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Zespół badawczy chemii (bio)kolooidów

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**Opracowanie metodologii do kompleksowej analizy biokolooidów
oraz ich wykorzystanie na potrzeby przemysłu mleczarskiego**

Rozprawa doktorska wykonana pod kierunkiem

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**„Development of methodology for a comprehensive study of
biocolloids and their application in dairy industry”**

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- Dofinansowanie badań w ramach VI edycji konkursu IDUB/granty dla doktorantów (mobilność i badania) „Evaluation the impact of ultrafiltration process on the glycosylation heterogeneity in bovine lactoferrin”.
- Dofinansowanie badań w ramach IX edycji konkursu IDUB/granty dla doktorantów (mobilność i badania) „The comprehensive investigation on lactoferrin-ruthenium complex formation: insight into isothermal and kinetic studies”.



Spis treści

Wykaz skrótów.....	7
Wprowadzenie.....	9
1. Definicja układów koloidalnych oraz ich przykłady w mleczarstwie.....	13
2. Aktualne problemy przemysłu mleczarskiego.....	15
3. Fortyfikacja produktów mleczarskich.....	19
3.1. Globalny problem niedoboru witaminy D oraz sposoby jej dostarczania do organizmu.....	19
3.2. Nowe podejście fortyfikacji żywności w witaminę D.....	20
3.3. Wpływ profilu kwasów tłuszczowych na właściwości funkcjonalne żywności....	22
4. Kazeina: struktura, stabilność oraz rola biologiczna białka.....	24
4.1. Układ micelarny kazeiny oraz jego stabilność pod wpływem różnych czynników.....	24
4.2. Metody izolacji kazeiny.....	26
4.3. Problem zasolenia białek.....	28
4.4. Kazeina – idealny nośnik cząsteczek bioaktywnych.....	28
4.5. Rola jonów metali w funkcjonalności białek.....	29
4.5.1. Związki srebra: aktywność biologiczna, zastosowanie w medycynie oraz problem cytotoksyczności.....	29
4.5.2. Modyfikacja kazeiny srebrem jako sposób otrzymywania biokompatybilnych środków antyseptycznych.....	31
5. Laktoferyna – białko o unikatowej strukturze oraz funkcjonalności.....	33
5.1. Struktura trójwymiarowa bLF.....	35
5.2. Właściwości fizykochemiczne LF o różnym stopniu nasyceniu Fe(III).....	35
5.3. Rola glikozylacji w LF: aspekt strukturalny oraz biologiczny.....	36
5.4. Metody izolacji oraz oczyszczania LF.....	37
5.5. Oddziaływanie LF z jonami metali.....	39
5.5.1. Właściwości biologiczne kompleksów rutenu.....	39
5.5.2. Projektowanie kompleksów LF-Ru jako potencjalne podejście w terapii celowanej.....	40
6. Bibliografia.....	45
Cel pracy.....	57

Pełne wersje publikacji:

[P1] Immobilization of Silver Ions onto Casein.....	59
[P2] The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties.....	76
[P3] Impact of Ultrafiltration on the Physicochemical Properties of Bovine Lactoferrin: Insights into Molecular Mass, Surface Morphology, and Elemental Composition.....	109
[P4] The Impact of Lactiplantibacillus Plantarum on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition.....	130
[P5] Lactoferrin as an Active Coordination Scaffold for Ruthenium(III).....	154
Podsumowanie i wnioski	196
Streszczenie	200
Abstrakt	202
Dorobek naukowy	204
Oświadczenia współautorów	209

Wykaz skrótów

- ATR-FTIR** – spektroskopia osłabionego całkowitego odbicia w podczerwieni z transformacją Fouriera (*ang. attenuated total reflectance Fourier transform infrared spectroscopy*)
- BZT** – biologiczne zapotrzebowanie na tlen (*ang. biochemical oxygen demand*)
- bLF** – laktoferyna bydlęca (*ang. bovine lactoferrin*)
- CCP** – koloidalny fosforan wapnia (*ang. colloidal calcium phosphate*)
- CD** – spektroskopia dichroizmu kołowego (*ang. circular dichroism spectroscopy*)
- ChZT** – chemiczne zapotrzebowanie na tlen (*ang. chemical oxygen demand*)
- CN** – kazeina (*ang. casein*)
- DLS** – dynamiczne rozpraszanie światła (*ang. dynamic light scattering*)
- DLVO** – teoria Derjaguina-Landaua-Verweya-Overbeeka (*ang. Derjaguin-Landau-Verwey-Overbeek theory*)
- D_{max}** – maksymalny wymiar cząstki (*ang. maximum particle dimension*)
- DNA** – kwas deoksyrybonukleinowy (*ang. deoxyribonucleic acid*)
- EDS** – spektroskopia dyspersji energii promieniowania rentgenowskiego (*ang. energy-dispersive X-ray spectroscopy*)
- GC-MS** – chromatografia gazowa sprzężona ze spektrometrią mas (*ang. gas chromatography-mass spectrometry*)
- GMP** – glikomakropeptyd (*ang. glycomacropeptide*)
- HSAB** – teoria twardych i miękkich kwasów i zasad (*ang. Hard and Soft Acids and Bases theory*)
- K_D** – stała dysocjacji (*ang. dissociation constant*)
- LAB** – bakterie kwasu mlekowego (*ang. lactic acid bacteria*)
- LDL** – lipoproteiny o małej gęstości (*ang. low-density lipoprotein*)
- LM** – mikroskopia świetlna (*ang. light microscopy*)
- MALDI-TOF/MS** — spektrometria mas z desorpcją/ionizacją laserową wspomaganą matrycą z analizatorem czasu przelotu (*ang. matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*)
- MD** – dynamika molekularna (*ang. molecular dynamics*)
- MS** – spektrometria mas (*ang. mass spectrometry*)
- MTT** – bromek 3-(4,5-dimetylotiazol-2-yl)-2,5-difenylo-tetrazoliowy (*ang. 3-(4,5-di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide*)
- MUFA** – jednonienasycone kwasy tłuszczowe (*ang. monounsaturated fatty acids*)

OECD-FAO – Organizacja Współpracy Gospodarczej i Rozwoju oraz Organizacja Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (*ang. Organisation for Economic Co-operation and Development-Food and Agriculture Organization of the United Nations*)

pI – punkt izoelektryczny (*ang. isoelectric point*)

PTM – modyfikacje potranslacyjne (*ang. post-translational modifications*)

PUFA – wielonienasycone kwasy tłuszczowe (*ang. polyunsaturated fatty acids*)

q_{max} – maksymalna pojemność sorpcyjna (*ang. maximum sorption capacity*)

R_g – promień bezwładności (*ang. radius of gyration*)

RNA – kwas rybonukleinowy (*ang. ribonucleic acid*)

ROS – reaktywne formy tlenu (*ang. reactive oxygen species*)

RP-HPLC – wysokosprawna chromatografia cieczowa w odwróconym układzie faz (*ang. reverse-phase high-performance liquid chromatography*)

SAXS – małokątowe rozpraszanie promieniowania rentgenowskiego (*ang. small-angle X-ray scattering*)

SDS-PAGE – elektroforeza w żelu poliakrylamidowym w warunkach denaturacyjnych (*ang. sodium dodecyl sulfate polyacrylamide gel electrophoresis*)

SEM-EDS – skaningowa mikroskopia elektronowa z dyspersją energii promieniowania rentgenowskiego (*ang. scanning electron microscopy with energy-dispersive X-ray spectroscopy*)

SFA – nasycone kwasy tłuszczowe (*ang. saturated fatty acids*)

