentatni strani v večji meri zastajajo LF, LPO in IgG, medtem ko manjši proteini (aLA in bLG) lažje prehajajo membrano. To vodi do znižane koncentracije LF in LPO v permeatu po opravljeni filtraciji sirotke, kar se kaže v nižjem masnem izkoristku za LF in LPO. Z namenom dviga izkoristka filtracije smo opravili več primerjalnih filtracij in diafiltracij kisle in sladke sirotke, pri čemer smo vrednotili vpliv koncentracijskega faktorja ter v primeru diafiltracije, vpliv dodatka prečiščene vode, ki jo med filtracijo dodajamo na retentano stran, na masni izkoristek. Kot glavni zaključek lahko izpostavimo, da med masnimi izkoristki filtracije ali diafiltracije ni bilo bistvenih razlik. Izkazalo se je, da ima na izkoristek nekoliko večji vpliv koncentracijski faktor filtracije, pri čemer se z njegovim večanjem dviga tudi sam masni izkoristek postopka.

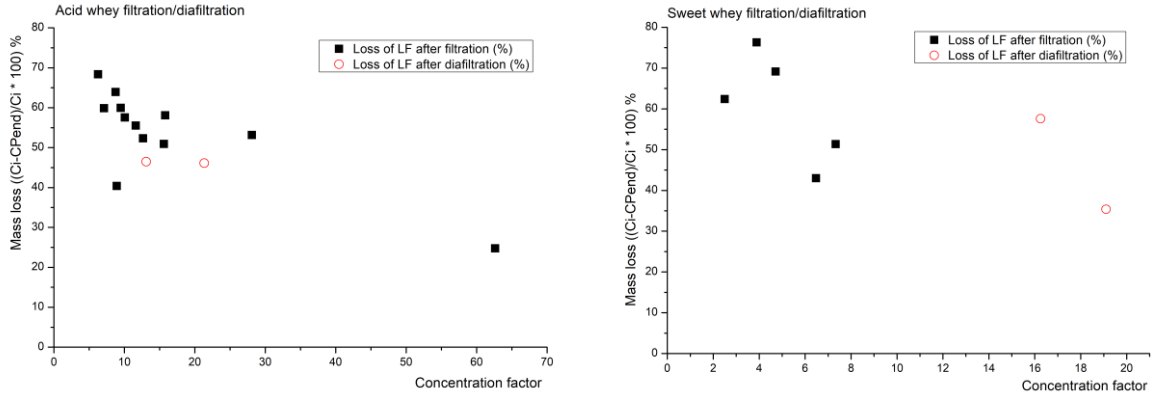
Ključne besede:filtracija, diafiltracija, sirotka, masni izkoristek, laktoferin, laktoperoksidaza

Uvod
Za potrebe kromatografske izolacije proteinskih izolatov (LF/LPO) z uporabo CIM kromatografske kolone (Bia Separations, Sartorius) iz sirotke, je le-to potrebno predhodno filtrirati. S filtracijo preko filtra s porami ustrezne velikosti, se iz sirotke odstrani velika večina mikroorganizmov, večji proteinski agregati in mlečna maščoba. S tem se prepreči zamašitev kromatografske kolone (2 µm pore) in tako ohrani njeno stabilno in ustrezno delovanje. Stranski pojav filtracije sirotke je, kljub relativno velikim poram filtra 0.5 µm (JIUWU HI-TECH, membrana: CMF19040, ne-gradientna), delno zadrževanje ciljnih proteinov (LF in LPO) na retentatni strani filtra in s tem onemogočena razpoložljivost za kromatografsko izolacijo. Z namenom povečanja masnega pretoka omenjenih proteinov preko membrane in povečanja masnega izkoristka filtracije, smo testirali različne pristope filtracije in diafiltracije sirotke. Z uporabo različnih analitskih tehnik smo sledili koncentraciji sirotkinih proteinov ter ovrednotili oba pristopa.

Rezultati
Izvedli smo dvanajst filtracij in dve diafiltraciji kisle ter pet filtracij in dve diafiltraciji sladke sirotke. Vse filtracije in diafiltracije sirotke so bile opravljene pri enakih nastavitvah filtracijske naprave in v primerljivem temperaturnem območju (5-8°C). Na **Sliki 1** je predstavljen primer spremljanja različnih parametrov med posamezno filtracijo, poleg katerih smo spremljali še druge, ki v prispevku niso prikazani. Izkazalo se je, da v primeru filtracije na retentatni strani v večji meri zastajajo LF, LPO in imunoglobulin G (IgG), medtem ko manjši proteini alfa-laktalbumin (aLA) in beta-laktoglobulin (bLG) lažje prehajajo membrano (Heidebrecht, H. J., Kulozik, U., 2019). To vodi do znižane koncentracije LF in LPO v permeatu po opravljeni filtraciji sirotke. Z dodajanjem prečiščene vode v zadnji fazi filtracije smo poizkusili masni izkoristek filtracije za LF in LPO dvigniti. Spremljanje masnega toka sirotkinih proteinov, predvsem LF, je pokazalo, da med obema pristopoma k filtraciji sirotke ni razlike. Izkazalo se je celo, da na sam masni izkoristek LF (**Slika 2**) vpliva predvsem koncentracijski faktor, tj. razmerje med končnima volumnoma permeata in retentata in tako višje doseženo razmerje vodi v večji izkoristek. Zanemarljiva razlika med filtracijo in diafiltracijo sirotke na omenjenem filtrskem sistemu in pomembnejši vpliv koncentracijskega faktorja na izkoristek lahko povezujemo s pojavom sekundarne plasti na površini ne-gradientne keramične membrane. Ta nastane med filtracijo in je sestavljena iz mešanice mikroorganizmov, maščob, proteinskih agregatov in netopnih soli (Piry A. et. al, 2012). Le-ta dodatno omeji prehod sirotkinih proteinov preko membrane in predstavlja sekundarno membrano, ki se jo zaradi svoje nepredvidljivosti težko nadzira. Z namenom dodatne optimizacije filtracije smo zato tekom projekta izvedli še poskusne filtracije na drugih tipih membran, ki zaradi gradienta por omogočajo bolj nadzorovano filtracijo sirotke.



Slika 1: Primer spremljanja specifičnega pretoka in koncentracij sirotkinih proteinov med diafiltracijo sladke sirotke. Slika je tudi splošen primer za vse izvedene eksperimente filtracije/diafiltracije sirotke, le da se v primeru filtracije na retentatno stran ni dodalo prečiščene vode kot je sicer prikazano.



Slika 2: Masne izgube LF tekom filtracije/diafiltracije glede na vhodno maso LF v sirotki v odvisnosti od koncentracijskega faktorja filtracije in tipa filtracijskega procesa.

Zahvala

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Literatura

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PiryA.,HeinoA., Kühnl W.,GreinT., RippergerS., and Kulozik U. (2012). Effectof membrane length, membrane resistance, and filtration conditions on the fractionation of milk proteins by microfiltration. J. Dairy Sci. 95 :1590–1602

Whey filtration and diafiltration – optimization of mass yield for lactoferrin and lactoperoxidase

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Abstract
Prior to chromatographic isolation of lactoferrin (LF) and lactoperoxidase (LPO) on the chromatographic column, whey must be filtered to protect the column and its functionality. Therefore, in order to perform isolations at the pilot level, we also regularly performed whey filtration on the pilot filtration system (JIUWU HI-TECH) and monitored the mass transfer of whey proteins (LF, LPO, IgG, aLA and bLG). It was observed that LF, LPO and immunoglobulin G (IgG) had been retained on the retentate side to a greater extent, while smaller proteins, like alpha-lactalbumin (aLA) and beta-lactoglobulin (bLG), cross the membrane more easily. This leads to a reduced concentration of LF and LPO in the permeate whey, which is reflected in lower mass yield for LF and LPO. In order to increase the filtration efficiency, we performed several comparative filtrations and diafiltrations of acid and sweet whey, evaluating the influence of the concentration factor on mass recovery and in the case of diafiltration, the influence of purified water added to the retentate side on mass recovery. As the main conclusion, we can point out that there were no significant differences in recovery efficiency between filtration or diafiltration processes. It turned out that the concentration factor of filtration has a slightly greater influence on the efficiency, and with its increase, the mass efficiency of the process itself increases.

Keywords:filtration, diafiltration, whey, mass yield, lactoferrin, lactoperoxidase

Introduction
For the purpose of chromatographic isolation of whey proteins (LF/LPO) using CIM chromatographic column (Bia Separations, Sartorius), the whey must be pre-filtered. Filtration of whey through a filter with pores of the appropriate size removes the majority of microorganisms, larger protein aggregates and milk fat. This prevents clogging of the chromatographic column (2 µm pores) and thus maintains its stable and proper functionality. The side effect of whey filtration was, despite the relatively large filter pores of 0.5 µm (JIUWU HI-TECH, membrane: CMF19040, non-gradient), partial retention of target proteins (LF and LPO) on the retentate side of the filter, which were consequently not available for chromatographic isolation. In an attempt to increase the mass transfer of these proteins through the membrane and consequently increase the mass efficiency of filtration, we tested different approaches to filtration and diafiltration of whey. Using various analytical techniques, we monitored the concentration of whey proteins and evaluated both approaches.

Results
Twelve filtrations and two diafiltrations of acid and five filtrations and two diafiltrations of sweet whey were conducted. All experiments were performed at the same filtration conditions and in a comparable temperature range (5-8°C). **Figure 1** shows an example of monitoring various parameters during each filtration, while some we also monitored are not shown in the paper. It turned out that LF, LPO and IgG are retained toa greater degree on the retentate side, while smaller proteins (aLA and bLG) cross the membrane more easily (Heidebrecht, H. J., Kulozik, U., 2019). This leads to a reduced concentration of LF and LPO in the permeate after filtration of the whey. By adding purified water in the last stage of filtration, we tried to increase the mass yield of filtration for LF and LPO. Monitoring the mass flow of whey proteins, especially LF, showed no difference between the two approaches, filtration and diafiltration. It has even been shown that the concentration factor mainly influences the mass efficiency of LF (**Figure 2**), i.e. the ratio between the final volumes of permeate and retentate, so the higher ratio leads to higher efficiency. A negligible difference between the filtration and diafiltration of whey on the said filter system and the more significant influence of the concentration factor on the recovery efficiency can be related to the formation of a secondary layer on the surface of the non-gradient ceramic membrane. It is formed during filtration and consists of a mixture of microorganisms, fats, protein aggregates and insoluble salts (Piry A. et al., 2012). This further restricts the passage of whey proteins across the membrane and thus represents a secondary membrane, which, however, is difficult to control due to its unpredictable formation and characteristics. In order to further optimize filtration, we, therefore, performed experimental filtrations on other types of membranes during the project, which, due to the pore gradient, enable more controlled whey filtration.

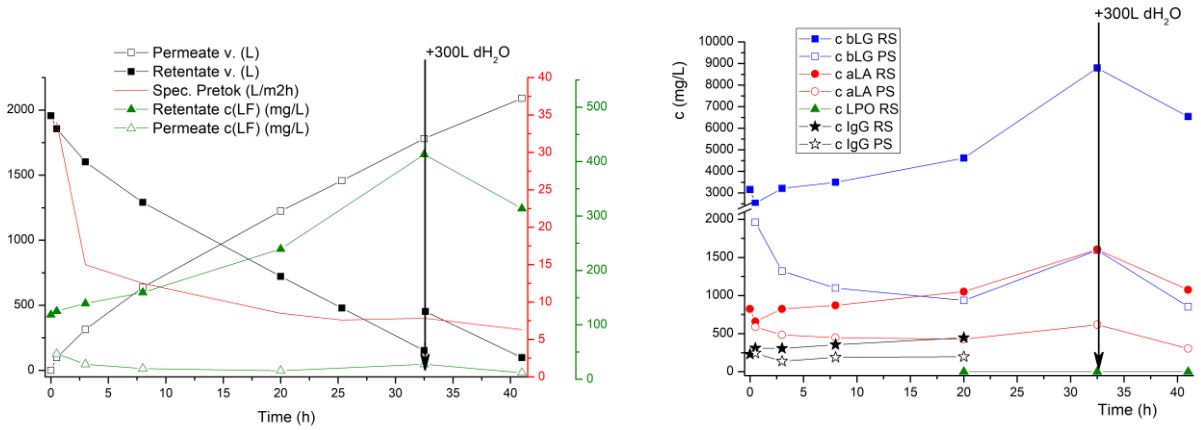
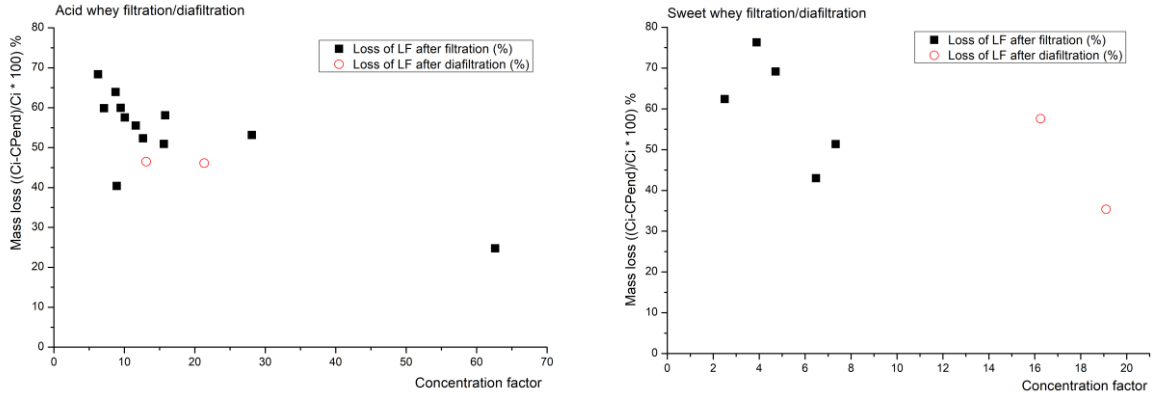


Figure 1: Example of monitoring specific flow and whey protein concentrations during sweet whey diafiltration. The figure is also a general example for all performed whey filtration / diafiltration experiments, except that in thecase of filtration, no purified water was added to the retentate side.



Slika 2: Mass loss during filtration / diafiltration in relation to the input mass of LF in whey, depending on the concentration factor of filtration and the type of filtration process.

Acknowledgements
This research was co-funded by the project LAKTIKA - Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements (OP20.03521) in the frame of the Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020 and the project LIFE for Acid Whey - Reuse of waste acid whey for the extraction of bioactive proteins with high added value (LIFE16 ENV/SI/000335) of the European financial instrument LIFE. We thank Celeia Dairy for the supply of whey.

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Primerjava učinkovitosti selektivne precipitacije α-laktalbumina iz različnih virov sirotke

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Povzetek
V zadnjem času, predvsem zaradi povečanega povpraševanja po grškem tipu jogurta, narašča količina proizvedene kisle sirotke, ki je stranski produkt mlekarske industrije. Podobno kot sladka sirotka, ki nastaja pri proizvodnji sira, ta v mlekarnah največkrat predstavlja odpadek, ki je zaradi biološke in kemijske potrebe po kisiku obremenjujoč za okolje, vendar ponuja številne možnosti za uporabo predvsem zaradi visoke dodane vrednosti prisotnih beljakovin, bioaktivnih fosfolipidov in organskih kislin. α-laktalbumin predstavlja približno 20% celokupnih beljakovin v kisli sirotki in je zanimiv zaradi ugodnega vpliva na zdravje ljudi. Iz kisle in sladke sirotke smo iz nevezane frakcije sirotke po izolaciji laktoferina z ionsko-izmenjevalno kromatografijo na monolitni koloni želeli s selektivno precipitacijo izolirati α-laktalbumin. Po izvedbi poskusov izolacije in optimizaciji pogojev reakcije na laboratorijski skali, smo postopek izvedli tudi na pilotnem nivoju.

Ključne besede: kisla sirotka, sirotkine beljakovine, bioaktivne molekule, α-laktalbumin, vpliv na zdravje

Uvod
Celovita izraba sirotke je ključnega pomena za zmanjšanje vpliva mlekarske industrije na okolje. Hkrati pa je bogat vir biološko aktivnih komponent, ki so zanimive z vidika vpliva na prehrano in zdravje ljudi. Ena od beljakovin z visoko dodano vrednostjo je α-laktalbumin (Rocha-Mendoza et al., 2021). Ta ima ugodno aminokislinsko sestavo, saj vsebuje esencialne aminokilsine cistein, metionin, lizin, triptofan in leucin. Z oleinsko kislino tvori komplekse z antikarcinogenim učinkom (Rammer et al., 2010), v otroških formulah ugodno vpliva na imunski odziv in povečuje absorbcijo hranil, predvsem železa in cinka. Pri proteolizi α-laktalbumina v prebavilih nastajajo bioaktivni peptidi s protimikrobnimi in prebiotičnimi lastnostmi (Layman et al., 2018). Za izolacijo α-laktalbumina iz kisle ali sladke sirotke je mogoče uporabiti več metod, kot so kationsko izmenjevalna kromatografija ali selektivna precipitacija pri nizkem pH.

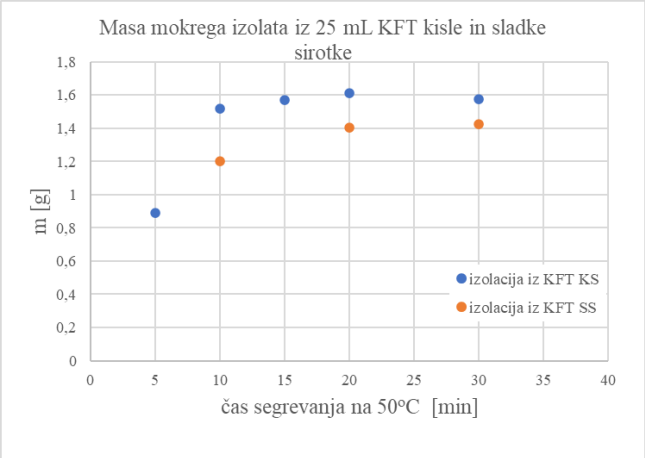
Selektivna precipitacija α-laktalbumina iz zgoščenih nevezanih frakcije sirotke
Za surovino za izolacijo smo uporabili nevezano frakcijo (FT) kisle (KS) in sladke sirotke (SS) po postopku izolacije laktoferina in laktoperoksidaze na CIMmultus monolitni koloni za kationsko-izmenjevalno kromatografijo. Zaradi smotrne izrabe reagentov, večje celokupne koncentracije beljakovin in lažje manipulacije zaradi manjše prostornine, smo FT predhodno zgostili do 10% prostornine z ultrafiltracijo skozi membrano z velikostjo por 10 kDa in pridobili koncentrat FT (KFT). Po optimiziranem postopku, povzetem po Haller in Kulozik (2020) in eksperimentih, opravljenih na Inštitutu za mlekarstvo in probiotike, smo v raztopini z dodatkom citrata in citronske kisline vrednost pH znižali na 3,5 in reakcijsko mešanico segreli do 50°C. Reakcijo smo prekinili s hitrim ohlajanjem vzorcev in s centrifugiranjem izločili oborino.
Z namenom predstavitve možnosti za izvedbo postopka pri optimiziranih pogojih na pilotnem nivoju, smo izvedli še eksperiment z večjo količino kisle sirotke KFT, pri čemer smo za ločbo oborine uporabili tubularno centrifugo.