SRCD – spektroskopia dichroizmu kołowego z wykorzystaniem promieniowania synchrotronowego (*ang. synchrotron radiation circular dichroism*)

STEM – skaningowa transmisyjna mikroskopia elektronowa (*ang. scanning transmission electron microscopy*)

TEM-EDS – transmisyjna mikroskopia elektronowa z dyspersją energii promieniowania rentgenowskiego (*ang. transmission electron microscopy with energy-dispersive X-ray spectroscopy*)

TF – transferyna (*ang. transferrin*)

UV-Vis – spektroskopia w zakresie ultrafioletu oraz światła widzialnego (*ang. ultraviolet-visible spectroscopy*)

UVB – promieniowanie ultrafioletowe B (*ang. ultraviolet B radiation*)

WPC – koncentrat białek serwatkowych (*ang. whey protein concentrate*)

WPI – izolat białek serwatkowych (*ang. whey protein isolate*)

ζ – potencjał zeta (*ang. zeta potential*)

Wprowadzenie

Przemysł mleczarski należy do najważniejszych sektorów gospodarki rolno-spożywczej, jednak jego dalszy rozwój wiąże się obecnie z koniecznością sprostania licznym wyzwaniom o charakterze ekonomicznym, technologicznym oraz środowiskowym. Do kluczowych problemów należą rosnące koszty produkcji, wynikające przede wszystkim ze wzrostu cen energii, surowców oraz utrzymania infrastruktury, którym często towarzyszy spadek opłacalności wytwarzania produktów mleczarskich. Sytuacja ta wymusza poszukiwanie nowych rozwiązań pozwalających zwiększyć wartość dodaną produktów, poprawić efektywność wykorzystania surowca oraz wzmocnić konkurencyjność przedsiębiorstw na rynku. Dodatkowym wyzwaniem pozostaje powstawanie dużych ilości produktów ubocznych, takich jak serwatka, permeaty czy inne frakcje poprodukcyjne, które z jednej strony stanowią istotne obciążenie środowiskowe, a z drugiej są cennym źródłem składników bioaktywnych możliwych do odzysku i dalszego zagospodarowania.

W szerszej perspektywie ekonomicznej współczesne mleczarstwo coraz wyraźniej odchodzi od modelu opartego wyłącznie na wolumenie produkcji i sprzedaży standardowych wyrobów masowych. Prognozy Organizacji Współpracy Gospodarczej i Rozwoju oraz Organizacji Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. Organisation for Economic Co-operation and Development-Food and Agriculture Organization of the United Nations – OECD-FAO) wskazują, że światowa produkcja mleka będzie nadal rosła w perspektywie dekady, natomiast w Unii Europejskiej (UE) spodziewana jest raczej stagnacja podaży przy utrzymującym się popycie na sery oraz inne produkty o wysokiej wartości dodanej. Komisja Europejska równolegle podkreśla, że to właśnie produkty wysokowartościowe, a nie sam surowiec, w największym stopniu wzmacniają pozycję konkurencyjną unijnego sektora na rynkach międzynarodowych. Oznacza to, że o przewadze przedsiębiorstw mleczarskich będzie w coraz większym stopniu decydować zdolność do frakcjonowania mleka, standaryzacji jego jakości i projektowania wyspecjalizowanych ingrediencji funkcjonalnych [1–3].

Znaczenie tego kierunku szczególnie dobrze widać na rynku polskim. Utrzymywanie silnej pozycji eksportowej przez krajowy sektor mleczarski opiera się nie tylko na skali produkcji, lecz również na strukturze sprzedaży zagranicznej, w której istotny udział mają sery, twarogi, mleko płynne, śmietana oraz tłuszcze mleczne. Dane Krajowego Ośrodka Wsparcia Rolnictwa (KOWR) pokazują, że w 2025 roku rosły zarówno dostawy surowca do skupu, jak i wartość eksportu produktów mlecznych, co z jednej strony potwierdza potencjał sektora, z drugiej jednak wzmacnia presję na poszukiwanie segmentów bardziej odpornych na wahania cen

energii, kosztów logistyki i koniunktury surowcowej. W praktyce oznacza to potrzebę przechodzenia od sprzedaży produktu standardowego do budowania portfela produktów premium, specjalistycznych i silniej zróżnicowanych funkcjonalnie [4,5].

Z tego względu coraz większego znaczenia nabiera projektowanie produktów wzbogacanych, których skład oraz właściwości odpowiadają aktualnym potrzebom żywieniowym oraz zdrowotnym społeczeństwa. Szczególne znaczenie ma to w odniesieniu do związków, których niedobór stanowi istotny problem zdrowia publicznego, takich jak witamina D. Odpowiednio dobrane matryce mleczarskie mogą sprzyjać stabilizacji oraz biodostępności związków lipofilowych, a zastosowanie biologicznych metod wzbogacania, opartych na przykład na aktywności metabolicznej bakterii kwasu mlekowego, pozwala na rozwijanie bardziej naturalnych oraz technologicznie atrakcyjnych strategii fortyfikacji żywności.

Równocześnie wzrasta znaczenie modelu gospodarki obiegu zamkniętego, w którym mleko i jego frakcje traktowane są jako surowiec wielostrumieniowy. Najnowsze opracowania wskazują, że serwatka, permeaty, maślanka oraz frakcje uzyskiwane w procesach membranowych mogą stanowić źródło białek, peptydów bioaktywnych, laktozy, substratów fermentacyjnych, a nawet materiałów do wytwarzania aktywnych opakowań i komponentów bio-based. W takim ujęciu o atrakcyjności technologii decyduje nie tylko wydajność pojedynczej operacji, lecz zdolność do kaskadowego wykorzystania surowca – od odzysku składników odżywczych, przez projektowanie matryc funkcjonalnych, aż po wytwarzanie produktów o zastosowaniach żywieniowych, farmaceutycznych oraz biomateriałowych. Badania nad biokoloïdami pochodzenia mleczarskiego wpisują się więc bezpośrednio w realne potrzeby rynku związane z wysoką waloryzacją surowca [6–11].

Na tym tle szczególnie wyraźnie rysuje się nowość naukowa niniejszej rozprawy. Surowce mleczne nie są tu analizowane wyłącznie jako klasyczne składniki żywności, lecz jako dynamiczne układy koloidalne, których funkcjonalność może być programowana na poziomie struktury, ładunku powierzchniowego, dostępności grup donorowych i zdolności do transportu cząsteczek aktywnych. Takie podejście odpowiada najnowszym trendom badań nad przebudowanymi micelami kazeinowymi, biomateriałami na bazie kazeiny oraz wielofunkcyjnymi kompleksami laktoferyny, w których właściwości biologiczne stają się bezpośrednim skutkiem kontrolowanej organizacji molekularnej. W rezultacie przedstawione badania tworzą pomost pomiędzy analityką, chemią koordynacyjną, technologią mleczarską i projektowaniem materiałów funkcjonalnych [12–17].