Rezultati
Iz vsebnosti α-laktalbumina (α-LA) in β-laktoglobulina (β-LG) v izvornem KFT smo izračunali teoretično razpoložljivo maso obeh beljakovin iz 25-mL alikvota KFT, ki smo ga uporabili za obarjanje. Na podlagi rezultatov vsebnosti posameznih beljakovin v vzorcih supernatantov in raztopljenih precipitatov smo naredili masno bilanco za ta dva proteina. Med eksperimentom smo spremljali maso usedline po centrifugiranju. Pričakovali smo, da je premo sorazmerna količini oborjenega α-laktalbumina (Slika 1). Iz tega grafa je razvidno, da se od 15. minute dalje količina oborine več bistveno ne spreminja. Izkazalo se je, da smo iz kisle sirotke pridobili več α-laktalbumina kot iz sladke sirotke. Tudi čistost surovega izolata iz kisle sirotke (73 %) je bila boljša od čistosti izolata iz sladke sirotke (24%). V surovem izolatu so prisotni še reagenti, ki povečajo količino suhe snovi in je delež beljakovin glede na delež suhe snovi v primeru obravnave kisle sirotke 49,6%, v primeru sladke pa 53,5%. S spiranjem surovega precipitata z vodo pa smo delež beljakovin glede na suho snov v obeh primerih bistveno povečali (89,1% pri kisli sirotki in 85,9% pri sladki sirotki). Tako smo s postopkom ultrafiltracije nevezane frakcije sirotke, selektivne precipitacije in enega koraka spiranja surovega

precipitata pridobili pretežno beljakovinsko frakcijo, bogato z α-laktalbuminom, v kateri je bil delež β-laktoglobulina pod mejo detekcije.

Tabela 1: Količina izoliranega α-laktalbumina in β-laktoglobulina iz 25 mL KFT.

		precipitat		supernatant				
	čas [min]	m β-LG [mg]	m α-LA [mg]	m β-LG [mg]	m α-LA [mg]	recovery β-LG	recovery α-LA	Kromaotgrafska čistost α-LA v precipitatu
KFT KS	10	16,6	91	405	10,3	100,0%	85,1%	67,9%
m(α-LA)= 119,00 mg	20	16	85,4	401,6	8,2	99,0%	78,7%	71,2%
m(β-LG) = 421,75mg	30	15,7	87,6	401,5	6,1	98,9%	78,7%	72,9%
KFT SS	10	11,54	18,71	253,25	2,95	105,8%	112,5%	24,3%
m(α-LA)= 19,25 mg	20	13,06	20,39	253,69	2,11	106,6%	116,9%	24,8%
m(β-LG) = 250,25mg	30	12,37	21,21	256,76	2,43	107,5%	122,8%	24,7%



Slika 1: Masa izolata po selektivni precipitaciji α-laktalbumina.

Zaključki

S postopkom ultrafiltracije nevezane frakcije sirotke, selektivne precipitacije in enega koraka spiranja surovega precipitata smo pridobili pretežno beljakovinsko frakcijo, bogato z α-laktalbuminom, v kateri je bil delež β-laktoglobulina pod mejo detekcije. V primeru izolacije α-laktalbumina iz kisle sirotke, je bila kromatografska čistost v netretiranem izolatu skoraj 73%, v primeru izolacije iz sladke sirotke pa le 24%. V poskusu, izvedenem na pilotnem nivoju, smo po precipitaciji in ločbi izolata na tubularni centrifugi pridobili pastozen produkt, kjer je bila kromatografska čistost α-laktalbumina 78%.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey - Ponovna uporaba odpadne kisle sirotke za ekstrakcijo bioaktivnih beljakovin z visoko dodano vrednostjo (LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA - Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil (OP20.03521) Operativnega programom za izvajanje evropske kohezijske politike v obdobju 2014 – 2020.

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Comparison of the efficiency of selective precipitation of α-lactalbumin from different whey sources

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Abstract
Due to the increasing demand for Greek type yogurt, the amount of acid whey produced has recently increased. Like the sweet whey produced during cheese making, it is the most common waste in dairies and is a burden on the environment due to its high chemical and biological oxygen demand. However, it offers many possibilities for use, mainly since it contains proteins with high added value, bioactive phospholipids, and organic acids. α-lactalbumin accounts for about 20% of total acid whey proteins and is of interest since the beneficial effects on human health have been reported. The aim of our research was to isolate α-lactalbumin from acid and sweet whey by selective precipitation from the unbound fraction after isolation of lactoferrin by ion-exchange chromatography on a monolithic column. After performing isolation experiments and optimising reaction conditions on a laboratory scale, the procedure was also performed on a small production scale.

Keywords: acid whey, whey proteins, bioactive molecules, α-lactalbumin, health benefits

Introduction
The efficient use of whey is crucial to reduce the environmental impact of the dairy industry. At the same time, it is a rich source of biologically active compounds that can significantly affect human health and nutrition. One of the proteins with high added value is α-lactalbumin. It has a favorable amino acid composition, as it contains the essential amino acids cysteine, methionine, lysine, tryptophan, and leucine. It forms complexes with oleic acid, which has been reported to have an anticarcinogenic effect (Rammer et al., 2010). It is added to infant formula since it impacts the immune response and absorption of nutrients, especially iron and zinc. Proteolysis of α-lactalbumin in the gastrointestinal tract produces bioactive peptides with antimicrobial and prebiotic properties (Layman et al., 2018). Various methods can be used to isolate α-lactalbumin from acidic or sweet whey, such as cation-exchange chromatography or selective precipitation at low pH.

Selective precipitation of α-lactalbumin from concentrated flow-through fractions of whey
The flow-through fraction (FT) of acid whey (AW) and sweet whey (SW) after chromatographic lactoferrin and lactoperoxidase isolation procedure on a CIMmultus monolithic column for cation exchange chromatography, was used as starting material for the isolation of α-lactalbumin. To minimize the use of reagents, increase total protein concentration, and facilitate handling, FT was pre-concentrated to 10% of the starting volume by ultrafiltration on a membrane with a pore size of 10 kDa, and FT concentrate was prepared (CFT). Following an optimized procedure based on Haller and Kulozik (2020) and experiments performed at the Institute of Dairy Science and Probiotics, the pH value was lowered to 3,5 by adding citrate and citric acid, and the reaction mixture was heated to 50 ° C. The reaction was quenched by rapid cooling, and the precipitate was removed from the mixture by centrifugation. To demonstrate the possibility of carrying out the process under optimized conditions at a small production level, we also experimented with a larger amount of CFT from acid whey, using a tubular centrifuge to separate the precipitate.

Results
From the content of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) in the CFTs, we calculated the available mass of both proteins from an aliquot of 25,0 mL of CFT, used for precipitation. We observed the distribution of both proteins between the supernatant and the precipitate fractions. During the experiment, we monitored the mass of the precipitate after centrifugation, which was expected to be directly proportional to the amount of precipitated α-lactalbumin (Figure 1). It also shows that the mass of the precipitate does not change significantly after 15 minutes of reaction duration. The amount of α-lactalbumin obtained from AW CFT was found to be significantly higher than from SW CFT. At the same time, the purity of the crude isolate is higher and is about 73% in the case of acid whey and only 24% in the case of sweet whey. Reagents remaining in the precipitate increase the amount of dry matter in the isolate. Proteins account for 49,6% of the dry matter in the acid whey isolate and 53,5% in the sweet whey isolate. After one step of washing the crude precipitate with distilled water, the protein content in dry matter was significantly increased in both cases (89,1% for acid whey and 85,9% for sweet whey). Thus, the process of ultrafiltration of the flow-through fraction, selective

precipitation, and one step of rinsing the crude precipitate with distilled water yielded a predominantly α-lactalbumin-rich protein fraction of whey in which the β-lactoglobulin content was below the detection limit.

Table 1: The amount of α-lactalbumin and β-lactoglobulin from 25 mL of CFT.

		precipitat		supernatant				
	time [min]	m β-LG [mg]	m α-LA [mg]	m β-LG [mg]	m α-LA [mg]	recovery β-LG	recovery α-LA	Purity of α-LA in isolate
CFT AW	10	16,6	91	405	10,3	100,0%	85,1%	67,9%
m(α-LA)= 119,00 mg	20	16	85,4	401,6	8,2	99,0%	78,7%	71,2%
m(β-LG) = 421,75mg	30	15,7	87,6	401,5	6,1	98,9%	78,7%	72,9%
CFT SW	10	11,54	18,71	253,25	2,95	105,8%	112,5%	24,3%
m(α-LA)= 19,25 mg	20	13,06	20,39	253,69	2,11	106,6%	116,9%	24,8%
m(β-LG) = 250,25mg	30	12,37	21,21	256,76	2,43	107,5%	122,8%	24,7%

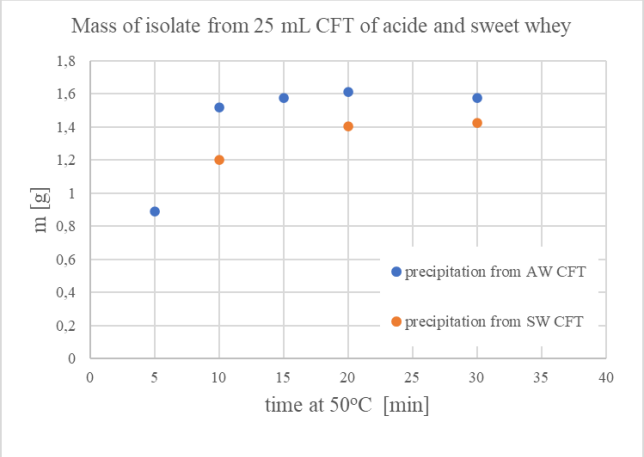

Figure 1: Mass of the isolate after selective precipitation of α-lactalbumin.

Conclusion

By the process of ultrafiltration of flow through whey fractions, selective precipitation and one step of washing the crude precipitate, we obtained an α-lactalbumin-rich protein fraction in which the β-lactoglobulin content was below the detection limit. When α-lactalbumin was isolated from acid whey concentrate, the chromatographic purity was 72% in the untreated isolate and only 24% when isolated from sweet whey. In a pilot-scale experiment, after precipitation and separation of the isolate in a tubular centrifuge, a pasty product was obtained in which the chromatographic purity of α-lactalbumin was 78%.

Acknowledgements

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Spremljanje rasti laktokokov in tvorbe nizina z uporabo različnih virov sirotke kot gojišča

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Povzetek
Nevezano frakcijo sirotke po izolaciji laktoferina in laktoperoksidaze smo želeli izkoristiti kot medij za gojenje mlečnokisllinskih bakterij in produkcijo nizina, ki je zanimiv zaradi svojih protimikrobnih lastnosti (Malvido s sod., 2019). V ta namen so bili izdelani stekleni bioreaktorji in prirejena bioreaktorska tehnika, ki omogoča gojenje v treh litrih gojišča. Za kultivacijo *Lactococcus lactis* subsp. *lactis* smo uporabili več različnih frakcij sirotke z dodanimi hranili in permeat izrabljenega gojišča po gojenju kefirnih zrn. Med procesom fermentacije, ki je potekala pri različnih pogojih, smo spremljali porabo laktoze, koncentracijo nastale mlečne kisline in bakteriocinsko aktivnost v bioreaktorski gošči. Po gojenju smo s centrifugiranjem in filtriranjem (mikrofiltracija, ultrafiltracija) na laboratorijskem filtracijskem sistemu poskusili z iz gošče odstraniti nastalo biomaso in pridobiti frakcijo z visoko bakteriocinsko aktivnostjo.

Ključne besede: mlečnokislinska fermentacija, *Lactococcus lactis* subsp. *lactis,* bakteriocin, nizin, sirotka, laktoza, mlečna kislina

Uvod
Z namenom celostne izrabe sirotke po kromatografski izolaciji posameznih beljakovin, smo nevezano frakcijo sirotke (FT), ki vsebuje laktozo, preostanek beljakovin, ki se na kromatografski nosilec ne vežejo, minerale in mlečno kislino poskusili uporabiti kot gojišče za bioprocesno pridobivanje nizina. Glede na razultate raziskav na laboratorijski ravni, opravljenih na Inštitutu za mlekarstvo in probiotike, smo poskusili postopek kultivacije laktokokov prenesti na večji nivo, in sicer v bioreaktorje s prostornino 3L. Za gojišče smo uporabili permeat po ultrafiltraciji FT (velikost por 10 kDa). To smo imenovali deproteinizirana FT frakcija, saj je v njej prisotnih približno petkrat manj beljakovin, kot v neobdelanem FT. Pričakovali smo, da bo zaradi manjše celokupne vsebnosti beljakovin v gojišču, možnost izolacije ali koncentriranja nizina po fermentaciji lažja in da bi s tem lahko vplivali na čistost pridobljenega produkta. Permeat FT (PKFT) smo pripravili iz sladke (SS) in kisle sirotke (KS). Kasneje smo poskusili enak proces peljati še v izrabljenem gojišču po gojenju kefirnih zrn, ki je bil prefiltriran skozi membrano z velikostjo por 30 kDa, saj je koncentracija laktoze tudi po tej fermentaciji ostala precej visoka. V vseh primerih je bilo pripravljeno gojišče pasterizirano in dekantirano od nastale oborine, pH pa umerjen na 6,5. V večini eksperimentov je bil v gojišče dodan kvasni ekstrakt v koncentraciji 2,5%. Bioprocese smo vodili šaržno, in sicer z ali brez regulacije pH med fermentacijo.

Slika 1: Steklena bioreaktorska posoda z regulacijo pH vrednosti med fermantacijo (levo) in laboratorijski filtracijski sistem za mikro in ultrafiltracijo (desno).

Tabela 1: Poskusi gojenja laktokokov v PKFT frakcijah kisle in sladke sirotke in izrabljenega gojišča po gojenju kefirnih zrn.

GOJIŠČE	pH regulacija	ZAČETNI pH	KONČNI pH	MLEČNA KISLINA (g/100g)	LAKTOZA (g/100g)	BAKT.AKT. (BA/mL)
PKFT SS	ne	6,39	5,45	0,03	2,70	0
PKFT SS + KE	ne	6,36	4,41	0,20	2,45	51200
PKFT SS + KE	ne	6,26	4,38	0,20	2,64	102400
PKFT KS + KE	ne	6,5	4,6	0,84	3,08	102400
PKFT KS + KE	ne	6,5	4,6	0,85	3,17	102400
PKFT KS + KE	da	6,5	6,5	2,33	0,33	409600
PKFT KS + KE	da	6,5	6,5	2,37	0,10	409600
PIKG (KS)	da	6,4	6,5	1,51	1,95	102400
PIKG (KS)	da	6,4	6,5	1,82	1,12	102400

Rezultati

Potek fermentacije smo spremljali z vidika doseganja čim višje bakteriocinske aktivnosti v bioprocesni brozgi in čim boljše izrabe laktoze v gojišču. V predhodnih poskusih smo ugotovili, da dodatek kvasnega ekstrakta (KE) v koncentraciji 2,5% bistveno vpliva na kinetiko rasti in produkcijo bakteriocina. Podobno smo potrdili tudi v teh eksperimentih (Tabela 1). V primeru uporabe sladke sirotke je bila koncentracija mlečne kisline, proizvedene med fermentacijo, nizka (do 0,2 g/100g). Dodatek KE je bistveno spremenil bakteriocinsko aktivnost v bioprocesni gošči. Pri uporabi kisle sirotke je bila koncentracija mlečne kisline po fermentaciji bistveno večja kot pri sladki sirotki (0,85 g/100g), bakteriocinska aktivnost pa primerljiva.

Najboljše rezultate smo dobili pri uporabi PKFT KS z dodatkom KE in regulacijo pH med procesom fermentacije, kjer je bila na koncu procesa ugotovljena najvišja BA, količina preostale laktoze je bila minimalna (približno 0,3 g/100g), koncentracija mlečne kisline pa večja kot v ostalih poskusih (2,3 g/100g). Pri fermentaciji v permeatu izrabljenega gojišča po gojenju kefirnih zrn in regulacijo pH med fermentacijo, je bila bakteriocinska aktivnost primerljiva s tistimi, izmerjenimi v PKFT SS in KS z dodatkom KE brez regulacije.

Za izbrane bioprocesne gošče smo po končani fermentaciji skušali ločiti na več frakcij, pri čemer smo pričakovali, da bomo lahko pridobili frakcijo z izrazito povečano bakteriocinsko aktivnostjo in drugo frakcijo, kjer bi le ta bila bistveno zmanjšana. Najprej smo s centrifugiranjem iz gošče odstranili biomaso in oborjene beljakovine, kasneje pa še z mikro in ultrafiltracijo supernatanta na laboratorijski tangencialni filtraciji pridobili posamezne frakcije retentatov in permeatov. Uporabili smo 2 membranska modula proizvajalca WaterSep z velikostjo por 750 in 10 kDa. Pri tem smo pričakovali, da nizin ne bo kvantitativno prehajal por velikih 10 kDa in se bo v večji meri zadržal v retentatni frakciji po ultrafiltraciji, in da bo zato permeatna frakcija imela bistveno nižjo bakteriocinsko aktivnost, vendar je bilo razporejanje bakteriocina bistveno drugačno v primerjavi z ultrafiltracijo, narejeno z uporabo ultrafiltracijskih centrifugirk Amicon na manjši skali.

Zaključki

Vzpostavili smo bioreaktorski proces in določili pogoje za mlečnokislinsko fermentacijo v gojišču na osnovi kisle sirotke. Najboljše rezultate smo dobili v permeatu po ultrafiltraciji kisle sirotke, ki mu je bil dodan kvasni ekstrakt, med fermentacijo s startersko kulturo *Lactococcus lactis* subsp. *lactis* pa smo uravnavali vzdrževali pH. Tako smo dosegli najvišjo bakteriocinsko aktivnost, ki je posledica proizvedenega nizina. S procesi selektivnih filtracij pa še nismo uspeli pridobiti frakcije, kjer bi bil nizin skoncentriran in ločen od večine drugih sestavin gojišča.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey (št. pogodbe LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (št. pogodbe OP20.03521) Operativnega programom EKP 2014 – 2020.

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Monitoring lactococcal growth and nisin formation using different whey sources as cultivation medium

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Abstract
Flow-through fraction of whey after the isolation of lactoferrin and lactoperoxidase was used as a growth medium for the cultivation of lactic acid bacteria and the production of nisin, which is interesting because of its antimicrobial activity (Malvido et al., 2019). For this purpose, glass bioreactors were made and adapted for the fermentation conditions that enable cultivation in three liters of growth medium. For the cultivation of the *Lactococcus lactis* subsp. *lactis*, we used several whey fractions with added nutrients and permeate of spent growth medium after cultivation of kefir grains under different fermentation conditions. During the fermentation process, lactose consumption, lactic acid concentration, and antimicrobial activity in the bioreactor broth were monitored. After cultivation, we tried to remove the resulting biomass from the broth by centrifugation and filtration with a laboratory filtration system (microfiltration, ultrafiltration) and obtain a fraction with high antimicrobial activity.

Keywords: lactic acid fermentation, bacteriocin, whey, lactose, lactic acid

Introduction
In order to fully utilize whey after chromatographic isolation of individual proteins, we used the flow-through fraction of whey (FT), which contains lactose, residual proteins that do not bind to the chromatographic monolith, minerals, and lactic acid, as a medium for the biotechnological production of nisin. Based on the results of laboratory-level research conducted at the Institute of Dairy and Probiotics, we tried to transfer the process of lactococci cultivation to a higher level, namely to bioreactors with a volume of 3L. After FT ultrafiltration (pore size 10 kDa), the permeate was used for the growth medium. We called this the deproteinized FT fraction, as it contains about five times less proteins than the untreated FT. We expected that due to the lower total protein content in the medium, the possibility of isolating or concentrating of nisin after fermentation would be easier and that the purity of the product obtained might be affected. The permeate of FT (PCFT) was prepared from sweet (SW) and acid whey (AW). Later, we tried to carry out the same process in the spent growth medium left after the cultivation of kefir grains (SGMK), which was filtered on a membrane with a pore size of 30 kDa, since the lactose concentration remained relatively high even after this fermentation. In all cases, the prepared medium was pasteurized and decanted from the precipitate formed, and the pH was calibrated to 6.5. In most experiments, a yeast extract (YE) was added to the medium at a concentration of 2.5%. Bioprocesses were carried out in batch mode with or without pH regulation during fermentation.

Figure 1: Glass bioreactor vessel with pH adjustment during fermentation (left) and laboratory filtration system for micro and ultrafiltration (right).

Table 1: Experiments of lactococcal fermentation in PCFT fractions of acid and sweet whey and spent medium after cultivating kefir grains.

GROWTH MEDIUM	pH regulation	INITIAL pH	FINAL pH	LACTIC ACID (g/100g)	LACTOSE (g/100g)	ANTIMICROB. ACT. (BA/mL)
PCFT SW	no	6,39	5,45	0,03	2,70	0
PCFT SW + YE	no	6,36	4,41	0,20	2,45	51200
PCFT SW + YE	no	6,26	4,38	0,20	2,64	102400
PCFT AW + YE	no	6,5	4,6	0,84	3,08	102400
PCFT AW + YE	no	6,5	4,6	0,85	3,17	102400
PCFT AW + YE	yes	6,5	6,5	2,33	0,33	409600
PCFT AW + YE	yes	6,5	6,5	2,37	0,10	409600
SGMK (AW)	yes	6,4	6,5	1,51	1,95	102400
SGMK (AW)	yes	6,4	6,5	1,82	1,12	102400

Results

The course of fermentation was monitored in terms of detecting the highest bacteriocin activity in the bioprocess broth and the highest utilization of lactose in the medium. Previous experiments have shown that the addition of yeast extract (YE) at a concentration of 2.5% significantly affected the growth kinetics and production of bacteriocin, which was also confirmed in these experiments (Table 1). In the case of sweet whey, the lactic acid concentration produced during fermentation was low (up to 0.2 g / 100 g). The addition of YE significantly increased antimicrobial activity in the fermentation broth. When acid whey was used, the lactic acid concentration after fermentation was significantly higher than in sweet whey (0.85 g / 100 g), while the antimicrobial activity was comparable.

The best results were obtained using PCFT AW with the addition of YE and pH regulation during the fermentation process. Here, the highest BA was obtained at the end of the process, the amount of residual lactose was minimal (about 0.3 g / 100 g), and the lactic acid concentration was much higher (2.3 g / 100 g) compared to other experiments. In the case of fermentation in the permeate of the spent kefir grain cultivation medium and pH regulated during fermentation, BA was comparable to those values obtained in PKFT SW and AW with the addition of YE without regulation.

We tried to separate selected broths into several fractions to obtain a fraction with significantly increased BA and another fraction where it would be significantly reduced. First, biomass and precipitated proteins were removed from the broth by centrifugation. Later, individual fractions of retentates and permeates were obtained by microfiltration and ultrafiltration of the supernatant on a laboratory tangential filtration system. We used two membrane modules from WaterSep with pore sizes of 750 and 10 kDa. We expected that nisin would not quantitatively pass through the 10 kDa pores and would remain in the retentate fraction after ultrafiltration and that therefore the permeate fraction would have a significantly lower BA. Still, the distribution of bacteriocin was significantly different from that obtained by ultrafiltration with Amicon ultrafiltration centrifuges on a smaller scale.

Conclusions

We set up the biotechnological process and determined the conditions for lactic acid fermentation in a growth medium based on acid whey. We achieved the best results with the permeate of ultrafiltration of acid whey, to which yeast extract was added, and the pH was regulated during fermentation with the starter culture *Lactococcus lactis* subsp. *lactis*. In this way, the maximum antimicrobial activity, resulting from the production of nisin was achieved. However, selective filtration procedures have not yet allowed us to obtain a fraction in which the nisin is concentrated and separated from the bulk of the growth medium.

Acknowledgements

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Optimizacija gojenja kefirnih zrn v sirotki

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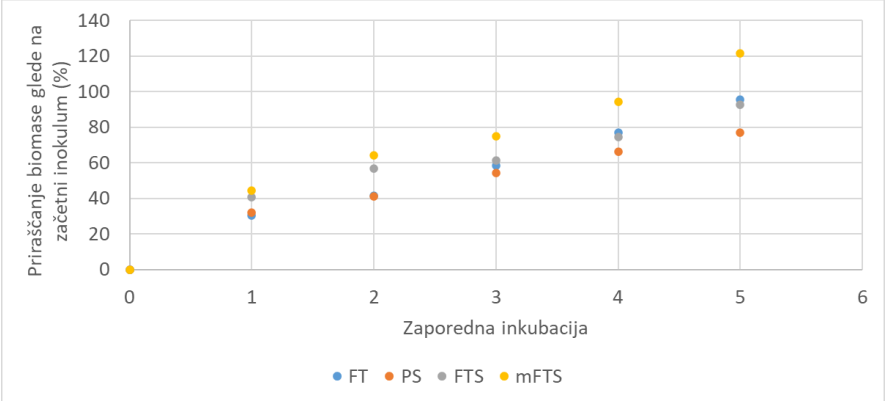
Povzetek
Optimizirali smo postopek gojenja kefirnih zrn v sirotki za doseganje visoke stopnje priraščanja z namenom predelave biomase kefirnih zrn in izrabljenega gojišča v uporabne produkte. Kot gojišča smo preizkusili različne frakcije kisle in sladke sirotke, ki preostanejo po procesu izolacije proteinov. Eksperimentalno smo določili optimalne pogoje gojenja kefirnih zrn v laboratorijskih pogojih. Preizkusili smo načine zamrzovanja in oživljanja zamrznjenih kefirnih zrn s ciljem optimalnega vzdrževanja matične kulture. Po optimizaciji gojenja kefirnih zrn na laboratorijskem nivoju, smo gojenje prenesli na pilotni nivo.

Ključne besede: kefirna zrna, fermentacija, pilotni nivo, kisla sirotka, sladka sirotka

Uvod
Kefirna zrna so kompleksni skupki mlečno kislinskih bakterij, kvasovk in včasih ocetno kislinskih bakterij v matriki iz polisaharidov in proteinov (Garotte in sod., 2010). Kot naravna starterska kultura v ustreznem gojišču vršijo fermentacijo, kjer razgrajujejo v gojišču prisotne sladkorje. Večinoma se kot gojišče uporablja mleko, preizkušena pa je bila tudi sirotka, predvsem sladka. Po selektivni izolaciji sirotkinih proteinov, ki smo jih izvajali v okviru projekta LIFE in LAKTIKA, je bila v ostankih sirotke prisotna še vsa laktoza in druge hranilne snovi, ki so bistvenega pomena za rast in razvoj mikroorganizmov. Zaradi vsebnosti številnih mikroorganizmov, metabolitov in raznolikih bioaktivnih snovi, ki nastajajo tekom fermentacije ter številnih pozitivnih vplivov na zdravje človeka, predstavljajo kefirna zrna in sirotka velik potencial v razvoju funkcionalnih živil in prehranskih dopolnil. Namen naše raziskave je bil optimizirati način gojenje kefirnih zrn za doseganje čim večje prirasti kefirne biomse na laboratorijskem in pilotnem nivoju z uporabo ostankov sirotke iz predhodnih postopkov predelave ter s tem tudi prispevati k zmanjšanju količin odpadne sirotke ter negativnih vplivov na okolje.

Optimizacija gojenja kefirnih zrn na laboratorijskem nivoju
V laboratoriju smo izvajali poskuse za opredelitev optimalnih pogojev rasti kefirnih zrn, načrtovali parametre za prenos gojenja v večje merilo in vzdrževali vitalno kefirno biomaso. Vplive na priraščanje biomse smo vrednotili z vidika načina priprave gojišča, začetnega pH, deleža inokuluma aktiviranih kefirnih zrn, trajanja in temperature inkubacije. Priraščanje kefirnih zrn smo spremljali v različnih frakcijah kisle in sladke sirotke, ki so preostajale v postopkih selektivne izolacije sirotkinih proteinov. Prirast biomase smo vrednotili z odcejanjem kefirnih zrn v dogovorjenem časovnem intervalu ter tehtanjem sveže mase zrn. Poteku fermentacije smo sledili z merjenjem pH in temperature gojišča. V poskusih smo uporabljali vitalna kefirna zrna. Za vitalna kefirna zrna smo opredelili tista, ki so imela >20 % dnevno prirast. Vitalnost kefirnih zrn smo dosegali z rednimi zaporednimi fermentacijami. Vitalna kefirna zrna, ki so dosegla primerno stopnjo priraščanja, smo v optimalnih pogojih in rednih intervalih shranjevali in oživljali z namenom vzdrževanja zadostne biomase za gojenje na laboratorijskem in pilotnem nivoju. Optimizirali smo tudi postopek oživljanja kefirnih zrn po zamrzovanju ter vrednotili stabilnost kefirnih zrn v zamrznjenem stanju z in brez krioprotektantov.

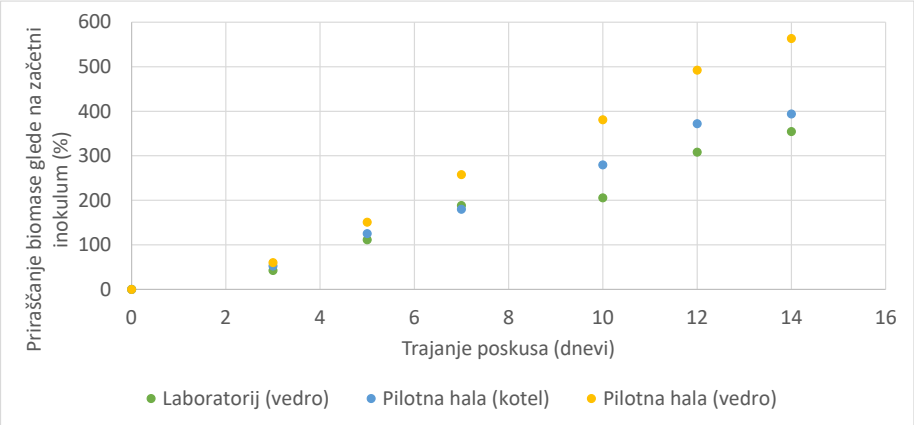


Slika 2: Primerjava povečevanja relativne kefirne biomase, preračunane na začetni inokulum gojenja kefirnih zrn v različnih gojiščih (FT in PS kisle sirotke ter FT sladke sirotke (FTS) in FT sladke sirotke s korigirano vsebnostjo mlečne kisline (mFTS).

Frakciji kisle in sladke sirotke, ki sta se izkazali kot primeren substrat za gojenje kefirnih zrn, sta bili permeatna frakcija sirotke (PS frakcija) po opravljeni mikrofiltraciji ter nevezana frakcija sirotke po opravljenem kromatografskem postopku izolacije sirotkinih proteinov (FT frakcija). Kefirna zrna so najbolje uspevala v FT frakciji sladke sirotke po korekciji vrednosti mlečne kisline, lepo so priraščala in ohranila vitalnost tudi v FT kisle sirotke, v FT sladke sirotke ter v PS kisle sirotke.

Prenos gojenja kefirnih zrn na pilotni nivo

Po optimizaciji gojenja kefirnih zrn na laboratorijskem nivoju smo gojenje prenesli na pilotni nivo. V prispevku je predstavljen poskus primerjave stopnje priraščanja kefirnih zrn iste matične linije na laboratorijskem in pilotnem nivoju. Na laboratorijskem nivoju smo zrna gojili v 10 L posodah. Gojenje na pilotnem nivoju je potekalo v prilagojenem fermentacijskem kotlu z maksimalno kapaciteto 150 L. Uporabili smo zamrznjena kefirna zrna, ki smo jih najprej oživljali v pasterizirani PS frakciji kisle sirotke do doseganja 25 % dnevnega prirasta. Poskus je potekal enajst dni z 48 do 72-urnimi inkubacijami oziroma menjavami gojišča. Potek fermentcije smo spremljali z merjenjem pH vrednosti gojišča in prirastka mase kefirnih zrn po vsaki inkubaciji. Pasterizacija gojišča na pilotni stopnji je potekala neposredno v feremntacijskem kotlu, v labotaroriju pa smo gojišče pasterizirali v posodah. Pasterizirano gojišče iz kotla smo uporabili tudi za dodatno linijo gojenja v laboratoriju z namenom preverjanja vpliva pasterizacije v kotlu na stopnjo priraščanja kefirnih zrn.



Slika 2: Primerjava stopnje priraščanja biomase kefirnih zrn (preračunane glede na začetni inokulum) na laboratorijskem in pilotnem nivoju.

Rezultati primerjave gojenja kefirnih zrn so pokazali zadosten in primerljiv trend priraščanja kefirnih zrn na laboratoriskem in pilotnem nivoju, s čimer smo pokazali uspešen prenos gojenja kefirnih zrn na pilotni nivo.

Zaključki

Uporaba preostankov frakcij kisle in sladke sirotke se je izkazala kot primeren substrat za gojenje kefirnih zrn s ciljem uporabe kefirnih zrn in preostanka gojišča za oblikovanje novih funkcionalnih živil in prehranskih dopolnil. Najugodnejše priraščanje kefirnih zrn smo dosegli v frakcijah sirotke z odstranjeno začetno startersko kulturo mlečnokislinskih bakterij, odstranjenim delom sirotkinih proteinov in korekcijo pH. Z izvedenim poskusom primerjave prirasti kefirnih zrn na laboratorijskem in pilotnem nivoju smo pokazali, da optimiziran postopek gojenja kefirnih zrn iz laboratorijskega lahko prenesemo na pilotni nivo.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid Whey - Ponovna uporaba odpadne sirotke za ekstrakcijo bioaktivnih beljakovin z visoko dodano vrednostjo (LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA - Frakcioniranje in oplemenitenje sirotkinih proteinov ter izraba preostanka za oblikovanje novih funkcionalnih živil in prehranskih dopolnil (OP20.03521) Operativnega programom za izvajanje evropske kohezijske politike v obdobju 2014 – 2020. Za vire sirotke se zahvaljujemo mlekarni Celeia.

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Optimization of kefir grains cultivation in whey

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Abstract
We have optimized the process of growing kefir grains in whey to achieve a high growth rate in order to process the biomass of kefir grains and spent medium into valuable products. Different fractions of acid and sweet whey that remain after the protein isolation process were tested as a growth media. The optimal conditions for growing kefir grains in the laboratory were determined. To achieve optimal maintenance of the original culture, we tested different freezing procedures and revival of frozen kefir grains. After optimal conditions for the cultivation of kefir grains were established in the laboratory, the cultivation of kefir grains was transferred to the pilot scale.

Keywords: kefir grains, fermentation, pilot scale, acid whey, sweet whey

Introduction
Kefir grains are complex groups of lactic acid bacteria, yeasts, and sometimes acetic acid bacteria in a polysaccharide-protein matrix (Garotte et al., 2010). As a natural starter culture in a suitable growth medium, they perform fermentation where they break down the sugars present in the medium. Milk is most frequently used as a growth medium, but whey, especially sweet, has also been tested. After selective isolation of whey proteins, carried out within the projects LIFE and LAKTIKA, all lactose and other nutrients essential for the growth and development of microorganisms remain in whey. Due to the presence of many microorganisms, metabolites, and various bioactive substances produced during fermentation and many positive effects on human health, kefir grains and whey represent great potential in developing functional foods and food supplements. The purpose of our study was to optimize the cultivation of kefir grains to achieve maximum growth of kefir biomass at the laboratory and pilot-scale using whey residues from previous processes and thus contribute to reducing whey waste and negative environmental impacts.

Optimization of kefir grain cultivation at the laboratory level
In the laboratory, we performed experiments to determine the optimal conditions for the growth of kefir grains and maintained vital kefir biomass. We planned parameters for transferring kefir grain cultivation to a larger scale. The effects on kefir grains growth were evaluated in terms of the method of growth medium preparation, initial pH, inoculum content of activated kefir grains, duration, and incubation temperature. The growth of kefir grains was monitored in different fractions of acid and sweet whey, which remained after the selective isolation of whey proteins. The growth rate of kefir grains was determined by draining kefir grains in a specific time interval and weighing fresh grain mass. The pH and temperature of the medium were monitored during fermentation. In our experiments, vital kefir grains were used. Vital kefir grains were defined as those with > 20 % daily growth. The vitality of kefir grains was achieved by regular successive fermentations. Vital kefir grains, which have reached the desired level of growth, were frozen and revived in optimal conditions and at regular intervals to maintain sufficient biomass for cultivation at the laboratory and pilot scale. We also optimized the process of reviving kefir grains after freezing and evaluated the stability of kefir grains in the frozen state with and without cryoprotectants.

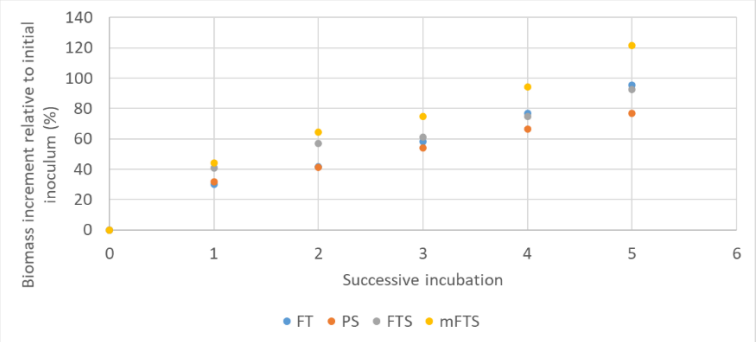

Figure 1: Comparison of the relative increase of the kefir grain biomass, calculated on the initial inoculum of kefir grains grown in different media (FT and PS of acid whey and FT of sweet whey (FTS) and FT of sweet whey with adjusted lactic acid content (mFTS).

The fractions of acid and sweet whey that prove to be a suitable substrate for cultivating kefir grains were the permeate whey fraction (PS fraction) obtained after microfiltration and the unbound whey fraction received after chromatographic isolation of whey proteins (FT fraction). Kefir grains thrived best in the FT fraction of sweet whey after adjusting the value of lactic acid. Furthermore, they also grew well and maintained vitality in FT of acid whey, in FT of sweet whey, and PS of acid whey.

Transfer of kefir grain cultivation to the pilot-scale

After optimizing the cultivation of kefir grains at the laboratory scale, the cultivation was transferred to the pilot scale. In this paper, the results of comparing the growth rate of kefir grains from the same line at the laboratory and pilot-scale are presented. At the laboratory scale, the grains were cultivated in 10 L buckets. At the pilot scale, kefir grains were cultivated in an adjusted fermentation kettle with a maximum capacity of 150 L. We used frozen kefir grains, which were first revived in the pasteurized PS fraction of acid whey to achieve 25 % daily gain. The experiment lasted eleven days with 48 to 72-hour incubations of kefir grains. After each incubation, the growth medium was changed. The fermentation process was monitored by measuring the pH value of the medium and the increase in kefir grains weight after each incubation. The pasteurization of the medium at the pilot-scale took place directly in the fermentation kettle, and in the laboratory, we pasteurized the medium in buckets. Pasteurized medium from the fermentation kettle was also used for an additional cultivation line in the laboratory to verify the effect of pasteurization in the kettle on the growth rate of kefir grains.

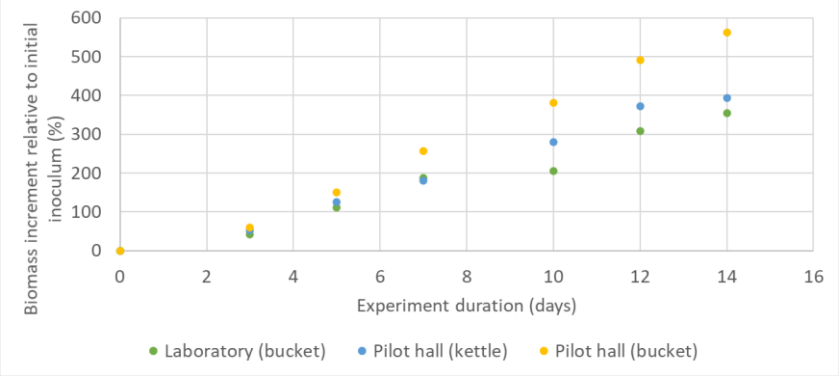

Figure 2: Comparison of kefir grain growth rate (calculated according to the initial inoculum) at laboratory and pilot scale.

The comparison of kefir grain cultivation showed a sufficient and comparable trend of kefir grains growth at the laboratory and pilot scale. A successful transfer of kefir grain cultivation to the pilot scale has been achieved.

Conclusions

The use of residues of acid and sweet whey has proven to be suitable as a growth media for cultivating kefir grains with the aim of using the grains and spent medium to form new functional foods and food supplements. The most favourable growth of kefir grains was achieved in whey fractions with a removed initial starter culture of lactic acid bacteria, removed parts of whey proteins and pH correction. Furthermore, the experiment comparing the growth of kefir grains at the laboratory and pilot level has shown that it is possible to transfer the optimized process of kefir grains cultivation from the laboratory to the pilot level.