Równolegle coraz większe znaczenie zyskują badania nad ukierunkowaną modyfikacją białek mleka w celu nadania im nowych lub ulepszonych właściwości funkcjonalnych. Jednym z

najbardziej obiecujących kierunków jest funkcjonalizacja białek jonami metali, która może prowadzić do zmian w organizacji strukturalnej biocząsteczek, a jednocześnie umożliwiać projektowanie nowych układów nośnikowych oraz bioaktywnych. W niniejszej pracy do projektowania tego rodzaju układów wykorzystano dwa białka mleka, tj. kazeinę oraz laktoferynę. W przypadku kazeiny szczególne znaczenie ma jej amfifilowy charakter, który stwarza szerokie możliwości modyfikacji chemicznej oraz wykorzystania tego białka jako nośnika w projektowaniu układów o nowych właściwościach funkcjonalnych, w tym o aktywności przeciwbakteryjnej oraz ograniczonej cytotoksyczności względem wolnych jonów metali. W przypadku laktoferyny jej unikatowe cechy strukturalne, szeroki zakres aktywności biologicznej oraz zdolność do swoistego oddziaływania z receptorami komórkowymi sprawiają, że może ona stanowić obiecującą platformę do projektowania kompleksów metalobiałkowych o potencjale biomedycznym. Takie podejście otwiera nowe możliwości wykorzystania składników mleka nie tylko jako substancji odżywczych, lecz również jako funkcjonalnych materiałów do zastosowań zaawansowanych.

Złożoność układów mleczarskich oraz wielokierunkowy charakter ich możliwych modyfikacji sprawiają, że ich badanie wymaga zastosowania kompleksowego podejścia metodologicznego. Konieczne jest jednocześnie uwzględnienie właściwości strukturalnych, koloidalnych, fizykochemicznych oraz biologicznych, a także ocena wpływu kontrolowanej modyfikacji na funkcjonalność badanych układów.

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Na rozprawę doktorską składa się pięć prac naukowych opublikowanych w latach 2023-2026.

[P1] Tetiana Dyrda-Terniuk, Oleksandra Pryshchepa, Katarzyna Rafińska, Mateusz Kolankowski, Adrian Gołębiowski, Michał Gloc, Renata Dobrucka, Krzysztof Kurzydłowski, Paweł Pomastowski. Immobilization of Silver Ions onto Casein. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **2023**, 667, 131390.

(IF = 5.4; 70 pkt. MNiSW)

[P2] Tetiana Dyrda-Terniuk, Paweł Pomastowski. The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties. *Journal of Agricultural and Food Chemistry*, **2023**, 71, 20500–20531.

(IF = 6.2; 140 pkt. MNiSW)

[P3] Tetiana Dyrda-Terniuk Paweł Pomastowski. Impact of Ultrafiltration on the Physicochemical Properties of Bovine Lactoferrin: Insights into Molecular Mass, Surface Morphology, and Elemental Composition. *Journal of Dairy Science*, **2024**, 107, 10280–10298.

(IF = 4.4; 200 pkt. MNiSW)

[P4] Tetiana Dyrda-Terniuk, Viorica Railean, Aleksandra Florkiewicz, Justyna Walczak-Skierska, Mateusz Kolankowski, Joanna Rudnicka, Dorota Białczak, Paweł Pomastowski. The Impact of Lactiplantibacillus Plantarum on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition. *International Dairy Journal*, **2025**, 161, 106118.

(IF = 3.4; 100 pkt. MNiSW)

[P5] Tetiana Dyrda-Terniuk, Michalina Ehlert, Kacper Roszak, Grzegorz Trykowski, Kamil Szpotkowski, Łukasz Skowroński, Oleksandra Pryshchepa, Rudi van Eldik, Katarzyna Mizgalska, Wayne Guida, Aleksandra Karolak, Riddhi Sainda, Prafulla K Jha, Paweł Pomastowski. Lactoferrin as an Active Coordination Scaffold for Ruthenium(III). *Inorganic Chemistry Frontiers*, **2026** (doi.org/10.1039/d6qi00255b)

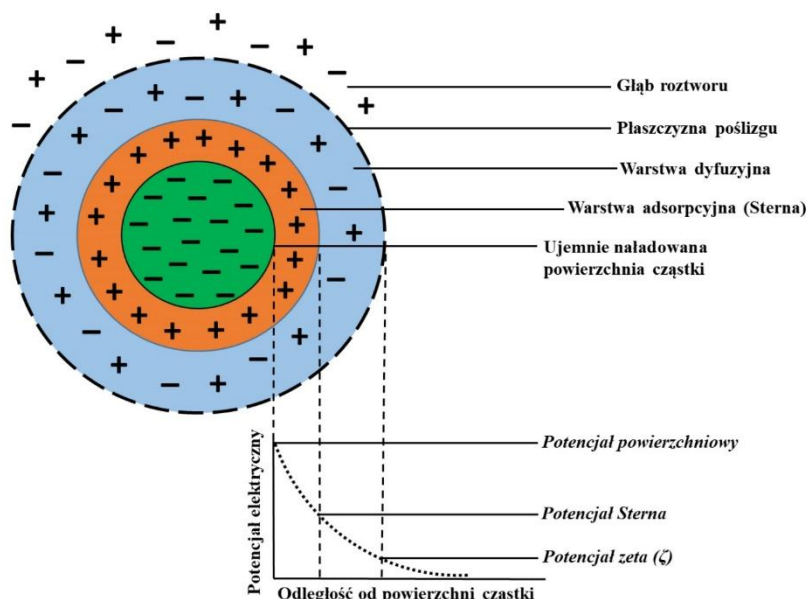
(IF = 6.4; 140 pkt. MNiSW)

$\sum \text{IF} = 25.8$ $\sum \text{MNiSW} = 650 \text{ pkt.}$

1. Definicja układów koloidalnych oraz ich przykłady w mleczarstwie

Koloidami nazywane są heterogeniczne (niejednorodne) układy dyspersyjne, stanowiące stan pośredni pomiędzy roztworem właściwym a zawiesiną. Wielkość cząstek fazy rozproszonej w układach koloidalnych mieści się zazwyczaj w zakresie od 1 nm do 1 μ m. Jednym z rodzajów układów koloidalnych są biokoloidy. Biokoloidy definiuje się jako układy koloidalne, w których fazę rozproszoną stanowią obiekty pochodzenia biologicznego, takie jak białka, bakterie, wirusy, algi czy pierwotniaki [18].

Cząstki koloidalne posiadają ładunek, który na ogół pochodzi od zjonizowanych grup funkcyjnych znajdujących się na ich powierzchni oraz od jonów adsorbowanych z roztworu. Tutaj warto wprowadzić pojęcie „podwójnej warstwy elektrycznej”. Podwójna warstwa elektryczna tworzy się na granicy faz pomiędzy cząstką koloidalną a ośrodkiem dyspersyjnym oraz odgrywa kluczową rolę w stabilności układu koloidalnego (**Rysunek 1**). Istnieją kilka modeli, które opisują budowę podwójnej warstwy elektrycznej, w tym model Helmholtza, model Gouya-Chapmana oraz model Sterna. Podstawową koncepcję podwójnej warstwy elektrycznej po raz pierwszy wprowadził Hermann von Helmholtz w drugiej połowie XIX wieku. Według tego modelu podwójna warstwa elektryczna składa się z warstwy ładunku cząstki oraz przeciwjonów, które tworzą nieruchomą, zwartą warstwę przy jej powierzchni. Model ten miał jednak pewne ograniczenia, ponieważ nie uwzględniał ruchów termicznych ani dyfuzji jonów w fazie ciekłej. Na początku XX wieku Gouy i Chapman przedstawili udoskonaloną wersję modelu podwójnej warstwy elektrycznej, w której uwzględniono ruchliwość jonów. Zgodnie z tym modelem przeciwjony nie są unieruchomione przy powierzchni naładowanej cząstki, lecz są rozproszone w otaczającym roztworze, tworząc warstwę rozmytą (dyfuzyjną). Model ten nie uwzględniał jednak zjawiska specyficznej adsorpcji ani tworzenia się zwartej warstwy jonów bezpośrednio przy powierzchni cząstki. Łącząc założenia obu wcześniejszych modeli, w 1924 roku Stern zaproponował nowy model podwójnej warstwy elektrycznej. Model Sterna zakłada, że podwójna warstwa elektryczna składa się z warstwy przeciwjonów przylegających do powierzchni cząstki (warstwa adsorpcyjna) oraz z warstwy rozproszonych jonów, czyli warstwy dyfuzyjnej. W oparciu o wymienione modele, w latach 1940-ch Derjaguin, Landau, Verwey oraz Overbeek przedstawili teorię DLVO w której zostały omówione siły wpływające na stabilność układów koloidalnych w roztworach wodnych [19]. Zgodnie z tą teorią stabilność koloidalną definiuje wzajemne oddziaływanie dwóch rodzajów sił: sił przyciągania van der Waalsa oraz sił elektrostatycznego odpychania podwójnej warstwy elektrycznej. Przewaga sił przyciągania powoduje koagulację cząstek koloidalnych, natomiast przewaga sił odpychania zapewnia stabilność danego układu.