Acknowledgements

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Proizvodnja bioplina iz sirotke skozi daljše obdobje bi lahko bila motena zaradi pomanjkanja mikrohranil

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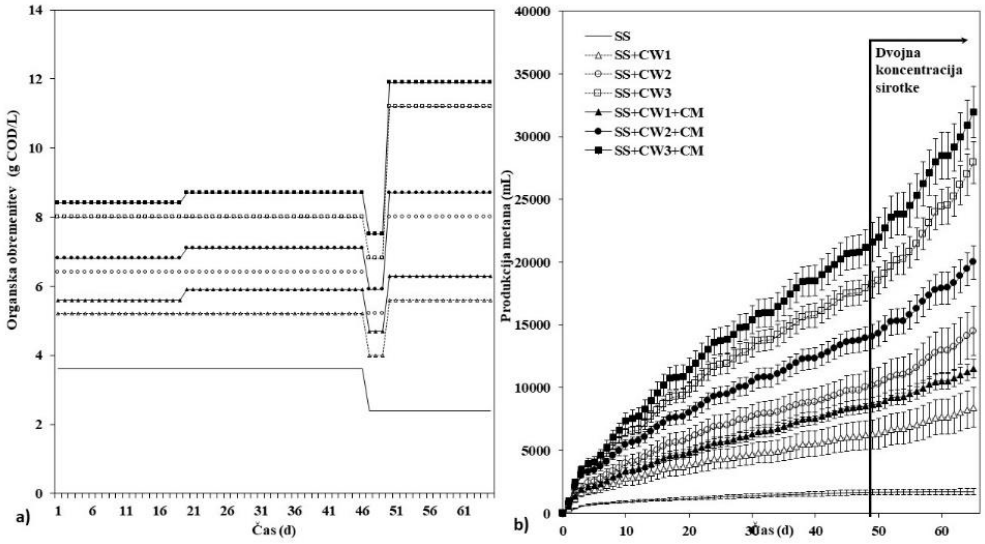
Povzetek
V okviru poskusa smo izvedli semikontinuiran test (68 dni, 38°C) biometanskega potenciala s pomočjo avtomatskega sistema za merjenje metanskega potencial (AMPTS II) iz primarnega blata (PB), sirotke (SI) in kravje gnojevke (KG). Uporabljenih je bilo sedem različnih kombinacij substratov z različnimi organskimi obremenitvami in hidravličnimi zadrževalnimi časi. Tekom testa smo spremljali fizikalno-kemijske parametre. Po 50 dneh stabilne produkcije smo podvojili količino SI (do 65 dne). Stabilna produkcija metana je bila zaznana pri vseh kombinacijah substratov in je korelirala s količino dodane SI. Opazili smo, da je SI glavni vir kratkoverižnih maščobnih kislin, KG pa prispeva k stabilnosti proizvodnje s povečano pufersko kapaciteto. Opazili smo, da bi lahko stabilnost proizvodnje na daljši rok bila porušena, zaradi pomanjkanja mikrohranil, zaradi razredčevanja in izpiranja iz reaktorjev.

Ključne besede: sirotka, mikrohranila, AMPTS II, semikontinuiran proces

Uvod
Sirotka (SI) nastaja kot stranski produkt pri proizvodnji sira. Na globalni ravni je tako na leto proizvedene 40,5 milijona ton SI. Glavni problem vključevanja SI v procese anaerobne digestije (AD) nastopijo zaradi nizkega pH in nizke alkalitete. V okviru tega dela smo preverili ali obstaja možnosti izkoriščanja SI v procesu anaerobne ko-digestije s primarnim blatom (PB) in kravjo gnojevko (KG), hkrati pa preverili koristi in slabosti takega procesa.

Materiali in metode Za test metanskega potenciala smo uporabili AMTS II (Bioprocess Control, Sweden), nadgrajen s 5 L reaktorji na semikontinuiran način (Kolbl in sod., 2014; Kolbl in sod., 2016; Murovec in sod., 2015). AD PB je bila uporabljena kot kontrolni eksperiment. Uporabljene so bile tri različne kombinacije SI, PB in tri različne kombinacije SI, PB in KG (slika 1a). AD je potekala v mezofilnem območju (38°C) v vodni kopeli z občasnim mehanskim mešanjem (1 min na vsakih 5 min). Po 50 dnevu smo podvojili količino SI (do 65 dne). Spremljali smo fizikalno-kemijske parametre (pH, amonijski dušik, KPK, mikrohranila (XRF) in razmerje med kratkoverižnimi maščobnimi kislinami (KMK) in alkaliteto (VOA/TIC)).



Slika 1. Uporabljene organske obremenitve reaktorjev (a) in kumulativa pridobljenega CH_4 (b).

Rezultati in zaključki

Po prvih 50 dneh je bilo v kontrolnem reaktorju proizvedenega 1682±204 mL CH_4. Dnevni dodatek 55 ml SI, je povečal kumulativno proizvodnjo CH_4 za približno 16000 ml (SS+CW3). Dnevni dodatek 10 ml KG pa je proizvodnjo še dodatno povečal za 20000 ml (SS+CW3+CM). Podvojitev volumna dodane SI, je povečal proizvodnjo CH_4 za od 33 % do 53 %. Največ CH_4 je bilo proizvedenega v reaktorju z razmerjem PB:SI:KG 70:55:10 (70:110:55 po 50 dnevu) (slika 1b).

Parameter, ki določa KMK, je padal do 50. dne v vseh variantah in je začel naraščati po podvojitvi SI v vseh eksperimentih z dodano SI. pH v reaktorjih s SI je konstantno padal. Sirotka kot taka je bila glavni vir KMK in je bila razlog za povečanje proizvodnje CH_4. Na drugi stani, je parameter alkalitete bil višji v reaktorjih z dodano KG, kar je dokaz, da je ta glavni vir povišane puferske kapacitete.

Opazili smo odstopanje v vsebnosti mikrohranil v reaktorjih z dodano SI. S pomočjo napovedovanja z linerano regresijo, smo poskušali določiti časovno območje, kdaj bi lahko prišlo do pomanjkanje le teh, kar bi rezultiralo v inhibiciji AD. Do te bi lahko prišlo zaradi stradanja metanogenih arhej. Tako bi lahko prišlo do pomanjkanja Fe med 130-150 dnevi, Mn med 120-220 dnevi, Zn med 160-210 dnevi in Cu med 180-270 dnevi. Večja organska obremenitev (ORL) bi lahko prej rezultirala v inhibiciji zaradi hitrejšega razredčevanja. Za boljši potek procesa, bi morali razmisliti o možnostih za uravnavanje in preprečitev take inhibicije, kar pa bi bilo potrebno preveriti na testih metanskega potenciala na daljša časovna obdobja.

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Gas production from cheese whey over longer periods may be interrupted by micronutrient restriction

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Abstract
A semi-continuous test of the biomethane potential of sewage sludge (SS), cheese whey (CW), and cow manure (CM) was investigated using an automated methane potential system (AMPTS II) over 68 days (38 °C). Seven different combinations of substrates with different organic loadings and hydraulic retention times were used. During the test, we performed measurements of physicochemical parameters. After 50 days of stable methane production, we doubled the CW concentration (until day 65). Stable methane production was observed with all combinations of substrates. Methane yield correlated with the amount of CW added. According to our observations, CW was the main source of SCFAs in the system, while CM contributed to the stability of the process with increasing buffer capacity. We observed potential problems with process stability when the process was extended, as the concentrations of some micronutrients in the digestion mixture decreased due to washout and dilution.

Keywords: cheese whey, micronutrients, AMPTS II, semi-continuous process

Introduction
Cheese whey (CW) is a byproduct of cheese production and one of the energy-rich products that can boost biogas production. The annual production of CW is more than 40.5 million tons worldwide. The main problems of CW are low pH and alkalinity, which can lead to inhibition of anaerobic digestion process (AD). In this work, we investigated the feasibility of using CW in the anaerobic co-digestion of mixtures of sewage sludge (SS) and cow manure (CM) by estimating the benefits or negative effects on methane production.

Materials and Methods
The automated methane potential test (AMPTS II; Bioprocess Control, Sweden), upgraded to a 5-litre size, was used in a semi-continuous experiment as previously described (Kolbl et al., 2014; Kolbl et al., 2016; Murovec et al., 2015). We ran AD of SS as a control experiment. Three different combinations of SS and CW and three different combinations of SS, CW and CM were used for co-digestion analysis (Fig. 1a). The reactors were kept under mesophilic conditions (38°C) in a water bath and mechanically stirred for 1 min every 5 min. After day 50, we doubled the concentrations of CW (until day 65). Various physicochemical analyses were performed during the experiment (pH, ammonium nitrogen, COD (TOC), microelements (XRF), SCAFs and alkalinity (VOA/TIC)).

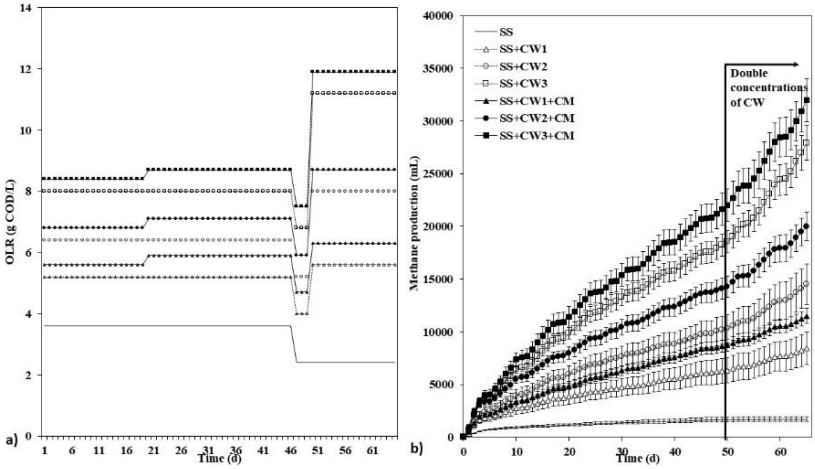

Figure 1: (a) Organic loading rates in seven different combinations of AD and (b) cumulative production of CH_4.

Results and Conclusions

During the first 50 days, 1682±204 mL of CH_4 was produced in the control reactor. Addition of 55 mL CW increased methane production by approximately 16000 mL (SS+CW3). Daily addition of 10 mL CM to co-digest CW and SS increased CH_4 yield by 20000 mL by day 50 (SS+CW3+CM). Doubling the CW concentration increased methane production by 33% to 53% in only 15 days. The highest CH_4 yields were observed in the digestion mixture with a volume ratio of SS, CW and CM of 70:55:10 (70:110:10 after day 50) (Fig. 1b).

The VOA parameter (SCFA) decreased until day 50 in all experimental variants and started to increase after doubling the CW in all experiments except the control experiment, suggesting that CW is the main source of SCFAs and this could also be a reason for the increased methane yields in the co-digestion experiments (pH was also decreased in the reactors with CW). On the other hand, TIC values (alkalinity) were higher in the experiments with CM.

Variations in microelements were observed in all experimental variants with CW. Using a linear forward projection regression analysis, we attempted to determine the critical time for inhibition of the process of anaerobic co-digestion of SS, CW, and CM due to starvation of methanogenic archaea. This could be problematic between 130-150 days (Fe), 160-210 days (Zn), 120-220 days (Mn), and 180-270 days (Cu). In reactors with higher organic loading, there is a higher probability of starvation due to the higher dilution. This could be remedied by adding these trace elements to the digestion mixture to extend the sustainability of the process. However, this should be verified in further experiments with a longer period of co-digestion.

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Možnosti uporabe kisle sirotke v proizvodnji bioplina v kombinaciji z odpadnim biološkim blatom

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Povzetek
Namen raziskave je bil izmeriti biometanski potencial (BMP) sirotke v kombinaciji z mešanico biološkega blata iz primarnega in sekundarnega usedalnika Centralne čistilne naprave Domžale-Kamnik d.o.o. pri mezofilnih pogojih, ugotoviti največjo količino dodane kisle sirotke v pilotni bioplinski digestor, ki še omogoča nemoteno delovanje procesa in z metodo TRFLP ugotoviti, ali dodana sirotka vpliva na spremembe v sestavi mikrobne združbe. Ugotovili smo, da je sirotka ustrezen substrat za hkratno anaerobno razgradnjo z odpadnim biološkim blatom iz čistilnih naprav, saj dodatek sirotke poveča proizvodnjo bioplina.

Ključne besede: kisla sirotka; biološko blato; bioplin; metan

Uvod
Sirotka je tekoč stranski produkt mlekarske industrije, ki nastaja pri predelavi mleka v sir in druge fermentirane mlečne izdelke. Ločimo kislo in sladko sirotko. Uporaba neobdelane kisle sirotke ni zelo razširjena, največ se je porabi za krmljenje živali in v živilski industriji. Večina kisle sirotke tako še vedno konča v odpadnih vodah, kjer zaradi visoke vsebnosti organske snovi predstavlja veliko obremenitev. V že obstoječih bioplinskih napravah lahko v procesu anaerobne presnove sirotka predstavlja dodatni substrat, pri čemer vpliv njene nizke alkalinitete in pH na anaerobni proces ni podrobno raziskan. Na čistilnih napravah, kjer v anaerobnih digestorjih presnavljajo biološko blato zaradi zmanjšanja obratovalnih stroškov z njegovim odstranjevanjem, lahko z dodatkom sirotke prav tako povečajo proizvodnjo bioplina, saj takšni digestorji običajno obratujejo pri nizki stopnji organske obremenitve (Aichinger in sod., 2015; Chow in sod, 2020).

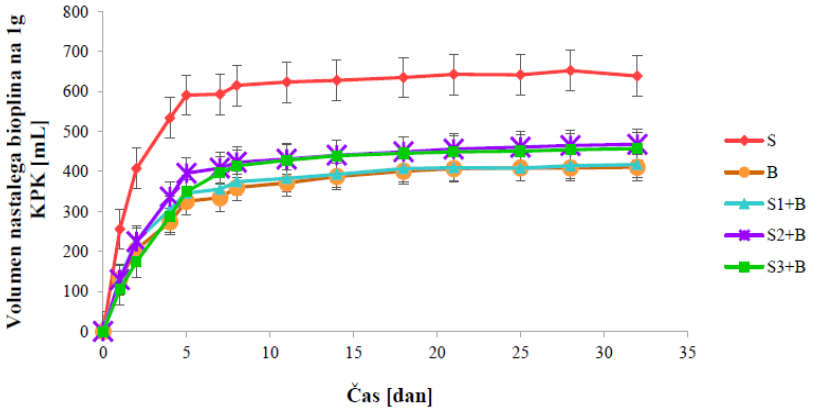
Izvedba poskusov

Test biometanskega potenciala (BMP)
Test BMP smo izvajali 32 dni pri 39 °C v litrskih testnih steklenicah v treh ponovitvah in ugotavljali, kako dodatek sirotke v kombinaciji z mešanico primarnega in sekundarnega biološkega blata vpliva na proizvodnjo bioplina in delež metana v njem. V poskusne steklenice smo poleg mešanice primernega in sekundarnega blata (0,3 g KPK_{blata}/1 g $OS_{mikrobne\ biomase}$) (B) dodali še različne koncentracije kisle sirotke (0,1, 0,3 in 0,5 g $KPK_{sirotke}$/1 g $OS_{mikrobne\ biomase}$) (S1+B; S2+B; S3+B). Med testom smo spremljali proizvodnjo bioplina, vsebnost kratkoverižnih maščobnih kislin (KMK) in analizirali sestavo bioplina s plinsko kromatografijo. Na začetku in ob zaključku testa BMP smo izmerili pH in kemijsko potrebo po kisiku (KPK).

Poskus v pilotnih digestorjih
Poskus na pilotnem nivoju smo izvajali 238 dni v dveh 200-litrskih digestorjih pri 39 °C (R1 in R2). V oba digestorja smo dnevno dodajali 2,25 L primarnega in 2,25 L sekundarnega blata, v eksperimentalni digestor (R2) smo vsakodnevno v petih različnih obdobjih dodajali še različno količino sirotke (1 L, 2L, 3 L, 4 L ali 5,5 L). Dnevno smo pri vzorcih izmerili vrednost pH, vsebnost KMK in alkaliniteto. Enkrat tedensko smo izmerili še KPK, suho snov, organsko snov, vsebnost amonijskega dušika, vsebnost Kjeldahlovega dušika in analizirali sestavo bioplina s plinskim kromatografom. Ob koncu vsakega obdobja dodajanja enake količine sirotke smo v obeh digestorjih določali še sestavo KMK in spreminjanje mikrobne združbe z metodo TRFLP (Panjičko in sod., 2017).

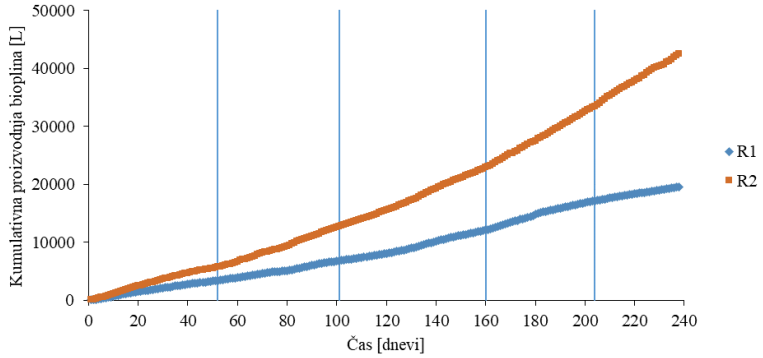
Rezultati in zaključki

Ob koncu testa BMP smo največ bioplina (Slika 1) in metana izmerili v mešanicah s samo sirotko. Delež metana v proizvedenem bioplinu je bil 65 %. V testu izmerjen biometanski potencial kisle sirotke znaša 384 mL CH_4/g KPK. Dodatek srednje količine sirotke k biološkem blatu (S2+B) je v primerjavi z mešanicami s samim blatom najbolj povečal proizvodnjo bioplina, in sicer za 14 %, v testu BMP pa v primerjavi z mešanicami s samim blatom (B) dodatek kisle sirotke k biološkem blatu ni povečal izplena metana.



Slika 1: Volumen nastalega bioplina (mL) na 1 g $KPK_{substrata}$ med testom BMP. S= kisla sirotka, B= primarno in sekundarno biološko blato, S1+B= najnižja koncentracija sirotke in biološko blato, S2+B= srednja koncentracija sirotke in biološko blato; S3+B= največja koncentracija sirotke in biološko blato

Dodatek sirotke v eksperimentalni digestor (R2) ni imel večjega vpliva na vrednost pH, vsebnost KMK, suhe in organske snovi ter sestavo bioplina. Opazen je bil vpliv faktorja redčenja pri alkaliniteti ter vsebnosti amonijevega in Kjeldahlovega dušika. Dnevna proizvodnja bioplina je bila v eksperimentalnem digestorju (R2) večja kot v kontrolnem (R1) (Slika 2). Dodatek sirotke k mešanici primarnega in sekundarnega blata je sicer povečal proizvodnjo bioplina v digestorju R2, a ni povečal izkoristka substrata. Izkoristek se je v R2 povečal v primerjavi z R1 le v zadnjem, petem obdobju. Analiza spreminjanja mikrobne združbe z metodo TRFLP je pokazala večje premike v arhejski kot tudi v bakterijski mikrobni združbi.



Slika 2: Spreminjanje kumulativne proizvodnje bioplina v digestorjih R1 in R2 po dnevih. Navpične črte ločujejo obdobja dodajanja enake količine sirotke v digestor R2 (1 L, 2 L, 3 L, 4 L in 5,5 L).

Zahvala

Avtorji se zahvaljujemo Javnemu podjetju centralna čistilna naprava Domžale-Kamnik za možnost opravljanja raziskav na področju proizvodnje bioplina iz kisle sirotke v pilotnih bioplinskih reaktorjih.

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Possibilities of using acid whey in biogas production in combination with sewage sludge

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Abstract
The aim of the study was to measure the biochemical methane potential (BMP) of acid whey in combination with a mixture of sewage sludge from the primary and secondary settling tank of the Central wastewater treatment plant Domžale-Kamnik d.o.o. at mesophilic conditions (39 °C), to determine the maximum quantity of acid whey added into the pilot biogas digester that still allows the stable process and to determine the impact of added acid whey on the microbial community profile by the TRFLP analysis. We concluded that acid whey is a suitable cosubstrate for anaerobic digestion in combination with sewage sludge from wastewater treatment plants, because the addition of whey increases biogas production.

Keywords: whey, sewage sludge, biogas, methane

Introduction
Acid whey is a liquid waste product of the dairy industry, which is produced by processing milk into cheese and other fermented dairy products. We distinguish between acid and sweet whey. The use of unprocessed acid whey is not very widespread, it is mostly used for animal feed and in the food industry. Therefore, it often ends up in wastewater, where it represents a high environmental burden because of its high organic matter content. Despite the mentioned fact it is the acid whey's structure that makes the liquid a great potential to increase biogas production and methane yield in anaerobic co-digestion process with sewage sludge, which is generated during wastewater treatment at wastewater treatment plants. In existing biogas plants, acid whey may be an additional substrate in the process of anaerobic digestion. The influence of its low alkalinity and pH on the anaerobic process has not been studied in detail yet. In wastewater treatment plants, where anaerobic digesters use sewage sludge to reduce operating costs by removing it, the addition of acid whey can also increase biogas production as such digesters typically operate at low organic load levels (Aichinger et al., 2015; Chow et al., 2020).

Experimental setup

Biochemical methane potential assay (BMP)
BMP assay was carried out in 1-L test bottles under mesophilic conditions (39 °C) for 32 days. Besides the mixture of the primary and secondary sludge (0,3 g COD_{sludge}/1 g $OS_{microbial\ biomass}$) (B), we also added different concentrations of acid whey (0,1, 0,3 and 0,5 g COD_{whey}/1 g $OS_{microbial\ biomass}$) (S1+B; S2+B; S3+B) to the test bottles. During the test, we measured the production of biogas, the concentration of short-chain fatty acids (SCFA's) and analysed the composition of biogas by gas chromatography. The amount of produced biogas was measured manually with a pressure gauge and water column. At the beginning and at the end of the BMP assay, we measured pH and chemical oxygen demand (COD).

The experiment in pilot digesters
The experiment was carried out for 238 days in two 200-L pilot digesters at mesophilic conditions (39 °C) (R1 and R2). A mixture of primary (2,25 L) and secondary sludge (2,25 L) was added daily to both digesters and a different amount of whey was added to the experimental digester (1 L, 2L, 3 L, 4 L, or 5,5 L) (R2). We measured pH, alkalinity, SCFAs, COD, total and volatile solids, ammonium and Kjeldahl nitrogen, biogas composition and, shifts within microbial communities. At the end of each period of adding the same amount of acid whey, the composition of SCFAs and shifts in microbial community profiles were determined in both digesters by the TRFLP method (Panjičko et al., 2017).

Results and conclusions

At the end of the BMP assay, the highest biogas (Figure 1) and methane production was measured in experimental mixtures with acid whey in comparison to mixtures with sewage sludge. The biomethane potential of the acid whey was 384 mL CH_4/g COD. The addition of the medium amount of whey to sludge (S2+B) increased biogas production by 14 % compared to mixtures with sewage sludge (B). The results of the BMP assay showed that the added acid whey increased the biogas production, but did not increase the methane yield.

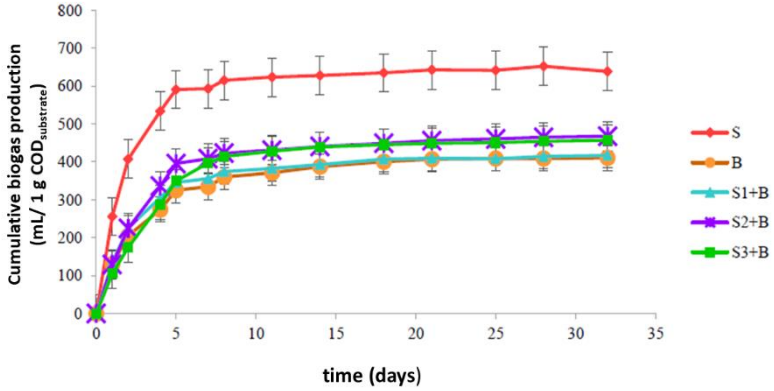

Figure 1: Cumulative biogas production (at standard conditions) per 1 g $COD_{substrate}$ during the BMP assay in mL. S= acid whey, B= primary, and secondary sludge, S1+B= the lowest whey concentration and sludge, S2+B= medium whey concentration and sludge; S3+B= the highest whey concentration and sludge

The addition of acid whey to the experimental digester (R2) did not have a significant effect on the pH, COD, total and volatile solids, biogas composition, and SCFAs. However, the influence of the dilution factor on the alkalinity, ammonium, and Kjeldahl nitrogen was observed. Daily biogas production in the experimental digester R2 was higher than in the control digester R1 (Figure 2). The addition of whey to the mixture of primary and secondary sludge increased the production of biogas in the R2 digester, but did not increase the biogas yield. The efficiency in R2 was increased in comparison to R1 only in the last, fifth period. The TRFLP profiles of microbial communities showed a greater shift in archaeal as in the bacterial community.

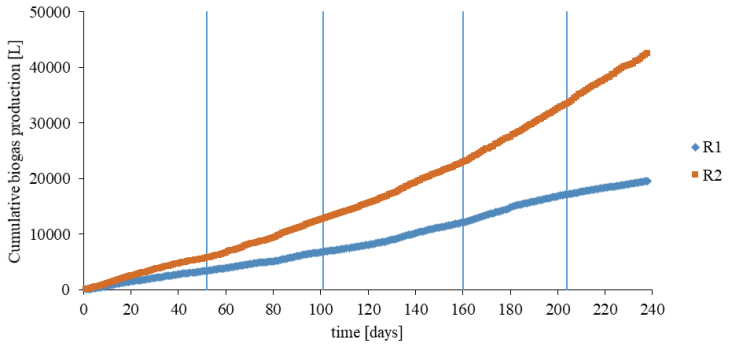

Figure 2: Cumulative biogas production in digesters R1 and R2. Vertical lines represent the periods of adding the same amount of acid whey to the digester R2 (1 L, 2 L, 3 L, 4 L and 5,5 L).

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Ločevanje sirotkinih frakcij z uporabo membranskih filtracij

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Povzetek
V industriji predelave mleka se mikrofiltracija pogosto uporablja za zmanjšanje bakterij in odstranjevanje maščob v mleku in sirotki ter za standardizacijo beljakovin in kazeina. Namen testiranja mikrofiltracijske membrane je bil optimizirati procese filtracije sirotke za doseganje visoke prepustnosti sirotkinih beljakovin (predvsem laktoferina) in zadrževanje mikroorganizmov in drugih sestavin sirotke, ki bi omejile uporabo sirotke v nadaljnjih procesih predelave. Vzpostavili smo najučinkovitejši način delovanja mikrofiltracijske keramične membrane in režim čiščenja. Najvišja prepustnost laktoferina je znašala 51 % pri tlaku 1 bar. Membrana je bila učinkovito očiščena v treh korakih. Permeat po mikrofiltraciji smo obdelali še z nanofiltracijo, s čimer smo popolnoma ločili laktozo iz sirotke.

Ključne besede: kisla sirotka, mikrofiltracija, laktoferin, nanofiltracija, laktoza

Uvod
Mikrofiltracija (MF) je široko uporabljen postopek s številnimi aplikacijami v industriji predelave mleka. Keramične membrane so edine, ki izpolnjujejo vse zahteve za uporabo v industriji predelave mleka, kot so: močna mehanska odpornost, ki omogoča uporabo visokih hitrosti recirkulacije viskoznih MF retentatov, široka toleranca na pH (0,5 do 13,5), ki omogoča njeno uporabo pri čiščenju namesto kavstične sode (do 3 %), toleranca na dušikove kisline (do 2 %) – vendar se je treba izogibati fosforjevi in fluorovodikovi kislini – in toleranca na natrijev hipoklorit zaradi higienskih zahtev. Glavna težava pri MF je mašenje, kar vodi do zmanjšanja pretoka permeata in selektivnosti membrane. Nekateri raziskovalci (Bowen in Gan, 1991) poročajo o adsorpciji beljakovin v notranji strukturi por kot o najbolj prevladujočem koraku pri pojavu zamašitve, medtem ko drugi (Jim in sod., 1992) domnevajo, da je upad pretoka večinoma posledica površinskega odlaganja in nastanka pogače, vključno z beljakovinskimi agregati. Številni raziskovalci so dokazali, da obstaja prehod pri mehanizmu mašenja. Zokaee in sodelavci (Zokaee in sod., 1999) poročajo o premiku od notranjega blokiranja k tvorbi pogače med kontinuirano membransko filtracijo pri filtriranju biološke raztopine. V tej študiji so raziskali učinke obratovalnih pogojev, kot sta obratovalni tlak in hitrost tekočine, na vsak posamezen upor pri stacionarni tangencialni MF sirotke z uporabo keramične cevaste membrane (velikost por 0,5 µm), ki ji je sledila nanofiltracija (NF).

Eksperimentalni del
Za poskuse smo uporabili keramično cevno MF membrano (velikost por 0,5 µm). Pred vsako serijo filtracije sirotke je bil izmerjen pretok vode (bazna linija 1), da se določi stopnja čiščenja membrane. Ko je bil zaznan stalen pretok permeata, je bilo doseženo ustaljeno stanje. Po filtraciji sirotke smo permeat nadalje obdelali z NF in sistem izprali z vodo in ponovno izmerili pretok vode (bazna linija 2). Nato smo membrano izpirali s permeatne in retentatne strani, izvedli povratno izpiranje vode in filtriranje vode. Sledilo je merjenje pretoka vode po čiščenju membrane z vodo (bazna linija 3). Po kemičnem čiščenju smo izmerili končni pretok vode (bazna linija 4) in ga primerjali z začetnim pretokom vode (bazna linija 1). Za določitev stanja čistosti membrane so potrebni postopki merjenja pretoka vode. Sistem je bil očiščen pri 80 % hitrosti črpalke in tlaku 0,5 bara. Izveden je bil poskus čiščenja membrane z različnimi koncentracijami čistilnih sredstev in različnimi pogoji delovanja (tabela 1). Določen in uporabljen je bil najučinkovitejši postopek čiščenja.

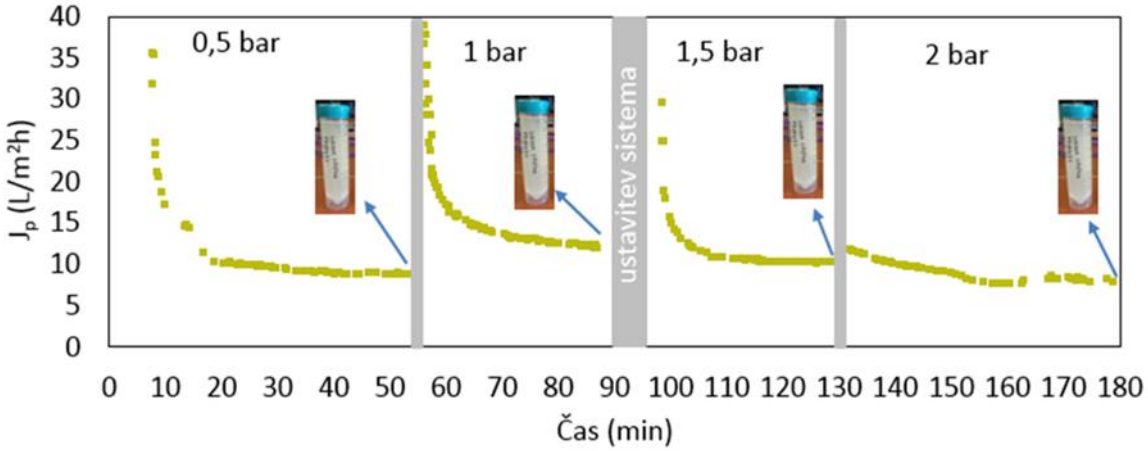
Tabela 1: Čistilna sredstva in pogoji med čiščenjem membrane.

Čistilno sredstvo	Koncentracija (%)	pH	Temperatura (°C)
NaOH	0,4 - 1	11,5	50 - 60
HNO3	0,3 - 1	1,3	50 - 60
NaClO	0,5	7,8	50 - 60
Divos 120CL	1	10	50

Za NF smo uporabili tankoslojno membrano Desal DL (Suez, ZDA). Material je poli piperidinamid z velikostjo por 340 Da, območje pH 2-11, temperatura do 50 °C in tlak 0.5 – 28 bar.

Rezultati in diskusija

Filtriranje sirotke je bilo izvedeno z retentatne strani rezervoarja, skozi keramično membrano v permeatno stran rezervoarja, s povečanjem tlaka retentata pri določeni hitrosti črpalke. Filtrirali smo 10 L kisle sirotke pri njenem pH (4,8) in 60 % hitrosti črpalke po naslednjem postopku (slika 1):



Slika 1: Pretok sirotke pri različnih tlakih.

Sprva opazimo hitro zmanjšanje pretoka v prvih minutah filtracije zaradi učinka koncentracijske polarizacije. Na tej sliki bi lahko upad pretoka razvrstili v dve različni fazi. "Faza I" in "Faza II" ločujeta močno zmanjšanje pretoka v prvih 5-10 minutah procesa od počasnega zmanjšanja do 20. minute in stabilnega stanja po tem.

Pri NF smo preizkusili različne transmembranske tlake od 10 bar do 40 bar in določili optimalne pogoje, pri katerih dosežemo največji pretok in najmanjše mašenje membrane. Optimalni pretok smo določili pri pH =3 in transmembranskem tlaku 20 bar, pri čemer smo dosegli 100 % zadržanost laktoze. Mlečna kislina je ostala v enakih deležih v permeatu in retentatu. Po sušenju z razprševanjem se lahko oba produkta uporabita kot dodatek kozmetičnim izdelkom.

Zaključek

Celotna odpornost filtriranja se je povečala, ker prevladuje proces zamašitve por. Odpornost na reverzibilno zamašitev narašča z naraščajočim tlakom. Reverzibilno mašenje je povezano predvsem s tvorbo filtrske pogače ali gela. Pri višjem tlaku je učinek doseganja nepopravljive zamašitve večji in ga ni mogoče odpraviti z uporabo povratnega izpiranja vode. Ireverzibilna zamašitev je povezana z zamašitvijo por, ki pri višjih tlakih poteka hitreje. Razlog za reverzibilno zamašitev so slabo vezane beljakovine znotraj por in tvorba beljakovinskih plasti na površini membrane. Po nanofiltraciji je laktozna frakcija ostala v koncentratu, medtem ko mlečne kisline ni bilo mogoče zadržati.

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Separation of whey fractions using membrane filtrations

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Abstract
In the dairy industry, microfiltration is widely used for bacteria reduction and fat removal in milk and whey as well as for protein and casein standardization. The purpose of microfiltration membrane testing was to optimize whey filtration processes, with the aim of achieving high permeability for whey proteins (especially lactoferrin) and retention of microorganisms and other whey components that would limit the use of whey in further processing operations. The most efficient mode of microfiltration ceramic membrane operation was established as well as the cleaning regime. The highest lactoferrin permeability was 51 % at a pressure of 1 bar. Membrane was effectively cleaned in three steps. Permeate was additionally treated with nanofiltration and lactose was totally retained.

Keywords: acid whey, microfiltration, lactoferrin, nanofiltration, lactose

Introduction
Microfiltration (MF) is a widely used process with many applications in dairy industry. Ceramic membranes are the only ones that satisfy all the requirements of the applications in the dairy industry i.e. a strong mechanical resistance which allows the use of high recirculation velocities of viscous MF retentates, a wide tolerance to pH (0.5 to 13.5) allowing its usage for cleaning in place of caustic soda (up to 3%), of nitric acid (up to 2%) – but phosphoric and hydrofluoric acids should be avoided – and of sodium hypochlorite for sanitation. The main problem with MF is fouling, which leads to reduce permeate flux and membrane selectivity. Protein adsorption in the internal structure of the pores has been reported as the most dominant step in fouling phenomena by some researchers (Bowen and Gan, 1991), while others suppose that flux decline results mostly from surface deposition of a cake including protein aggregates (Jim *et al*., 1992). Many researchers proved that there is a transition in the fouling mechanism. Zokaee et al. (Zokaee Ashtiani *et al*., 1999) reported a shift from the internal blocking to cake formation during a continuous membrane filtration of a biological solution. In this study, the effects of operating conditions, such as the operating pressure and fluid velocity, on each individual resistance in the steady-state crossflow MF of whey were investigated using ceramic tubular membrane (pore size 0.5 μm) followed by nanofiltration (NF).

Experimental part
For experiments the ceramic tubular MF membrane (pore size 0.5 μm) was used. Before each batch of whey filtration, water flow (Baseline 1) was measured to determine the degree of membrane cleaning. When a constant permeate flow was detected, a steady state was reached. After filtration of the whey, the permeate was further treated by NF and the system was washed with water and the water flow was measured again (Baseline 2). Then, the membrane was washed from the permeate and retentate sides, performed the backwash of water and the filtration of water. This was followed by measuring the water flow after cleaning the membrane with water (Baseline 3). After chemical cleaning, the final water flow (Baseline 4) was measured and compared to the initial water flow (Baseline 1). Water flow measurement procedures are required to determine the membrane cleanliness status.The system was cleaned at 80% pump speed and 0.5 bar of pressure. An experiment was made to clean the membrane with different concentrations of cleaning agents and different operating conditions (Table 1). The most effective cleaning procedure was determined and used.

Table 1: Cleaning agents and conditions during membrane cleaning.

Cleaning agent	Concentration (mas %)	pH	Temperature (°C)
NaOH	0,4 - 1	11,5	50 - 60
HNO3	0,3 - 1	1,3	50 - 60
NaClO	0,5	7,8	50 - 60
Divos 120CL	1	10	50

TFC NF membrane Desal DL (Suez, USA) was applied, made of poly-piperazine amide with pore size 340 Da, applicable for pH range 2-11, temperature up to 50 °C and pressure range 0.5 – 28 bar.

Results and discussion

Whey filtration was performed from the retentate side of the tank, through the ceramic membrane into the permeate side of the tank, by increasing the retentate pressure at a specified pump speed. We filtered 10 L of acidic whey at its pH (4.8) and 60% of the pump speed according to the following procedure (Fig.1):

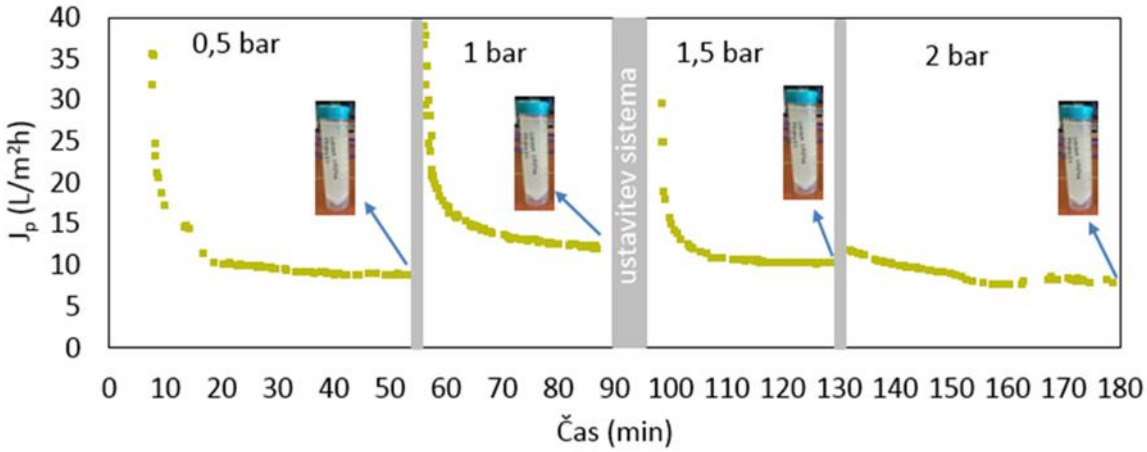

Figure 1: Whey flux at different pressures.