Rysunek 1. Micela koloidu.

Jednym z parametrów charakteryzujących podwójną warstwę elektryczną jest potencjał zeta. Potencjał zeta (ζ) definiuje się jako potencjał elektrokinetyczny występujący w płaszczyźnie poślizgu, która oddziela warstwę elektrolitu związaną z powierzchnią cząstki od swobodnie poruszającej się masy cieczy. Uzyskana wartość potencjału zeta determinuje o stabilności koloidalnej badanego układu. Przyjmuje się, że układ, którego wartość bezwzględna potencjału zeta przekracza 30 mV, jest stabilny; wówczas uzyskany potencjał zeta z poza tego zakresu informuje o przewadze sił przyciągających oraz tendencji danego układu do tworzenia agregatów. Wartość potencjału zeta może się zmieniać w zależności od warunków środowiskowych, na przykład, pH, siły jonowej, rodzaju elektrolitu oraz lepkości rozpuszczalnika [20,21]. Zmiana pH wpływa na protonację i deprotonację grup funkcyjnych na powierzchni cząstek, a tym samym na wartość potencjału zeta. Wartość pH przy którym suma ładunków dodatnich i ujemnych cząsteczki jest równa zero jest zdefiniowana jako punkt izoelektryczny (pI). Poniżej punktu izoelektrycznego potencjał zeta ma zwykle wartości dodatnie, natomiast powyżej pI przyjmuje wartości ujemne [22]. W zakresie punktu izoelektrycznego jego wartość zbliża się do zera, co sprzyja agregacji cząstek wskutek osłabienia odpychania elektrostatycznego. Właśnie dlatego przypadku białek minimalną rozpuszczalność najczęściej obserwuje się w zakresie punktu izoelektrycznego. Poprzednio zaobserwowano spadek wartości bezwzględnej potencjału zeta biocząsteczek wraz ze wzrostem stężenia elektrolitu co może być związane ze zmniejszeniem grubości warstwy dyfuzyjnej [23]. Analogiczne zjawisko jest obserwowane w obecności jonów o wyższej wartościowości, które są zdolne do bardziej efektywnego ekranowania ładunku

powierzchniowej cząstki. Potencjał zeta jest bezpośrednio powiązany z ruchliwością elektroforetyczną (u) cząstki w polu elektrycznym. Zależność ta jest opisana równaniem Smoluchowskiego (1):

$$\zeta = \frac{u\eta}{\epsilon_0\epsilon} \quad (1)$$

gdzie: u – ruchliwość elektroforetyczna; η – lepkość ośrodka; ϵ_0 – przenikalność dielektryczna próżni oraz ϵ – przenikalność dielektryczna ośrodka.

Z praktycznego punktu widzenia potencjał zeta stanowi ważny parametr wykorzystywany w kontroli jakości produktów, doborze warunków procesowych podczas projektowania różnych formułacji oraz ocenie przepuszczalności, transportu komórkowego, biodostępności i biozgodności badanego układu koloidalnego. Wartość potencjału zeta decyduje o skłonności cząstek do agregacji, a tym samym wpływa na strukturę, teksturę i trwałość produktów spożywczych [22]. W przypadku białek może być także wykorzystywany do oceny ich właściwości funkcjonalnych, takich jak zdolność do emulgowania, pienienia, żelowania oraz oddziaływania z innymi składnikami żywności.

Mleko krowie jest znanym przykładem skomplikowanego układu koloidalnego. Jest to bogate źródło różnych składników odżywczych, takich jak białka, tłuszcze, węglowodany, witaminy, karotenoidy oraz minerały. Najcenniejszą grupę składników bioaktywnych znajdujących się w mleku krowim stanowią białka. Zawartość białek w mleku stanowi około 3-4%, z których 80% przypada na białka kazeinowe (α_{S1} -, α_{S2} -, β - and κ -kazeina) natomiast reszta 20% przedstawiają białka serwatkowe, w tym α -laktoalbumina, β -laktoglobulina, albumina surowicy bydlęcej, laktoferyna oraz immunoglobuliny [24]. Sole obecne w mleku odgrywają znaczącą rolę w stabilności białek. W mleku krowim występują one w postaci układu pozostającego w równowadze dynamicznej, obejmującego zarówno wolne jony w surowicy mleka, jak i kompleksy związane z różnymi składnikami mleka, takimi jak białka, peptydy, aminokwasy oraz węglowodany. Kluczowymi jonami występującymi w mleku to są kationy wapnia, magnezu, sodu oraz potasu a także aniony fosforanu, chlorku oraz cytrynianu. Zawartość składników mineralnych w mleku nie jest stała, lecz zmienia się pod wpływem różnych warunków, takich jak faza laktacji, sposób żywienia oraz czynniki genetyczne.

2. Aktualne problemy przemysłu mleczarskiego

Mleczarstwo jest uważane jednym z kluczowych sektorów przemysłowych w Polsce. Polska była piątym największym producentem mleka w Unii Europejskiej po Niemczech, Francji, Włoszech oraz Holandii w 2022 roku z udziałem około 8.8% [25]. Spośród produktów najwięcej były eksportowane ser, świeże mleko, śmietana oraz masło. Niemniej jednak z

każdym rokiem powstaje coraz więcej wyzwań do utrzymania stabilnej pozycji na europejskim rynku mleczarskim. Jednym z kluczowych problemów jest wysoki koszt produkcji spowodowany rosnącym kosztem energii, któremu równolegle towarzyszy spadek cen wyrobów mlecznych.

Analiza rynku europejskiego pokazuje, że przewaga konkurencyjna sektora będzie w coraz mniejszym stopniu zależeć od prostego wzrostu wolumenu surowca, a w coraz większym od produktywności, jakości i elastyczności zagospodarowania poszczególnych frakcji mleka. OECD-FAO prognozują dalszy wzrost globalnej produkcji mleka, ale jednocześnie wskazują, że w Unii Europejskiej dynamika ta będzie ograniczona przez presję kosztową, środowiskową i demograficzną. Dane Komisji Europejskiej potwierdzają, że UE pozostaje jednym z najważniejszych światowych eksporterów sera i produktów wysokowartościowych, co oznacza, że maksymalizacja wartości z litra mleka staje się strategicznie istotniejsza niż sama skala jego pozyskania [1,2].