Initially, a rapid decrease in flow is observed in the first minutes of filtration due to concentration polarization effect. The flux decline could be classified into two distinct phases in this figure. “Phase I” and “Phase II” separated the sharp flux reduction in the first 5-10 minutes of the process from the slow reduction up to the 20th minute and the steady-state condition after that.

Transmembrane pressure at NF was varied from 10 bar to 40 bar in order to determine optimal conditions with the lowest membrane fouling. The optimal flow was determined at pH =3 and transmembrane pressure 20 bar with total lactose retention. Lactic acid remained in quite similar shares in permeate and retentate. After spray drying both could be used in cosmetic industry as supplements.

Conclusion

The overall resistance of filtering was increased because the pore clogging process is dominant. The resistance of reversible clogging increases with increasing pressure. Reversible fouling is mainly associated with the formation of a filter cake or gel. At higher pressure, the greater is the effect of achieving irreversible clogging, which cannot be eliminated by the use of backwash of water. Irreversible clogging is related to clogging of pores, which take place more rapidly at higher pressures. The reason for reversible clogging is the poorly bound proteins within the pores and the formation of protein layers on the membrane surface. After nanofiltration the lactose fraction remained in concentrate while lactic acid could not be retained.

Acknowledgements

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Izbor mikrofiltracijskih keramičnih membran s ciljem doseganja minimalnega prehoda mikroorganizmov in maksimalnega prehoda laktoferina

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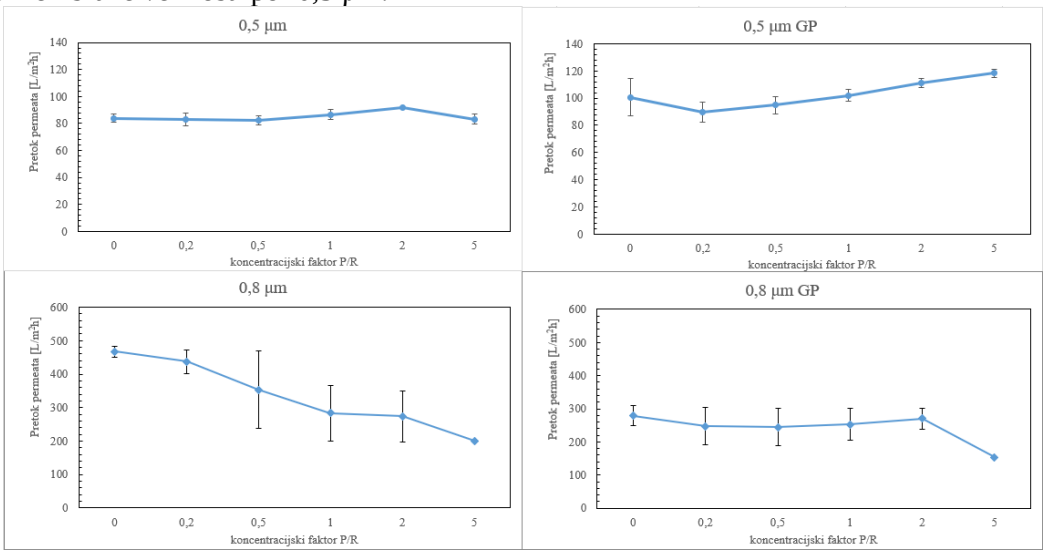
Povzetek
Pri optimalnih nastavitvah parametrov mikrofiltracije kisle sirotke smo na napravi MMS SW25 testirali keramične membrane z različno velikostjo por. Eksperimentalno delo je bilo namenjeno iskanju optimalnega razmerja med prehodnostjo laktoferina in zadrževanjem mikroorganizmov.

Ključne besede: mikrofiltracija, kisla sirotka, skupno število mikroorganizmov, laktoferin

Uvod
Na napravi MMS SW25 smo preizkušali keramične membrane proizvajalca Pall (Membralox) z velikostjo por 0,5 µm in 0,8 µm s ciljem odstranitve mikroorganizmov iz kisle sirotke in maksimalnega prepuščanja laktoferina. Preizkušali smo gradientno (GP) in negradientno različico, tako da smo skupaj testirali 4 membrane. Vsak poskus smo ponovili dvakrat.

Parametri mikrofiltracije
Optimalne nastavitve mikrofiltracije smo določili v ločenem poskusu s testiranjem različnih membran in različnih nastavitev naprave MMS SW25. Mikrofiltrirali smo 30 L kisle sirotke iz Mlekarne Celeia. Na filtracijskem sistemu smo nastavili prvo črpalko na 50 % hitrosti in drugo črpalko na 65 % hitrosti. Na permeatni peristaltični črpalki je bil transmembranski tlak (TMP) nastavljen na 0,9 bar.

Rezultati in diskusija
Namen eksperimentalnega dela je bil izbor optimalne keramične membrane, ki jo bomo kasneje uporabljali na pilotnem mikrofiltracijskem sistemu Bactocatch (TetraPak). Mikrofiltrirali smo 30 L kisle sirotke do volumskega preostanka retentata 5 L in s tem pridobilili 25 L permeata. Pri enakih nastavitvah je filtracija z uporabo keramičnih negradientnih membran z velikostjo por 0,8 µm potekla 3,7-krat hitreje kot z uporabo keramične negradientne membrane velikosti por 0,5 µm. Pri enakih nastavitvah je filtracija z uporabo keramičnih gradientnih membran velikosti por 0,8 µm potekla 2,5-krat hitreje kot z uporabo keramične gradientne membrane velikosti por 0,5 µm.



Slika 1: Specifični pretoki permeata pri določenem koncentracijskem faktorju.

Na Sliki 1 so prikazani specifični pretoki permeata (L/m^{2}h) kot povprečje obeh eksperimentov. Zanimalo nas je, kako membrane delujejo pri določenem koncentracijskem faktorju, ki je izračunan iz volumskega razmerja permeat : retentat. Pri obeh keramičnih membranah velikosti por 0,8 µm opazimo trend padanja pretoka oziroma mašenja membran tekom eksperimenta. Prav tako je standardna deviacija večja kot pri membranah velikosti por 0,5 µm. Večja napaka kaže na slabšo ponovljivost filtracije. Pri membranah velikosti por 0,5 µm je standardna deviacija manjša, pri 0,5 µm gradientni (GP) membrani se pretok permeata z naraščanjem

koncentracijskega faktorja celo veča, kar je idealno, sam graf pa nakazuje tudi zelo stabilno filtracijo, kjer ne prihaja do mašenja membran. Trend nakazuje, da bi lahko dosegli še višji koncentracijski faktor, kar bi pomenilo še višji masni izkoristek filtracije za LF.

V Preglednici 1 so predstavljene začetne vrednosti SŠMO v kisli sirotki (KE/mL) in končne vrednosti v permeatni frakciji po končani mikrofiltraciji. Vse membrane so izkazale dobro redukcijo mikroorganizmov, vendar je bil cilj doseči vrednosti SŠMO vsaj pod 100 KE/mL oziroma znižanje parametra SŠMO za log vsaj 4.

Preglednica 1: Skupno število mikroorganizmov (SŠMO) izraženih v koliformnih enotah na mililiter (KE/mL) v začetni frakciji sirotke pred mikrofiltracijo in permeatni frakciji sirotke po opravljeni mikrofiltraciji z uporabo keramičnih gradientnih (GP) in negradientnih membran z velikostjo por 0,5 in 0,8 µm.

MEMBRANA	0,5 µm	0,5 µm	0,5 µm GP	0,5 µm GP
IZVORNA SIROTKA (KE/mL)	770.000	490.000	210.000	230.000
PERMEAT (KE/mL)	10.000	2.900	*	40
MEMBRANA	0,8 µm	0,8 µm	0,8 µm GP	0,8 µm GP
IZVORNA SIROTKA (KE/mL)	3.600.000	2.200.000	1.900.000	3.300.000
PERMEAT (KE/mL)	300	80	710	75

*napaka meritve

Pri analizah vsebnosti laktoferina smo ugotovili, da je permeabilnost laktoferina pri membranah z večjimi porami nekoliko višja (Preglednica 2), a bi se s povečanjem koncentracijskega faktorja te razlike zelo verjetno zmanjšale, masni izkoristek za LF pa povečal. Filtracija je v vseh primerih potekala do zgornje meje koncentracijskega faktorja (25L:5L=5), ki jo, zaradi mrtvega volumna retentatne zanke, testni filtracijski sistem še dovoljuje.

Preglednica 2: Delež (%) laktoferina (LF) v permeatni frakciji po mikrofiltraciji kisle sirotke z uporabo keramičnih gradientnih (GP) in negradientnih membran z velikostjo por 0,5 in 0,8 µm.

MEMBRANA	% LF V PERMEATU
0,5 µm	68,54 ± 10,07
0,5 GP µm	79,14 ± 2,63
0,8 µm	85,43 ± 0,66
0,8 µm GP	88,82 ± 16,86

Zaključek

Cilj mikrofiltracije je bil odstraniti čim več mikroorganizmov in hkrati doseči dober izkoristek mikrofiltracije v smislu masne bilance za laktoferin. Ne glede na to, da je permeatni pretok gradientne keramične membrane z velikostjo por 0,5 µm nižji in s tem filtracija daljša, smo jo izbrali za nadaljnje postopke obravnave sirotke zaradi dobre prepustnosti za laktoferin, visoke učinkovitosti odstranjevanja mikroorganizmov in stabilnega delovanja pri različnih koncentracijskih faktorjih.

Zahvala

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Selection of microfiltration ceramic membranes to achieve minimum passage of microorganisms and maximum passage of lactoferrin

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Abstract
Ceramic membranes with different pore sizes were tested on the MMS SW25 filtration system at previously defined optimal settings of acid whey microfiltration parameters. The experimental work was intended to find the optimal relationship between lactoferrin recovery and microorganism retention.

Keywords: microfiltration, acid whey, the total number of microorganisms, lactoferrin

Introduction
On the MMS SW25 filtration system, we tested ceramic membranes from the manufacturer Pall (Membralox) with pore sizes of 0.5 µm and 0.8 µm to remove microorganisms from acid whey and achieve maximum lactoferrin passage. In addition, we tested the gradient (GP) and non-gradient versions, altogether four membranes. Each experiment was repeated twice.

Parameters of microfiltration
The optimal microfiltration settings were determined in a separate experiment by testing different membranes and different settings of the MMS SW25 filtration system. For each test, 30 L of acid whey from Celeia Dairy was used. On the filtration system, we set the first pump at 50% speed and the second pump at 65% of max. speed. The permeate peristaltic pump's trans-membrane pressure (TMP) was set to 0.9 bar.

Results and discussion
The purpose of the experimental work was to select the optimal ceramic membrane, which will later be used on the pilot microfiltration system Bactocatch (TetraPak). 30 L of acid whey were microfiltered to a retention volume of 5 L to give 25 L of permeate. At the same settings, filtration using ceramic non-gradient membranes with a pore size of 0.8 µm was 3.7 times faster than using ceramic non-gradient membranes with a pore size of 0.5 µm and a similar ratio (2.5x) was observed when gradient membranes were tested.

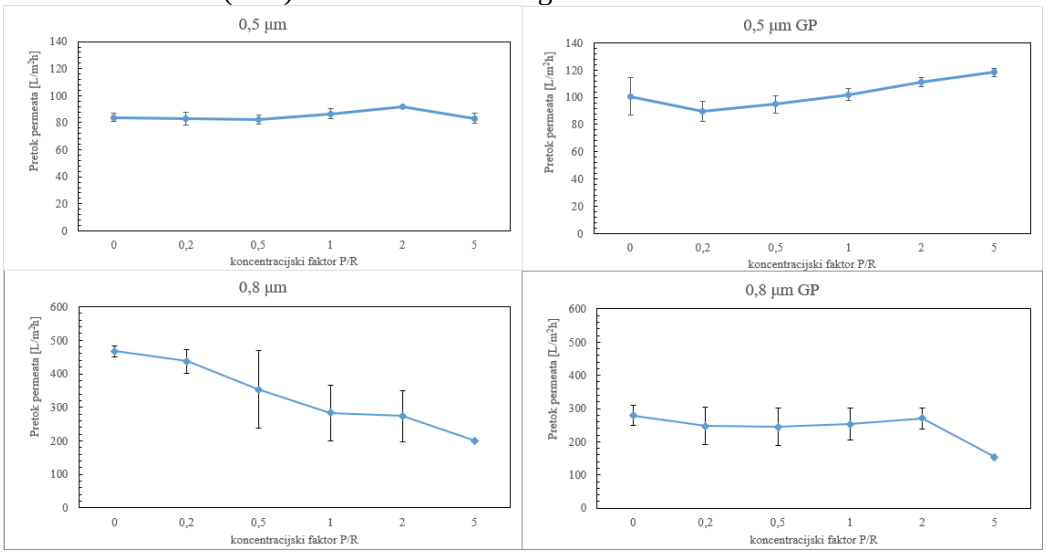

Figure 1: Specific permeate flow at a given concentration factor for each tested membrane.

Figure 1 shows the specific permeate flows (L/m^{2}h) as the average of the two experiments. We were interested in how membranes perform at a specific concentration factor, which is calculated from the permeate : retentate volume ratio. In both cases of ceramic membranes with larger pores (0.8 µm), a trend of decreasing specific permeate flow was observed. Also, the standard deviation was greater than that for 0.5 µm pore size membranes. A larger error indicates poorer filtration repeatability while decreasing specific permeate flow points to the growing clogging effect. Tests using 0.5 µm pore size membranes show that the standard deviation is low. In the case of a 0.5 µm gradient (GP) membrane, the permeate flow even increases with increasing

concentration factor, which indicates stable membrane performance with no effect of membrane clogging. This trend suggests that a higher concentration factor could be achieved, which would lead to even higher filtration mass efficiencies for LF.

In Table 1, the values of the total number of microorganisms (SŠMO) in acid whey and the final values of SŠMO in the acid whey permeate fraction after microfiltration are presented as CFU/mL. All membranes showed a high reduction of microorganisms, but the goal was to achieve SŠMO values at least below 100 CFU/mL or to reduce the SŠMO parameter by a log of at least 4.

Table 1: Total number of microorganisms (SŠMO) expressed in colony-forming units per millilitre (CFU/mL) in the initial whey fraction before microfiltration and the permeate whey fraction after microfiltration using a ceramic gradient (GP) and non-gradient membranes with a pore size of 0.5 and 0.8 µm.

MEMBRANE	0.5 µm	0.5 µm	0.5 µm GP	0.5 µm GP
FEED WHEY (CFU/mL)	770.000	490.000	210.000	230.000
PERMEATE (CFU/mL)	10.000	2.900	*	40
MEMBRANE	0.8 µm	0.8 µm	0.8 µm GP	0.8 µm GP
FEED WHEY(CFU/mL)	3.600.000	2.200.000	1.900.000	3.300.000
PERMEATE (CFU/mL)	300	80	710	75

*measurement error

Table 2: Proportion (%) of lactoferrin (LF) in the permeate fraction after microfiltration of acid whey using a ceramic gradient (GP) and non-gradient membranes with or 0.5 and 0.8 µm.

MEMBRANE	% LF IN PERMEATE
0.5 µm	68.54 ± 10.07
0.5 GP µm	79.14 ± 2.63
0.8 µm	85.43 ± 0.66
0.8 µm GP	88.82 ± 16.86

Conclusions

With microfiltration we aimed to remove almost all microorganisms and at the same time, to achieve good microfiltration efficiency in terms of mass balance for lactoferrin. Therefore, even though the permeate flow of the gradient ceramic membrane with a pore size of 0.5 µm was lower and thus the filtration was longer, it was chosen for further whey treatment procedures due to good permeability to lactoferrin, high efficiency for microorganism removal and its stable performance at various concentration factors.

Acknowledgements

This research was co-funded by the project LAKTIKA - Fractionation and processing of whey proteins and exploitation of the residue for the formation of new functional foods and food supplements (OP20.03521) in the frame of the Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020 and the project LIFE for Acid Whey - Reuse of waste acid whey for the extraction of bioactive proteins with high added value (LIFE16 ENV/SI/000335) of the European financial instrument LIFE.

Povečanje izplena laktoferina iz kisle sirotke s postopkom diafiltracije

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Povzetek
Diafiltracija je membranska filtracija pri kateri v fazi filtracije vključimo enkraten ali večkraten postopek redčenja retentata z vodo ali drugim topilom z namenom povečanja čistosti retentatne frakcije ali povečanja izplena molekul v permeatni frakciji. Namen poskusov je bil povečati izplen laktoferina v permeatni frakciji po končani mikrofiltraciji kisle sirotke. Z eksperimentom smo potrdili povečanje izplena laktoferina v permeatni frakciji z uporabo diafiltracijskega načina mikrofiltracije sirotke in s tem zmanjšali izgube laktoferina v postopku njegovega pridobivanja.

Ključne besede: diafiltracija, kisla sirotka, laktoferin

Uvod
Diafiltracija je membranska filtracija pri kateri v fazi filtracije vključimo enkraten ali večkraten postopek redčenja retentata z vodo ali drugim topilom. S tem procesom znižamo koncentracijo molekul v retentatu, ki lahko potujejo skozi pore membran, oziroma pridobimo večji izplen želenih molekul v permeatu. V postopku pridobivanja laktoferina iz kisle sirotke s kromatografsko ekstrakcijo, smo sirotko predhodno filtrirali z uporabo mikrofiltracijskih membran. Nemen mikrofiltracije siroke je odstranitev mikroorgaizmov, ki bi ovirali kromatografski postopek. Hkrati pa si želimo čim boljšo prehodnost laktoferina, katerega koncentracije v sirotki so nizke in izguba v posameznem proizvodnem koraku pomeni nižji končni izplen proizvodnje. V postopku mikrofiltracije sirotke nam je laktoferin zastajal v retentatni frakciji. Z dodanim korakom diafiltracije, ki je vkjučeval ponovitev mikrofiltracije po redčenju retentata, smo želeli doseči večji izplen laktoferina v permeatu.

Parametri diafiltracije
Diafiltracijo smo izvajali na napravi MMS SW25. Uporabili smo gradientno keramično membrano proizvajalca Pall z oznako EP1940 in z velikostjo por 0,5 µm. Mikrofiltrirali smo 30 L kisle sirotke, diafiltririali pa retentat, ki je predstavljal 10 % začetnega volumna kisle sirotke (t.j. 3 L). Sirotko so nam dobavili iz Mlekarne Celeia. Na napravi MMS SW25 smo nastavili prvo črpalko na 50 % hitrosti, drugo črpalko pa na 65 %. Na permeatni peristaltični črpalki smo nastavili transmembranski tlak (TMP) na 0,9 bar. Pri teh pogojih je potekala mikrofiltracija in diafiltracija. V prvem poskusu smo diafiltracijo izvajali z dodatkom mehke vode, v drugem poskusu pa z dodatkom »flow-through« (FT) frakcije sirotke. FT frakcija predstavlja tako imenovano nevezano frakcijo sirotke po njenem pretakanju skozi kromatografsko kolono in je osiromašena beljakovin, ki se vežejo na kromatografski nosilec.

Rezultati in diskusija
Po mikrofiltraciji 30 L kisle sirotke smo trem litrom retentata dodali 12 L mehke vode oziroma FT frakcije. Diafiltrirali smo do 3 L retentata in ponovno dodali 12 L mehke vode oziroma FT frakcijo. Parameter, ki nas je najbolj zanimal je bil »recovery« laktoferina oziroma dodaten izplen v določenem koraku diafiltracije.

V Preglednici 1 smo predstavili rezultate prvega poskusa, kjer smo diafiltracijo izvajali z dodatkom mehke vode. Z začetno mikrofiltracijo smo dosegli 85,13% izplen laktoferina v permeat. V nadaljnjih dveh korakih diafiltracije, ob dodatku 12 L mehke vode, je bil izplen laktoferina podoben, in sicer 89,16 % v prvem koraku diafiltracije in 85,28% ob drugem koraku diafiltracije. Z dodanima korakoma diafiltracije, oziroma dvakratnemu redčenju retentata mikrofiltracije, smo dosegli 93,9% izplen laktoferina. Pri tem je drugi korak diafiltracije prispeval le še 1,45% celokupne mase laktoferina.

V drugem poskusu smo v fazi diafiltracije dodajali retentatu prve mikrofiltracije FT frakcijo sirotke. Z začetno mikrofiltracijo smo dosegli 64,81 % izplen laktoferina, ki se je nahajal v 30 L kisle sirotke. Izplen laktoferina v procesu mikrofiltracije je bil za 20 % nižji kot v prvem poskusu. V nadaljnjih dveh korakih diafiltracije, ob dodatku 12 L FT frakcije, je bil izplen laktoferina v prvi diafiltraciji višji, in sicer 80,99 %, v drugem koraku diafiltracije pa podoben kot pri začetni mikrofiltraciji, 64,8%. Z obema korakoma diafiltracije, oziroma dvakratnemu redčenju retentata mikrofiltracije, smo dosegli 87,90% izplen laktoferina.

Preglednica 1: Vsebnosti laktoferina (LF) v retentatni in permeatni frakciji tekom diafiltracije z dodajanjem vode.

1. POSKUS	POTEK POSKUSA	Volumen (L)	LF (mg)	OZNAKA	% začetnega laktoferina	% izplena LF
KORAK 0	MIKROFILTRACIJA	30	5294,55	KISLA SIROTKA	100	85,13
		27	4507,65	PERMEAT 0	85,13	
		3	134,78	RETENTAT 0	2,54	
KORAK 1	DODATEK 12 L mehke vode	12	386,88	PERMEAT 1	7,3	89,15
		3	47,04	RETENTAT 1	0,88	
KORAK 2	DODATEK 12 L mehke vode	12	77,04	PERMEAT 2	1,45	85,27
		3	13,3	RETENTAT 2	0,25	
				SUM	93,9	

Preglednica 2: Vsebnosti laktoferina (LF) v retentatni in permeatni frakciji tekom diafiltracije z dodajanjem flow-through (FT) frakcije sirotke.

2. POSKUS	POTEK POSKUSA	Volumen (L)	LF (mg)	OZNAKA	% začetnega laktoferina	% izplena LF
KORAK 0	MIKROFILTRACIJA	30	4690,75	KISLA SIROTKA	100	65,81
		27	3040,35	PERMEAT 0	64,81	
		3	478,87	RETENTAT 0	10,2	
KORAK 1	DODATEK 12 L FT frakcije	12	738,77	PERMEAT 1	15,74	80,99
		3	173,3	RETENTAT 1	3,69	
KORAK 2	DODATEK 12 L FT frakcije	12	344,09	PERMEAT 2	7,33	64,81
		3	186,75	RETENTAT 2	3,98	
				SUM	87,9	

Zaključek

Postopek diafiltracije je v obeh eksperimentih potrdil možnost doseganja povečanja skupnega deleža laktoferina v permeatni frakciji sirotke in s tem zmanjšanja izgub laktoferina v postopku filtracije. Glede na to, da vsak dodaten korak filtracije predstavlja strošek v smislu porabe časa in energije, je potrebno ovrednotiti rentabilnost dodatnih korakov glede na vrednost pridobljenega produkta. V primeru redčenja z vodo, se je pokazal kot zadovoljiv že en korak redčenja. V primeru redčenja z FT frakcijo sirotke, so bili rezultati manj konsistentni in bi potrebovali več ponovitev za potrditev primernosti FT frakcije kot ustreznega topila v postopku diafiltracije.

Zahvala

Raziskava je bila sofinancirana s projektom LIFE for Acid (LIFE16 ENV/SI/000335) evropskega finančnega instrumenta LIFE in projektom LAKTIKA (OP20.03521) operativnega programa za izvajanje evropske kohezijske politike v obdobju 2014 – 2020. Za redne tedenske dobave sirotke v pilotnih fazah izvedbe projekta se zahvaljujemo Mlekarni Celeia.

Increase of lactoferrin yield from acid whey by the diafiltration process

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Abstract
Diafiltration is a membrane filtration in which single or multiple processes of diluting the retentate with water or another solvent are included in the filtration phase to increase the purity of the retentate fraction or to increase the yield of molecules in the permeate fraction. The purpose of the experiments was to test whether additional diafiltration steps would increase the recovery of lactoferrin in the permeate fraction after the microfiltration of acid whey. The experiment confirmed the increase in lactoferrin recovery in the permeate fraction using the diafiltration method and thus the reduction of lactoferrin in the process of its production.

Keywords: diafiltration, acid whey, lactoferrin

Introduction
Diafiltration is a membrane filtration in which single or multiple processes of diluting the retentate with water or another solvent is included in the filtration phase. With this process, we reduce the concentration of molecules in the retentate, which can travel through the pores of the membranes or obtain a higher yield of the desired molecules in the permeate. In the process of obtaining lactoferrin from acid whey by chromatographic extraction, the whey was pre-filtered using microfiltration membranes. The aim of microfiltration was to remove microorganisms that would interfere with the chromatographic process. At the same time, we wanted the best possible transfer of lactoferrin, whose concentrations in whey are low, and the loss in each production step means a lower final yield. In the process of whey microfiltration, lactoferrin was trapped in the retentate fraction. With the added step of diafiltration, which included the repetition of microfiltration after dilution of the retentate, we wanted to achieve a higher yield of lactoferrin in the permeate.

Parameters of diafiltration
Diafiltration was performed with MMS SW25 device made by a swiss company MMS. A gradient ceramic membrane from Pall with a pore size of 0.5 µm (EP1940) was used. With the microfiltration of 30 L of acid whey, we obtained 3 L of retentate which represented 10 % of the initial volume. This ammount of volume (3 L) of retentate was diafiltered. The whey was delivered from dairy company called Mlekarna Celeia. On the MMS SW25 device, we set the first pump to 50 % speed and the second pump to 65 %. The transmembrane pressure (TMP) was set to 0.9 bar on the permeate peristaltic pump. Under these conditions, microfiltration and diafiltration took place. In the first experiment, diafiltration was performed with the addition of soft water and in the second experiment with the addition of FT (flow-through) fraction. The FT (flow-through) fraction represents the unbound fraction of whey after it flows through the chromatographic column and is depleted of proteins that bind to the chromatographic carrier.

Results and discussion
After microfiltration of 30 L of acid whey, 12 L of soft water or FT fraction was added to three liters of retentate. After the first diafiltration, 12 L of soft water or FT fraction was added again. The parameter that interested us the most was the recovery of lactoferrin and additional yield in a certain step of diafiltration.

Table 1 shows the results of the first experiment. where diafiltration was performed with the addition of soft water. With initial microfiltration, we achieved 85.13 % yield of lactoferrin in the permeate fraction. In the next two steps of diafiltration, with the addition of 12 L of soft water in each step, the yield of lactoferrin was similar, amounting to 89.16 % in the first step of diafiltration and 85.28 % in the second step of diafiltration. With these two added steps of diafiltration, we achieved 93.9 % yield of lactoferrin. The second step of diafiltration contributed only 1.45 % of the total mass of lactoferrin.

In the second experiment (Table 2), the addition of soft water in the first experiment was replaced by FT (flow-through) fraction. With initial microfiltration, we achieved 65.81 % yield of lactoferrin in the permeate fraction. The yield of lactoferrin in the microfiltration process was 20 % lower than in the first experiment. In the next two steps of diafiltration, with the addition of 12 L FT fraction in each, the yield of lactoferrin in the first diafiltration was higher, amounting to 80.99 %, and in the second step of diafiltration, similar to the initial

microfiltration, 64.81 %. With both steps of diafiltration we achieved 87.90 % yield of the total mass of lactoferrin.

Table 1: Lactoferrin (LF) contents in retentate and permeate fraction during diafiltration with addition of soft water.

1. EXP.	PROTOCOL	Volume (L)	LF (mg)	LABEL	% initial lactoferrin	% recovery LF
LEVEL 0	MICROFILTRATION	30	5294.55	ACID WHEY	100	97.09
		27	4507.65	PERMEATE 0	85.13	
		3	134.78	RETENTATE 0	2.54	
LEVEL 1	ADDITION OF 12 L soft water	12	386.88	PERMEATE 1	7.3	89.15
		3	47.04	RETENTATE 1	0.88	
LEVEL 2	ADDITION OF 12 L soft water	12	77.04	PERMEATE 2	1.45	85.27
		3	13.3	RETENTATE 2	0.25	
				SUM	93.89	

Table 2: Lactoferrin (LF) contents in retentate and permeate fraction during diafiltration with addition of flow-through (FT) whey fraction.

2. EXP.	PROTOCOL	Volume (L)	LF (mg)	LABEL	% initial lactoferrin	% recovery LF
LEVEL 0	MICROFILTRATION	30	4690.75	ACID WHEY	100	65.81
		27	3040.35	PERMEATE 0	64.81	
		3	478.87	RETENTATE 0	10.2	
LEVEL 1	ADDITION OF 12 L FT fraction	12	738.77	PERMEATE 1	15.74	80.99
		3	173.3	RETENTATE 1	3.69	
LEVEL 2	ADDITION OF 12 L FT fraction	12	344.09	PERMEATE 2	7.33	64.81
		3	186.75	RETENTATE 2	3.98	
				SUM	87.9	

Conclusions

The diafiltration process in both experiments confirmed the possibility of increasing lactoferrin recovery in the permeate fraction of whey and thus a reduction in lactoferrin losses in the filtration process. Given that each additional filtration step represents a cost in terms of time and energy consumption, it is necessary to evaluate the profitability of additional steps in relation to the value of the obtained product. In the case of dilution with water, one dilution step has been shown to be satisfactory. However, in the case of dilution with FT whey fraction, the results were less consistent and more replicates would be needed to confirm the suitability of the FT fraction as a suitable solvent in the diafiltration process.

Acknowledgements

This research was co-funded by the project LIFE for Acid Whey (LIFE16 ENV/SI/000335) of the European financial instrument LIFE and the project LAKTIKA (OP20.03521) in the frame of the Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014 – 2020. We would also like to thank Mlekarna Celeia for regular weekly whey deliveries in the project's pilot phases.

Proučevanje možne rabe zgoščenega preostanka kisle sirotke po mikrofiltraciji

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Povzetek
V okviru nadaljnje rabe retentatne frakcije kisle sirotke, ki ostaja po mikrofiltraciji, smo proučili možnosti izrabe prisotne mikrobiološke kulture in velikega deleža beljakovin v tej frakciji. Iz neobdelanega retentata smo oblikovali izdelek za pospeševanje lakto-fermentacije organskih odpadkov. Na različne načine smo inaktivirali mikroorganizme v retentatu, ga posušili ter iskali možnosti uporabe tako dobljenega praška, bogatega z beljakovinami, v prehrani ljudi in živali. Zaključili smo, da inaktivirana posušena retentatna frakcija v prehrani ljudi ni primerna, ob primerni ceni pa je zelo zanimiva za uporabo kot krmni dodatek.

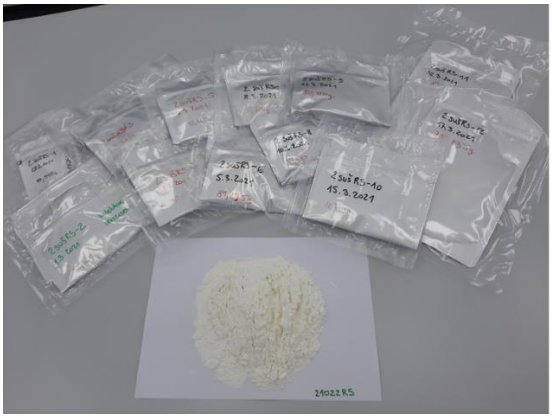
Ključne besede: retentatna frakcija kisle sirotke, mikrobiološka kultura, beljakovine, prehranski dodatki, krmni dodatki

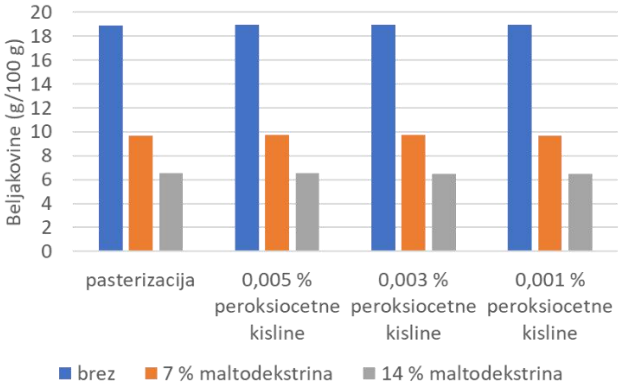
Uvod
Pri predelavi sirotke s ciljem izolacije proteinov ostane po mikrofiltraciji retentat (RS), ki predstavlja približno 10 % količine sirotke. RS poleg drugih snovi vsebuje veliko proteinov in primarno mlekarsko mikrobiološko kulturo. Predstavlja odpadek, ki se ga odpelje v obdelavo na bioplinarno. Nadaljnja uporaba RS bi pripomogla k zmanjševanju obremenjevanja okolja in zniževanju stroškov prevoza na bioplinarno.

RS je v okviru našega tehnološkega postopka vseboval povprečno 2,26 % beljakovin in je bil tako beljakovinsko najbolj bogata frakcija kisle sirotke. Beljakovine so pomemben del prehrane živali in ljudi. Za prehrano ljudi sta pomembni mikrobiološka in senzorična ustreznost produkta. Za uporabo kot krmni dodatek je bolj kot vonj in okus pomembna cena produkta.

Skupno število mikroorganizmov, ki ostanejo v RS frakciji kisle sirotke po mikrofiltraciji, je v povprečju 8,3 x 10^6. Prisotne mlečnokislinske bakterije v kombinaciji z laktozo, ki ostane v RS, lahko izkoristimo pri razgrajevanju organskih odpadkov, saj ga pospešujejo in usmerjajo v proces fermentacije, ki je mnogo koristnejši od drugih procesov bakteriološke razgradnje odpadkov.

Uporaba inaktivirane posušene retentatne frakcije
Preizkusili smo različne načine inaktivacije mikroorganizmov, prisotnih v RS, pred sušenjem z razprševanjem ali liofilizacijo. Ker prisotnost mlečne kisline v RS povzroča zlepljanje produkta, smo uporabili dodatek maltodekstrina, ki se v živilski industriji pogosto uporabljen za zmanjševanje zlepljanja suhih produktov in zmajševanje vsebnosti vlage v končnem produktu. V končnem produktu smo določili delež suhe snovi, število mikroorganizmov, kemijsko sestavo ter izvedli senzorični preskus. Najustreznejše lastnosti in ekonomsko upravičenost priprave sta pokazala vzorec RS inaktiviranega z 0,005 % peroksiocetno kislino brez maltodekstrina in vzorec RS inaktiviranega s pasterizacijo s 7 % maltodekstrinom, oba posušena z razprševanjem.





Slika 3: Levo - neobdelan retentat kisle sirotke brez dodatkov (bel prah) posušen z razprševanjem in zapakirani posušeni vzorci različno obdelanega retentata; desno - grafični prikaz vsebnosti beljakovin v vzorcih različno obdelanega retentata z ali brez dodatka maltodekstrina.

Proteinski dodatek v prehrani ljudi: Senzorično analizo vzorcev so izvedli na Inštitutu za mlekarstvo in probiotike. Pripravili so fermentiran mlečni izdelek z dodatkom RS. Izkazalo se je, da RS ni najbolj primeren kot dodatek k fermentiranim mlečnim izdelkov, ker že pri nižjem odstotku dodatka v mleko prispeva k nečistemu okusu končnega izdelka.

Proteinski dodatek v prehrani živali: Za uporabo kot krmni dodatek je zaželena čim večja vsebnost beljakovin v produktu. Največji delež beljakovin v izvedenem poskusu je bil v vzorcih brez dodanega maltodekstrina, nad 18 g beljakovin na 100 g produkta. Z dodatkom 7 % maltodekstrina je beljakovin manj kot 10 g/100 g, kar je še vedno mnogo več kot v sirotki v prahu, ki se običajno uporablja, a zaradi visoke cene le za občutljive kategorije živali - pujske in teleta, kjer cena krme prenese tak strošek. Produkt iz posušenega RS bi bil zato ob sprejemljivi ceni primeren za uporabo kot krmni dodatek.

Raziskali smo trg krmnih dodatkov in prišli do naslednjih zaključkov:

- Rejci prašičev bi bili zainteresirani za nakup sušenega koncentrata sirotke, seveda odvisno od cene. Zelo zainteresirani bi bili za nakup izolatov posameznih ali specifičnih (več skupaj) beljakovin sirotke domačega (slovenskega) proizvajalca.
- Vidimo možnost za sodelovanje z obrati za pridelovanje silaže za krmljenje predvsem mesnih pasem goveda v zvezi z možnostmi uporabe sirotke pri pridelavi oz. predelavi silaže.
- Beljakovinski produkt bi bil aktualen kot dopolnilno krmilo pri mačkah in psih, predvsem pri delovnih in športnih pasmah ter pri rehabilitaciji bolnih in podhranjenih živali. Zanimiv proizvod bi bili pasji priboljški z beljakovinskim dodatkom.
- Za konje bi v poštev prišla regeneracijska ploščica po tekmovanju ali daljši ježi.

Uporaba neobdelane retentatne frakcije

Mikrobiološko kulturo, prisotno v RS, smo uporabili za pripravo posipa za pospeševanje fermentacije organskih odpadkov, ki se predstavlja pod imenom LACTOPRO. Priprava je opisana v prispevku z naslovom »Izdelava posipa z aktivnimi mikroorganizmi iz ostankov sirotke za pospeševanje fermentacije organskih odpadkov in pripravo kompostne osnove«.

Slika 4: Poskusi, izvedeni z izrabo kulture mikroorganizmov iz zgoščene retentatne frakcije sirotke (RS). A - posip: posušen rastlinski material, inokuliran z mikrobiološko kulturo iz RS; B - s posipom pomešani organski odpadki v poskusu učinkovitosti posipa; C - bogatenje osiromašene zemlje s fermentiranimi organskimi odpadki; D - primerjava poganjkov v rastnem poskusu (levi so zrasli na obogateni zemlji, desni na osiromašeni).

Zaključki

S poskusi smo določili optimalni postopek inaktivacije mikroorganizmov in sušenja RS za pripravo praškastega produkta, ki je zelo bogat z beljakovinami. Za dodatek humani prehrani produkt sicer ni primeren zaradi neprijetnega okusa, raziskali smo pa možnosti uporabe kot krmni dodatek. Ob primerni ceni je zanimanja za takšen produkt na tržišču veliko.

S pripravo posipa za pospeševanje razgradnje organskih odpadkov smo pokazali, da lahko izkoristimo mikroorganizme, ki ostanejo v retentatni frakciji kisle sirotke po mikrofiltraciji in pripravimo uporaben produkt.

Zahvala

Raziskava je bila sofinancirana z evropskim finančnim instrumentom LIFE projekta LIFE16 ENV/SI/000335 Ponovna uporaba odpadne kisle sirotke za ekstrakcijo bioaktivnih beljakovin z visoko dodano vrednostjo, http://lifeforacidwhey.arhel.si . Za vire sirotke se zahvaljujemo mlekarni Celeia.

Studying the possible use of concentrated acid whey residue after microfiltration

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Abstract
We studied the possibilities of reusing the existing dairy starting culture from the primary fermentation and the large proportion of proteins from the retentate fraction of acid whey remaining after microfiltration. We designed a product to promote lacto-fermentation of organic waste from the untreated retentate. In addition, we inactivated the microorganisms in the retentate in various ways, dried it and searched for the possible use of the obtained protein-rich powder in human and animal nutrition. We concluded that the inactivated dried retentate fraction is unsuitable for human consumption but is very interesting for use as a feed additive at an acceptable price.

Keywords: retentate fraction of acid whey, microbiological culture, proteins, food additives, feed additives

Introduction
In whey processing with the aim of protein isolation, retentate (RS) remains after microfiltration. It represents about 10% of the amount of whey. In addition to other substances, RS contains a lot of proteins and primary dairy starter culture. It represents waste that is transported to a biogas plant for treatment. Further use of the RS would help reduce the environmental burden and the cost of transport to the biogas plant.