Współcześni konsumenci preferują żywność naturalną i tradycyjną, możliwie jak najmniej przetworzoną, o wyraźnie określonych walorach sensorycznych i potwierdzonej jakości. Fortyfikacja produktów mleczarskich stanowi istotne narzędzie poprawy konkurencyjności na rynku, ponieważ pozwala zwiększyć wartość odżywczą produktu, lepiej dopasować go do oczekiwań konsumentów oraz wyróżnić ofertę przedsiębiorstwa w segmencie żywności funkcjonalnej. Badania wskazują, że wzbogacone produkty wykazują wyższy potencjał konkurencyjny niż produkt standardowy, a przewaga ta wynikała przede wszystkim z lepszych wskaźników funkcjonalnych oraz większej akceptacji konsumenckiej [26]. Fortyfikacja żywności polega na dodawaniu odpowiednich mikroskładników odżywczych w celu zwiększenia ich biodostępności w organizmie. Wzbogacanie żywności w witaminy stanowi jeden z najbardziej perspektywicznych kierunków rozwoju żywności funkcjonalnej. Witaminy są mikroskładnikami niezbędnymi do prawidłowego funkcjonowania organizmu. Uczestniczą w procesach metabolicznych, regulują wzrost i rozwój, wzmacniają odpowiedź immunologiczną oraz pomagają w utrzymaniu homeostazy organizmu [27]. Warto podkreślić, że większość witamin nie jest syntetyzowana przez organizm człowieka, dlatego musi być dostarczana codziennie wraz z dietą.

Zmienność preferencji konsumenckich dodatkowo wzmacnia tę tendencję. Coraz większego znaczenia nabiera segment żywności funkcjonalnej, żywności wysokobiałkowej oraz produktów typu „clean label”, w których wartość rynkowa jest budowana nie tyle przez sam fakt fortyfikacji, ile przez wiarygodnie udokumentowany efekt biologiczny, bezpieczeństwo i naturalność zastosowanego rozwiązania. W konsekwencji przewagę uzyskują te technologie,

które integrują funkcję zdrowotną z samą matrycą produktu, zamiast polegać wyłącznie na prostym dodatku składnika syntetycznego. Taki kontekst rynkowy wzmacnia znaczenie biologicznych metod modulowania składu produktów mleczarskich oraz projektowania funkcjonalnych układów białkowych [28–30].

Dodatkowym wyzwaniem przemysłu mleczarskiego jest powstanie dużej ilości produktów ubocznych, które stanowią poważny problem ekologiczny. Odpady mleczarskie generalnie można podzielić na produkty uboczne przetwórstwa, które zwykle nadają się do dalszego zagospodarowania technologicznego (serwatka, permeat serwatkowy, mleko rozlane, mleko zepsute) oraz odpady eksploatacyjne, związane głównie z utrzymaniem linii produkcyjnej (osady poprodukcyjne, nieoczyszczone ścieki technologiczne) [6]. Produkty uboczne przemysłu mleczarskiego, w szczególności serwatka, stanowią bogate źródło białek, tłuszczu i laktozy, co wiąże się z wysokimi wartościami chemicznego (ChZT) i biologicznego (BZT) zapotrzebowania na tlen. Serwatka jest kluczowym odpadem przemysłu mleczarskiego która głównie powstaje jako produkt uboczny podczas produkcji sera (około 10 litrów serwatki powstaje w wyniku produkcji 1 kilograma sera) [7]. Utylizacja serwatki wymaga istotnych nakładów energii co również wiąże się ze stratą cennych składników odżywczych, t.j. białka serwatkowe, zawartość których w serwatce wynosi około 20% [8]. Białka serwatkowe mogą być stosowane do produkcji proszków serwatkowych, w tym izolatów (ang. whey protein isolate – WPI) oraz koncentratów (ang. whey protein concentrate – WPC) a także do formułowania napojów serwatkowych dla sportowców. Pomimo tego, proszki serwatkowe są często wykorzystywane jako dodatki do żywności, które poprawiają jej wartość odżywczą, trwałość a także pozytywnie wpływają na właściwości sensoryczne produktu końcowego. Innym potencjalnym kierunkiem zagospodarowania serwatki może być produkcja biopaliw (bioetanol, biogaz, biowodór), kwasów organicznych, biopolimerów, witamin oraz enzymów w wyniku fermentacji mikrobiologicznej [7]. W związku z tym, dla efektywnego wykorzystania jej potencjału odżywczego oraz ograniczenia szkodliwego wpływu na środowisko, konieczne jest wdrażanie ekonomicznie uzasadnionych oraz zrównoważonych metod zagospodarowania serwatki, ukierunkowanych na produkcję nowych, wartościowych produktów.

W wymiarze gospodarczym produkty uboczne mleczarstwa nie powinny być traktowane jako odpady o jednolitej negatywnej wartości, lecz jako strumienie surowcowe o zróżnicowanym potencjale ekonomicznym. Najnowsze przeglądy wskazują, że serwatka, kwaśny permeat serwatkowy, maślanka oraz frakcje powstające po rozdziale membranowym mogą stanowić podstawę produkcji koncentratów białkowych, peptydów bioaktywnych, fermentacyjnie

wytwarzanych związków przeciwbakteryjnych, materiałów opakowaniowych oraz specjalistycznych ingrediencji do żywności dla niemowląt. W praktyce oznacza to przesunięcie logiki technologicznej od kosztownej utylizacji do kaskadowej waloryzacji, w której każdy etap separacji powinien prowadzić do odzysku składnika o możliwie najwyższej wartości funkcjonalnej i rynkowej [9,11].

Jednym z najpoważniejszych problemów w mleczarstwie jest mastitis albo zapalenie gruczołu mlekowego. Najczęściej jest spowodowane przez zakażenie bakteryjne (głównie przez *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis*, *Escherichia coli*, *Klebsiella pneumoniae*), warunki utrzymania bydła mleczarskiego, higienę doju, urazy mechaniczne, choroby metaboliczne oraz czynniki genetyczne [31]. Rozwój mastitis negatywnie wpływa zarówno na zdrowie krów, jak i na jakość oraz przydatność technologiczną mleka. Stanu zapalnego towarzyszy wzrost liczby komórek somatycznych, pogorszenie składu mleka, zmiany smaku, konsystencji oraz barwy. W praktyce oznacza to istotne straty ekonomiczne zarówno dla producenta mleka, jak i dla zakładów mleczarskich, ponieważ surowiec taki może nie nadawać się do standardowych procesów przemysłowych. Wynikiem tego jest zmniejszenie ilości pozyskiwanego mleka, konieczność wycofania surowca z obrotu, a także wydzielenie dodatkowych kosztów na leczenie zwierząt. Jednocześnie mleko, które nie spełnia wymagań jakościowych dla konwencjonalnego przetwórstwa mleczarskiego, może stać się przedmiotem badań nad alternatywnym zagospodarowaniem, na przykład poprzez odzysk cennych biologicznie składników. Dotychczasowe badania wskazują, że w przebiegu stanu zapalnego wymienia obserwuje się wzrost stężenia laktoferyny – białka o szerokim zakresie aktywności biologicznej oraz istotnym potencjale prozdrowotnym, którego właściwości zostaną szerzej omówione w kolejnych rozdziałach [32].

Z punktu widzenia waloryzacji surowca istotne jest również to, że stany fizjologiczne i patologiczne zwierząt zmieniają jakościowy profil mleka. Wzrost stężenia laktoferyny w przebiegu mastitis nie jest wyłącznie markerem stanu zapalnego, lecz zarazem potwierdza, że mleko stanowi zmienną biologicznie matrycę, z której można pozyskiwać frakcje o szczególnej aktywności. Taki sposób myślenia przesuwą punkt ciężkości z klasycznej oceny „przydatności technologicznej” surowca na bardziej złożoną analizę jego potencjału frakcjonowania, diagnostycznego oraz biofunkcjonalnego, co ma znaczenie dla nowoczesnych modeli przetwórstwa opartego na selektywnym odzysku składników [32].