Retentate contained on average 2.26% protein in our technological process and was thus the most protein-rich fraction of acid whey. Proteins are an essential part of the animal and human diet. Besides, the microbiological and sensory suitability of the product is important for human consumption. For use as a feed additive, the product's price is more important than the smell and taste.

The total plate count of microorganisms remaining in the RS fraction of acid whey after microfiltration was on average 8.3 x 10^6. Lactic acid bacteria in combination with lactose remaining in the RS can be used in the decomposition of organic waste, as they accelerate and direct decomposition to the process of fermentation that is much more beneficial than other processes of bacteriological decomposition of waste.

Use of inactivated dried retentate fraction
We tested various inactivation methods of microorganisms present in the RS before spray drying or lyophilization. Since the presence of lactic acid in the RS potentially poses a problem due to the product's stickiness, we tried adding maltodextrin, which is often used in the food industry to reduce the stickiness of dry products and achieve lower moisture content in the final product. In the final product, we determined the dry matter content, the number of microorganisms, the chemical composition and performed a sensory test. The most suitable properties and economic justification presented RS inactivated with 0.005% peroxyacetic acid without added maltodextrin and RS inactivated with pasteurization with 7% maltodextrin addition, both spray-dried.

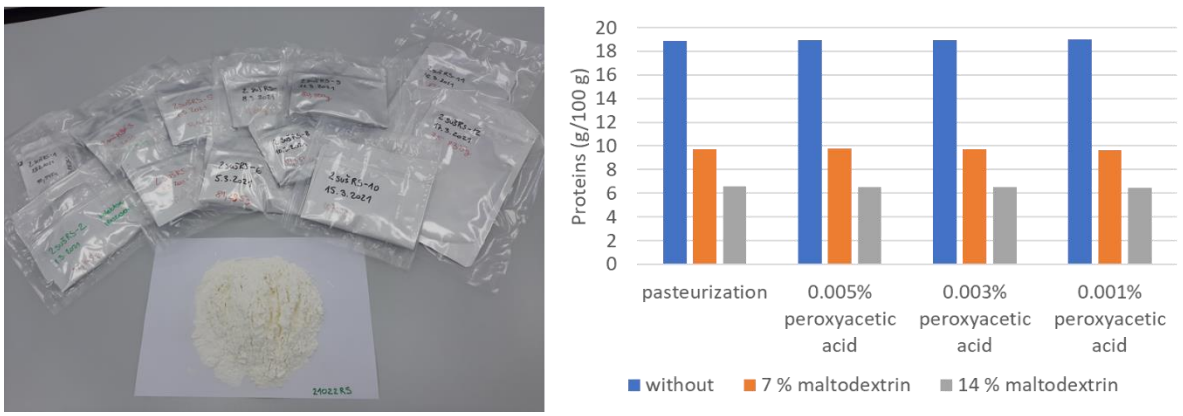

Figure 5: Left- spray-dried raw whey retentate without additives (white powder) and packaged dried samples of variously treated retentate; right-graphical representation of the protein content in samples of differently treated retentate with or without the addition of maltodextrin.

Protein additive in human nutrition: Sensory analysis of samples was performed by the Institute of Dairy Science and Probiotics. They prepared a fermented milk product with the addition of RS. It turned out that RS is not the most suitable as an additive to fermented dairy products because even with a lower percentage of additives in milk, it contributes to the impure taste of the final product.

Protein additive in animal nutrition: For use as a feed additive, the highest possible protein content in the product is desirable. In the experiment, the highest proportion of protein was in samples without added maltodextrin, above 18 g of protein per 100 g of product. With the addition of 7% maltodextrin, the protein content was less than 10 g / 100 g, which was still much more than in commonly used whey powders. The latter are to the high price only used for sensitive categories of animals - piglets and calves, where the feed price is worth the cost. Therefore, the dried RS product would be suitable as a feed additive at an affordable price.

We researched the market for feed additives and came to the following conclusions:

- Pig breeders would be interested in buying dried whey concentrate, of course, depending on the price. In addition, they would be interested in purchasing isolates of an individual or specific (several together) whey proteins from a domestic (Slovenian) producer.
- We see the possibility of cooperating with plants to produce silage for feeding mainly meat breeds of cattle in connection with the options of using whey in silage production or processing.
- The protein product would be suitable as a supplementary feed for cats and dogs, especially in working and sports breeds and in the rehabilitation of sick and malnourished animals. An interesting product would be dog treats with a protein additive.
- A regeneration bar after a race or a longer ride could be considered for horses.

Use of untreated retentate fraction

The residual of spent dairy starter culture in the RS was used to prepare a starter to promote the fermentation of organic waste, presented under the name LACTOPRO. The preparation procedure is described in the contribution entitled "Production of starter with active microorganisms from whey residues to promote fermentation of organic waste and preparation of compost base".

Figure 6: Experiments performed using the microbiological culture from the RS. A- starter: dried plant material inoculated with microbiological culture from RS; B - organic waste mixed with the starter to test its efficiency; C - enrichment of depleted soil with fermented organic waste; D - comparison of shoots in the growth experiment (the left grew on enriched soil, the right on depleted).

Conclusions

In the experiments, we determined the optimal procedures for the inactivation of microorganisms and drying of RS to prepare a protein-rich powder product. The product was not considered as suitable as a human food additive due to its unpleasant taste, but we have explored the possibility of using it as a feed additive. At a reasonable price, there is a lot of interest in such a product on the market.

With the produced starter for the acceleration of the decomposition of organic waste, we showed that we could use microorganisms that remain in the retentate fraction of acid whey after microfiltration and prepare a marketable product.

Acknowledgements

This research was co-funded by the project LIFE for Acid Whey - Reuse of waste acid whey for the extraction of bioactive proteins with high added value (LIFE16 ENV/SI/000335) of the European financial instrument LIFE. We thank Celeia Dairy for the delivery of whey.

Izdelava posipa z aktivnimi mikroorganizmi iz ostankov sirotke za pospeševanje fermentacije organskih odpadkov in pripravo kompostne osnove

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Povzetek
Razvita je bila inovativna rešitev uporabe ostanka kisle sirotke s pripravo posipa z aktivnimi mikroorganizmi za pospeševanje fermentacije organskih odpadkov in pripravo kompostne osnove. Izbran je bil primeren rastlinski nosilni material za obogatitev z aktivnimi mikroorganizmi iz predelave sirotke. Proces inokulacije in zorenja posipa je bil eksperimentalno optimiziran. Preizkušen je bil vpliv posipa na hitrost in smer fermentacije organskih odpadkov ter izvedeni rastni poskusi, ki so pokazali, da je dobljeni kompost primeren za obogatitev tal. Posip je bil izdelan v polindustrijskem obsegu in uspešno promoviran potencialnim kupcem.

Ključne besede: mlečnokislinska fermentacija, aktivni mikroorganizmi, ostanki sirotke, organski odpadki, posip za kompostiranje

Uvod
Eden od ciljev projekta LIFE for Acid Whey je bil poiskati možnosti popolne predelave ostanka kisle sirotke po selektivni izolaciji sirotkinih proteinov brez odpadkov po principu krožnega gospodarstva. V procesu predelave sirotke ostajajo in nastajajo raztopine, bogate z mlečnokislinskimi bakterijami in kvasovkami ter sladkorji. Ti mikroorganizmi so zmožni v kombinaciji s sladkorji pospešiti fermentacijo organskih odpadkov, ki je eden od načinov razgradnje organskih odpadkov, in usmerjati kompostiranje v večjo učinkovitost. Uveljavljena je tradicionalna japonska tehnika priprave fermentacijskega starterja Bokashi za spodbujanje fermentacije organskih odpadkov, kjer so potrebne tri surovine: voda, efektivni mikroorganizmi (komercialna proizvodnja) in vir sladkorja (običajno v obliki melase). Določeni ostanki sirotke predstavljajo vir vode, sladkorjev in efektivnih mikroorganizmov, ki lahko nadomestijo tri surovine pri pripravi posipa za pospeševanje fermentacije organskih odpadkov in pripravo kompostne osnove.

Razvoj produkta
Tekom izvajanja projekta smo razvili postopek uporabe kulture mikroorganizmov iz predelave sirotke za izdelavo posipa za pospeševanje fermentacije. Posip se predstavlja pod imenom LACTOPRO.

Razvoj produkta je potekal v več fazah:
1. Izbor rastlinskega nosilnega materiala, ki podpira nadaljnji razvoj mikrobne flore, dobro zadržuje tekočino in omogoča preživetje mikroorganizmov po sušenju.
2. Opredelitev priprave ostankov kulture in sirotke za inokulacijo rastlinskega materiala za doseganje čim višjega inokuluma.
3. Opredelitev načina zorenja inokuliranega posipa za čim bolj učinkovito rast koristnih mikroorganizmov.
4. Opredelitev načina sušenja zrelega posipa za doseganje čim večjega preživetja koristnih mikroorganizmov.
5. Testiranje vpliva posipa na hitrost in smer fermentacije organskih odpadkov.
6. Testiranje pridobljenega komposta z lakto-fermentacijskim posipom na rast rastlin v rastnih poskusih.
7. Proizvodnja posipa v polindustrijskem obsegu (predelava 330 kg celuloznega materiala).
8. Promocija produkta potencialnim strankam in anketiranje za ugotavljanje povpraševanja.
9. Pregled proizvodnih stroškov in izdelava poslovnega načrta.

Za testiranje trga smo končni produkt testne proizvodnje poimenovali “LACTOPRO - posip za biološke odpadke iz gospodinjstva”. Posip LACTOPRO se dodaja organskim odpadkom za pospeševanje in usmerjanje pretvorbe organskih odpadkov z mlečnokislinsko fermentacijo, ki poteka v zatesnjenih posodah. Posip lahko uporabimo tudi za pripravo bioloških odpadkov na kompostiranje v vrtnem kompostniku ali pa se uporablja za takojšnje izboljšanje kakovosti vrtnih tal ali rastlinskih substratov.

Glavni zaključki

Posebnost posipa LACTOPRO je celoten koncept priprave in namen uporabe, ki stremi k uporabi odpadnih surovin in s tem izboljševanju stanja okolja. Pri razvoju produkta smo združili uporabo stranskih proizvodov iz rastlinske pridelave, predelave lesa, mleka in sirotke. Zgoščeno sirotko z ostanki sladkorja in živo izrabljeno mlečnokislinsko kulturo smo uporabili kot kombiniran rastni substrat z vsebujočo startersko kulturo, s katero smo inokulirali in pripravili celulozno bazo starterja za pospeševanje fermentacije organskih odpadkov.

S testiranjem različnih celuloznih nosilcev smo ugotovili razlike v sposobnosti vezave vlage med žaganjem, peleti slame in sekanci. Največje vrednosti skupnega števila mikroorganizmov (SŠMO) na celuloznem nosilcu so bile dosežene ob uporabi zorenega retentata za inokulacijo in inkubaciji v anaerobnoh pogojih.

Inokulacija kuhinjskih odpadkov s pripravljenim posipom in inkubacija v zatesnjenih kompostnikih je pokazala, da se je na organskih nosilcih namnožilo dovolj mikroorganizmov, da so v anaerobnih pogojih pripomogli k fermentaciji organskih odpadkov. Po vkopavanju fermentiranih organskih ostankov v osiromašeno zemljo je razgradnja le-teh pripomogla k obogatitvi zemlje z organsko snovjo in hranili. Rastni poskus vzgoje kitajskega zelja na različno obravnavani zemlji je pokazal, da fermentirani organski ostanki, vkopani v zemljo, ugodno vplivajo na rast rastlin.

V pilotnem poskusu smo pripravili 400 vrečk po 800 g posipa za pretvorbo organskih odpadkov v rastlinski substrat. Predelali smo 330 kg žitnih plev, ki so predstavljale substrat za namnožitev koristnih mikroorganizmov, plesni in kvasovk, ter približno 135 L inokuluma, predvsem retentata kisle sirotke (pridobljenega iz približno 5000 L sveže kisle sirotke), nekaj tudi izrabljenih gojišč kefirnih zrn in samih kefirnih zrn. Primerjali smo različne načine priprave posipa in izbrali optimalni postopek glede na stroške in praktičnost priprave ter kakovost substrata, ocenjeno s stopnjo inokulacije.

Slika 7:Graf na levi- primerjava vrednosti skupnega števila mikroorganizmov ter plesni in kvasovk na substratu pred inokulacijo in na posušenem inokuliranem substratu, iz katerega smo z mletjem pripravili posip. Srednja slika- sušenje posipa. Desna- zapakiran produkt.

Način izdelave laktofermentacijskega posipa z uporabo zgoščene frakcije fermentirane kisle sirotke ali izrabljenih gojišč mlečnokislinskih mikroorganizmov gojenih v sirotki smo patentirali (patentna prijava P-202100089, Postopek izdelave posipa za pospešitev procesov fermentacije organskih odpadkov).

Produkt LACTOPRO je bil predstavljen na promocijskem dogodku v vrtnem centru, kjer je bilo med obiskovalce razdeljenih 328 vrečk posipa. Za izvedbo dogodka je bil pripravljen promocijski material. Iz rezultatov ankete, pripravljene za dogodek, sklepamo, da je pri kupcih v vrtnih centrih zanimanje za tovrsten posip veliko. Predvsem je kupce zanimalo pospeševanje kompostiranja v vrtnih kompostnikih. Večina vprašanih bi kupila posip LACTOPRO.

Zahvala

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Literatura

Zupančič Justin M, Čič M., Rozina T., Postopek izdelave posipa za pospešitev procesov fermentacije organskih odpadkov. Patentna prijava št P-202100089. Urad za intelektualno lastnino, 2021.

Production of starter with active microorganisms from whey residues to promote fermentation of organic waste and preparation of compost base

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Abstract
An innovative solution of the use of acid whey residue was developed by preparing a starter with active microorganisms for enhancing organic waste fermentation and preparation of a compost base. A suitable plant carrier material was chosen to be enriched with active microorganisms from whey processing. The process of inoculation and maturation of the starter was experimentally optimised. The influence of the starter on the rate and direction of fermentation of organic waste was tested, and growth experiments were conducted, which showed that the obtained compost is suitable for the enrichment of the soil. The starter was produced on a semi-industrial scale and successfully promoted to potential customers.

Keywords: lacto-fermentation, active microorganisms, whey residue, organic waste, compost starter

Introduction
One of the objectives of LIFE for Acid Whey project was to provide options for complete processing of acid whey residue after a selective extraction of whey proteins in a zero-waste manner based on the circular economy principle. During whey processing, solutions rich in lactic acid bacteria, yeasts and sugars remain and/or are formed. In combination with sugars, these microorganisms can accelerate the fermentation of organic waste, which is one of the ways of decomposing organic waste, and direct composting to greater efficiency. A traditional Japanese technique of preparing a Bokashi fermentation starter to promote the fermentation of organic waste is well known. Three raw materials are needed: water, effective microorganisms (commercial production) and a source of sugar (usually in the form of molasses). Certain whey residues are a source of water, sugars and effective microorganisms that can replace the three raw materials in the preparation of the starter to promote the fermentation of organic waste and preparation of compost base.

Product development
During the implementation of the project, we developed a process to use the culture of microorganisms from the whey processing in making a starter for the acceleration of fermentation of organic waste. The starter is presented under the name LACTOPRO.

Product development took place in several stages:
1. The selection of plant carrier material, which supports further development of the microbial flora, retains liquid well and maintains the survival of microorganisms after drying.
2. Defining the preparation of culture and whey residues for inoculation of plant material to achieve the highest possible inoculum.
3. Defining the maturation of the inoculated starter for the optimal development of beneficial microorganisms.
4. Definition of the method of drying the matured starter to achieve the highest possible survival of beneficial microorganisms.
5. Testing the influence of the starter on the rate and direction of fermentation of organic waste.
6. Testing of obtained compost with lacto-fermentation starter on plant growth in planting experiments.
7. Production of a starter on a semi-industrial scale (process 330 kg of cellulose material).
8. Promotion of the product to potential customers and surveying to determine demand.
9. Review of production costs and elaborate a business plan.

We named the final product of test production “LACTOPRO - bran for household bio-waste” for market testing. The LACTOPRO bran is added to organic waste as a starter media to accelerate and direct the transformation of organic waste through lactic acid fermentation, carried out in air-tightly closed containers. The starter can also prepare biological waste for composting in a garden composter or is applied to immediately improve garden soils or plant substrates.

Main conclusions

The entire concept of preparation of the starter and the purpose of the use is unique since it aims to use waste materials and thus improve the state of the environment. Within product development, we combined by-products from crop production, wood processing, milk, and whey processing. Concentrated whey with residual sugars and live spent lactic acid culture were used as a combined growth substrate with a containing starter culture with which we inoculated and prepared the cellulosic base of the starter to promote the fermentation of organic waste.

Initial experiments of testing the carrier materials showed that sawdust, straw pellets, and wood chips of two sizes have a different potential for moisture uptake. In addition, the development of the microbial flora on the carrier material was increased by pre-maturing the whey concentrates before application to the plant carrier material and by exposure of the carrier material to anaerobic incubation.

Inoculation of kitchen waste with the ready starter and incubation in closed containers showed that a sufficient number of microorganisms have multiplied on organic carriers to contribute to the fermentation of organic waste under anaerobic conditions. Mixing fermented organic residues in organic-matter depleted soil and their further decomposition enriched the soil with organic matter and nutrients. A growth experiment of growing Chinese cabbage on differently treated soil has shown that fermented organic residues buried in the soil have a beneficial effect on plant growth.

In a pilot experiment, we prepared 400 bags of 800 g of dry starter to be used for the acceleration of the conversion of organic waste into plant substrate. We processed 330 kg of cereal husks, the carrier material for the multiplication of beneficial microorganisms, moulds and yeasts, and about 135 L of inoculum, mainly concentrated residual of acid whey (obtained from about 5000 L of fresh acid whey). We also used spent media from kefir grains cultivation and kefir grains themselves in some batches. We compared different bran preparation methods and selected the optimal procedure according to the cost and practicality of preparation and the quality of the substrate, assessed by the degree of inoculation.

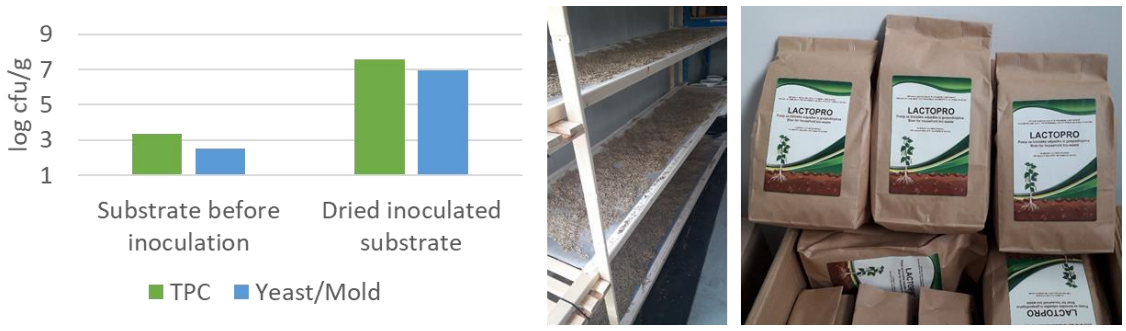

Figure 2: Left- total plate count, yeast and mould on the substrate before inoculation and on the dried inoculated substrate from which the starter was prepared. Middle-drying of the starter. Right- packaged product.

A method of making a dry lacto-fermentation starter using a concentrated fraction of fermented acid whey or spent media of lactic acid microorganisms grown in whey was patented (patent application P-202100089, Process for making a spread to accelerate the fermentation of organic waste).

The product LACTOPRO was presented at a promotional event at a garden centre, where 328 bags of the starter were distributed among the interested visitors. Promotional material was prepared for the event. Results of a survey, prepared for the event show that customers in garden centres are very interested in this product. Buyers were mainly interested in the acceleration of composting in garden composters. Most respondents would buy LACTOPRO starter.

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CLOSURE CONFERENCE OF THE PROJECT

LIFE16 ENV/SI/000335 LIFE for Acid Whey

Reuse of waste acid whey for the extraction of bioactive proteins with high added value