3. Fortyfikacja produktów mleczarskich

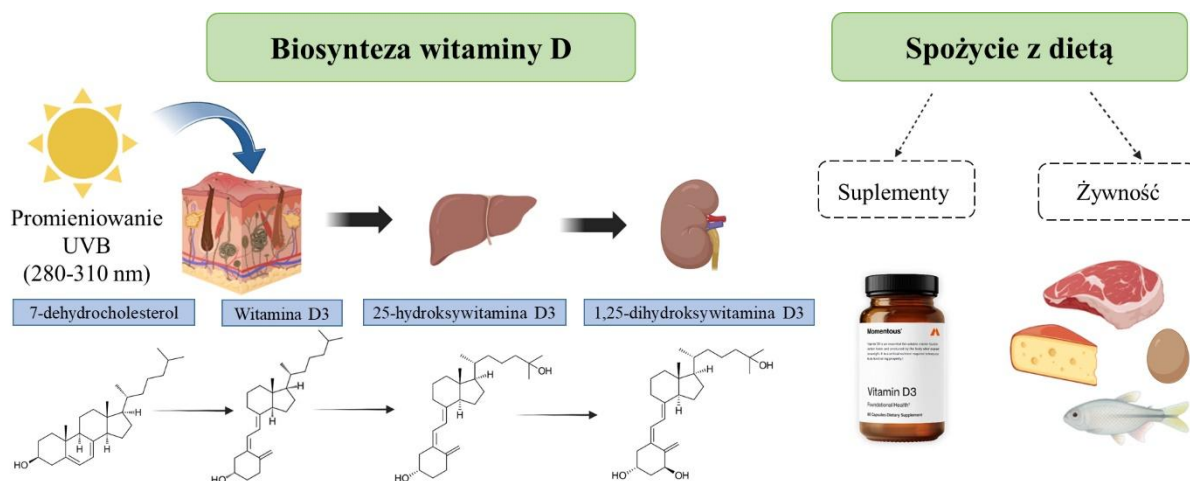
3.1. Globalny problem niedoboru witaminy D oraz sposoby jej dostarczania do organizmu

Niedobór witaminy D jest uznawany jako za jeden z najbardziej rozpowszechnionych problemów zdrowia publicznego – około 1 miliarda ludzi na całym świecie spotyka się z tym problemem [33]. Generalnie szereg różnych czynników może powodować niewystarczający poziom witaminy D w organizmie człowieka, na przykład niska ekspozycja na promieniowanie słoneczne związana spowodowana położeniem geograficznym oraz zmianami sezonowymi, a także styl życia oraz osobliwości diety ograniczające spożycie produktów bogatych w witaminę D. Witamina D pełni wiele istotnych funkcji biologicznych w organizmie, obejmujących utrzymanie homeostazy wapniowo-fosforanowej w surowicy krwi, a także działanie immunomodulujące oraz przeciwzapalne. Jej niedobór może być przyczyną rozwoju cukrzycy, nowotworów, chorób układu oddechowego oraz układu krążenia.

Witamina D jest znana jako witamina tłuszczorozpuszczalna, która może występować w dwóch formach, jako witamina D₂ oraz witamina D₃. Witamina D może być dostarczona do organizmu na dwa sposoby: endogenny (80%) oraz egzogeny (20%). W pierwszym przypadku biosynteza witaminy D następuje w naskórku po ekspozycji na fotony promieniowania UVB (280-310 nm). Podczas tego procesu zachodzi fotoliza 7-dehydrocholesterolu (prowitamina D₃) do prewitaminy D₃, która następnie ulega izomeryzacji termicznej do witaminy D₃ (cholekalcyferol) [34]. Następnie witamina D₃ ulega hydroksylacji w wątrobie do 25-hydroksywitaminy D₃ (25(OH)D₃), stanowiącej główną formę krążącą w organizmie, po czym w nerkach jest przekształcana do biologicznie aktywnej postaci, czyli 1,25-dihydroksywitaminy D₃ (1,25(OH)₂D₃) [35]. Pomimo tego witamina D może być otrzymana w sposób egzogeny, który obejmuje jej dostarczenie wraz z żywnością czy stosowaniem odpowiednich suplementów diety (**Rysunek 2**). Spośród produktów charakteryzujących się wysoką zawartością witaminy D₃ można wyróżnić tłustą rybę (tuńczyk, łosoś, makrela, sardynki, śledź), produkty mleczarskie (ser, masło), żółtko jajka, wątróbkę oraz mięso (czerwone, drobiowe). Z kolei witamina D₂ znajduje się w produktach pochodzenia roślinnego, głównie grzybach [36].

Z chemicznego punktu widzenia kluczowym etapem biosyntezy witaminy D₃ jest fotochemiczna transformacja 7-dehydrocholesterolu, której wydajność zależy od dostępu promieniowania, mikrośrodowiska lipidowego oraz konkurencyjnych reakcji izomeryzacji. W matrycach mlecznych szczególne znaczenie ma rozmieszczenie prekursora w fazie tłuszczowej, stan utlenienia lipidów oraz obecność składników powierzchniowo czynnych stabilizujących emulsję. Oznacza to, że sama zawartość tłuszczu nie przesądza jeszcze o

skuteczności wzbogacania; równie istotna jest organizacja fazy tłuszczowej, wielkość kuleczek tłuszczowych i lokalna dostępność sterolu dla reakcji fotokonwersji. Produkty takie jak śmietanka pełnią zatem nie tylko funkcję nośnika lipofilnej witaminy, ale również stanowią reaktywne środowisko, w którym właściwości koloidalne mogą w istotny sposób wpływać na efektywność procesu [29,33–37].



Rysunek 2. Sposoby dostarczania witaminy D do organizmu.

3.2. Nowe podejście fortyfikacji żywności w witaminę D

W czasach dzisiejszych stosuje się różne podejścia w celu rozwiązania problemu niedoboru witaminy D, na przykład większe spożycie produktów bogatych w witaminę D, stosowanie odpowiednich suplementów diety, wzrost ekspozycji na promieniowanie słoneczne oraz wzbogacanie (fortyfikacja) żywności. Wcześniej już zostały przeprowadzone praktyki fortyfikacji różnych produktów żywności w witaminę D, w tym mleka, serów, jogurtów, masła, margaryny, płatków śniadaniowych oraz soku pomarańczowego [38]. Pomimo że najczęściej stosowaną metodą jest bezpośredni dodatek syntetycznej witaminy D do produktów spożywczych, warto zwrócić uwagę również na wzbogacanie pośrednie, na przykład poprzez wspomaganą syntezę z udziałem bakterii kwasu mlekowego (ang. lactic acid bacteria – LAB). Do grupy LAB należą różne rodzaje bakterii na przykład, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* oraz *Bifidobacterium*. Bakterie kwasu mlekowego biorą udział w procesie fermentacji, podczas którego cukry są przekształcane w kwas mlekowy, czemu zazwyczaj towarzyszy obniżenie pH środowiska. Produktami metabolizmu LAB także mogą być różne związki, na przykład kwasy organiczne, krótkołańcuchowe kwasy tłuszczowe, egzopolisacharydy, bakteriocyny, peptydy, wolne aminokwasy oraz witaminy [39]. Liczne bakterie kwasu mlekowego są zaliczane się do mikroorganizmów probiotycznych. Do najważniejszych właściwości prozdrowotnych bakterii kwasu mlekowego należą korzystny

wpływ na przewód pokarmowy, odporność organizmu oraz działanie ochronne przeciwko wybranym infekcjom i chorobom. Pomimo ulepszenia cech odżywczych oraz funkcjonalnych, dodatek kultur LAB do żywności może poprawić smak, teksturę oraz wydłużyć termin ważności produktu. Zastosowanie mikroorganizmów produkujących witaminy jest bardziej naturalnym i opłacalnym sposobem wzbogacania żywności niż użycie syntetycznych form tych związków. Pozwala ono zwiększyć zawartość witamin w produktach bez istotnego wzrostu kosztów produkcji, przynosząc korzyści zarówno producentom, jak i konsumentom. Wcześniejsze prace wykazały, że bakterie kwasu mlekowego mogą uczestniczyć w biosyntezie serii witamin z grupy B (B2, B6, B9), w procesie fermentacji [27]. Właściwy dobór szczepów oraz ich warunków inkubacji stwarza możliwość wytwarzania nowej żywności o podwyższonej wartości odżywczej i prozdrowotnej [P4].

Najnowsze analizy systematyczne randomizowanych badań klinicznych wskazują, że mleko, mleko w proszku oraz fermentowane produkty mleczne wzbogacane w witaminę D istotnie zwiększają stężenie 25(OH)D3 w surowicy. Tym samym matryce mleczne nie są jedynie wygodnym nośnikiem technologicznym, ale stanowią potwierdzony klinicznie kanał interwencji żywieniowej. Z punktu widzenia rynku oznacza to, że rozwiązania rozwijane w mleczarstwie mogą jednocześnie poprawiać zdrowotną wartość produktu i wzmacniać jego pozycjonowanie w segmencie żywności funkcjonalnej, zwłaszcza wtedy, gdy wzbogacanie przebiega w sposób zgodny z trendem naturalności i ograniczonej liczby dodatków [28–30].

Kultury LAB stosuje się w przemyśle w postaci płynnej, mrożonej, suszonej rozpyłowo oraz liofilizowanej. Ostatnio coraz większą popularnością cieszą się liofilizowane kultury LAB, które oferują liczne zalety technologiczne i funkcjonalne [40]. Przede wszystkim cechują się wyższą przeżywalnością, większą stabilnością oraz dłuższą trwałością przechowalniczą niż biomasa bakteryjna, co sprzyja zachowaniu ich aktywności biologicznej; także charakteryzują się wygodnością w przechowywaniu oraz dawkowaniu co z kolei ułatwia standaryzację produktu. Ich stosowanie ułatwia również standaryzację procesu i precyzyjne dawkowanie kultur starterowych. Z innej strony ich wykorzystanie wiąże się z większymi wymaganiami technologicznymi, wyższym zużyciem energii oraz wydłużeniem procesu produkcyjnego. Śmietanka jest produktem mleczarskim stanowiącym odpowiednie środowisko do wzrostu bakterii kwasu mlekowego, gdyż zawiera składniki odżywcze, takie jak laktoza, białka, tłuszcze oraz sole mineralne, które sprzyjają namnażaniu i aktywności metabolicznej tych drobnoustrojów. Pomimo tego, śmietanka stanowi odpowiedni surowiec do wzbogacania w witaminę D3, ponieważ jako produkt o wysokiej zawartości tłuszczu zapewnia korzystną matrycę dla związków tłuszczorozpuszczalnych, wpływając na ich stabilność oraz potencjalną

biodostępność [33,37]. W pracy [P4] opracowano nowe podejście do wzbogacania śmietanki w witaminę D3 z wykorzystaniem szczepu *Lactiplantibacillus plantarum* B/00401. Szczep ten był dodawany do śmietanki w dwóch różnych formach – jako biomasę (świeża, niesuszoną masę komórek bakteryjnych w postaci zawiesiny) oraz jako liofilizat bakteryjny. Efektywność biosyntezy witaminy D3 przez dodany szczep oceniano w różnych warunkach inkubacji, obejmujących: 1) ekspozycję na światło lub ciemność, 2) mieszanie lub brak mieszania, 3) różny stosunek objętościowy śmietanki do *L. plantarum* B/00401 (1:1, 3:1 (v/v)) oraz 4) zastosowaną formę szczepu, tj. biomasę lub liofilizat. Uwzględnienie ekspozycji na światło i ciemność wynikało z kluczowej roli światła w fotoaktywacji 7-dehydrocholesterolu, będącego prekursorem witaminy D3, dlatego konieczne było sprawdzenie, czy jego obecność zwiększa wydajność tego procesu. Porównanie warunków mieszania i braku mieszania miało z kolei wykazać, czy korzystniejsze dla tworzenia witaminy D3 są warunki statyczne czy dynamiczne. W układzie bez mieszania kuleczki tłuszczowe oraz prekursor witaminy D3 mogą gromadzić się przy powierzchni śmietanki, gdzie są lepiej eksponowane na światło, natomiast mieszanie wpływa na rozmieszczenie fazy tłuszczowej i homogenność całego układu. Zastosowanie dwóch różnych stosunków śmietanki do bakterii umożliwia ocenę wpływu ilości dodanego szczepu na zmiany zawartości witaminy D3. Z kolei zastosowana forma szczepu może różnić się aktywnością metaboliczną oraz zdolnością do wspomagania biosyntezy witaminy D3. Zawartość zsyntezowanej witaminy D3 w próbkach śmietanki po 48-godzinnej inkubacji w określonych warunkach oznaczono metodą wysokosprawnej chromatografii cieczowej w odwróconym układzie faz (ang. reverse-phase high-performance liquid chromatography – RP-HPLC).

Na tym tle wykorzystanie *L. plantarum* jako czynnika biologicznie kształtującego skład śmietanki stanowi rozwiązanie jakościowo odmienne od klasycznej fortyfikacji addytywnej. W ujęciu chemicznym i technologicznym nie polega ono na prostym zwiększeniu stężenia gotowego związku, lecz na takiej modyfikacji mikrośrodowiska matrycy, która sprzyja powstawaniu, ochronie lub akumulacji pożądanego metabolitu. To właśnie ten element nadaje badaniom istotną nowość aplikacyjną: produkt mleczny staje się aktywnym układem biosyntezy i stabilizacji cząsteczek bioaktywnych, a nie biernym nośnikiem dodatku. W perspektywie przemysłowej może to tworzyć podstawę dla nowych produktów funkcjonalnych o bardziej naturalnym profilu technologicznym i marketingowym [28–30,37,39].

3.3. Wpływ profilu kwasów tłuszczowych na właściwości funkcjonalne żywności

Pomimo udziału w biosyntezie niezbędnych metabolitów, LAB mogą zmieniać profil kwasów tłuszczowych w produktach mleczarskich. W zależności od stosowanego szczepu następuje

zmiana długości łańcucha, stopnia nasycenia oraz odpowiednio biodostępności kwasów tłuszczowych [41]. Kwasy tłuszczowe mogą być klasyfikowane ze względu na długość łańcucha węglowego na krótko- ($C < 6$), średnio- ($C = 6-12$) oraz długołańcuchowe ($C > 12$). W zależności od liczby wiązań podwójnych węgiel-węgiel można wyróżnić nasycone (ang. saturated fatty acids – SFA), mononienasycone (ang. monounsaturated fatty acids – MUFA) oraz wielonienasycone (ang. polyunsaturated fatty acids – PUFA) kwasy tłuszczowe. SFA pełnią przede wszystkim funkcję energetyczną oraz strukturalną. Są składnikami błon komórkowych, ale ich nadmiar, w szczególności długołańcuchowych SFA, może sprzyjać wzrostowi stężenia cholesterolu LDL (tzw. „zły cholesterol”), rozwojowi stanu zapalnego oraz zaburzeniom metabolicznym [42]. Z kolei MUFA zapobiegają rozwojowi chorób sercowo-naczyniowych. Warto podkreślić, że PUFA, zwłaszcza kwas linolowy (C18:2) oraz kwas linolenowy (C18:3) przedstawiają najcenniejszą grupę kwasów tłuszczowych charakteryzujących szerokim zakresem właściwości prozdrowotnym. PUFA pełnią w organizmie istotne funkcje strukturalne, regulacyjne oraz metaboliczne. Uczestniczą one w modulowaniu procesów zapalnych oraz odpowiedzi immunologicznej, a także wpływają na regulację ciśnienia krwi i mogą zmniejszać ryzyko rozwoju chorób sercowo-naczyniowych. Ponadto wspierają zdrowie jelitowe, ponieważ sprzyjają utrzymaniu korzystnego składu mikrobioty oraz wzmacniają barierę jelitową. Zmiany w profilu kwasów tłuszczowych mogą w istotny sposób wpływać na trwałość produktów, ich właściwości sensoryczne oraz funkcjonalność [43]. W pracy [P4] oceniono zmiany w zawartości SFA, MUFA oraz PUFA w próbkach śmietanki inkubowanych w warunkach analogicznych do wcześniej opisanych dla oznaczania witaminy D3, uwzględniając dostęp światła, mieszanie, formę dodanego szczepu oraz stosunek śmietanki do *L. plantarum* B/00401. Warto zaznaczyć, że stosowane medium hodowlane może mieć istotny wpływ na aktywność metaboliczną bakterii oraz kształtowanie profilu kwasów tłuszczowych w badanych próbkach [44]. Dlatego dodatkowo przygotowano warianty eksperymentalne zawierające wyłącznie mieszaninę śmietanki i bakterii, bez dodatku medium, co umożliwiło porównanie wpływu samej obecności drobnoustrojów z efektem uzyskanym w warunkach suplementacji podłoża. Zmiany zawartości kwasów tłuszczowych w poszczególnych próbkach śmietanki było oznaczono metodą chromatografii gazowej sprzężonej ze spektrometrią mas (ang. gas chromatography-mass spectrometry – GC-MS). Z chemicznego punktu widzenia zmiany profilu kwasów tłuszczowych wywołane aktywnością LAB należy rozpatrywać równolegle w kontekście wartości żywieniowej oraz stabilności oksydacyjnej. Wzrost udziału kwasów nienasyconych może poprawiać profil prozdrowotny produktu, ale jednocześnie zwiększa liczbę wiązań podwójnych podatnych na autooksydację, a

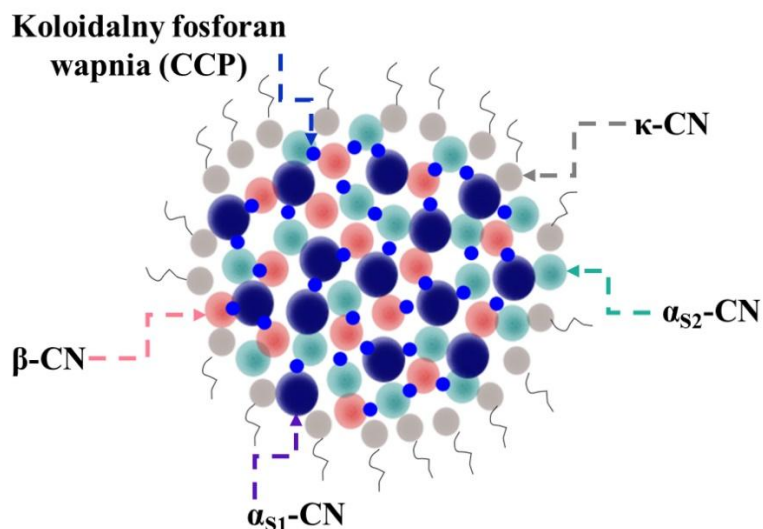
tym samym wpływa na trwałość sensoryczną i konieczność odpowiedniego doboru warunków przechowywania. Oceniając funkcjonalność tak zmodyfikowanej śmietanki, nie można więc ograniczyć się do samego oznaczenia składu lipidowego; konieczne jest również rozumienie konsekwencji kinetyki utleniania lipidów, tworzenia wtórnych produktów oksydacji oraz ich oddziaływania z białkami i witaminami rozpuszczalnymi w tłuszczach [41–44].

4. Kazeina: struktura, stabilność oraz rola biologiczna białka

4.1. Układ micelarny kazeiny oraz jego stabilność pod wpływem różnych czynników

Kazeina (CN) jako kluczowe białko znajdujące się w mleku spełnia rolę nośnika niezbędnych aminokwasów (fenyloalaniny, leucyny, izoleucyny, lizyny, metioniny, treoniny, waliny, histydyny), wapnia oraz fosforu, które są składnikami budulcowymi kości i zębów. W roztworze wodnym kazeina tworzy układy micelarne. Micele kazeinowe są supramolekularnymi strukturami powstającymi w wyniku asocjacji czterech rodzajów kazein, w tym α_{s1} -, α_{s2} -, β - oraz κ -kazeiny, występujących w przybliżonym stosunku 40:10:35:15 (w/w), z udziałem fosforanu wapnia (**Rysunek 3**). Fosforan wapnia łączy się frakcjami kazeinowymi przez fosforylowane reszty serynowe tworząc klastry, tak zwany koloidalny fosforan wapniowy (ang. colloidal calcium phosphate – CCP). Sformowany układ micelarny jest stabilizowany w wyniku szeregu różnych oddziaływań, w tym hydrofobowych, elektrostatycznych, wiązań wodorowych oraz disiarczkowych. Istotną rolę odgrywa także skład aminokwasowy kazein oraz wzajemne rozmieszczenie reszt hydrofilowych i hydrofobowych. Obecność zarówno obszarów hydrofilowych, jak i hydrofobowych sprawia, że kazeinę można zaliczyć do białek amfipatycznych [24]. Każda z frakcji kazeinowych charakteryzuje odmiennymi właściwościami fizykochemicznymi, które bardziej dokładnie są przedstawione w **Tabeli 1**.

Z chemicznego punktu widzenia o wyjątkowości kazeiny decyduje obecność licznych reszt fosfoserynowych zdolnych do koordynacji jonów Ca(II) i współtworzenia nanoklastrów fosforanu wapnia. To właśnie sprzężenie pomiędzy segmentami bogatymi w fosfoserynę, obszarami hydrofobowymi oraz dynamicznie wymienialną fazą mineralną odpowiada za „miękką”, lecz jednocześnie zaskakująco trwałą organizację miceli. Układ ten nie ma charakteru statycznego: zmiana pH, siły jonowej lub rodzaju kationów modyfikuje stopień ekranowania ładunku, uwodnienie powierzchni oraz równowagę pomiędzy repulsją elektrostatyczną a siłami przyciągania. W konsekwencji micela kazeinowa może być traktowana jako układ adaptacyjny, którego architektura jest podatna na świadome projektowanie chemiczne oraz fizykochemiczne [12,45–47].



Rysunek 3. Struktura miceli kazeinowej.

α_{S1} -CN, α_{S2} -CN, β -CN wykazują znacznie wyższy stopień fosforylacji w porównaniu do κ -CN, dlatego znajdują się one w części wewnętrznej miceli kazeinowej formując tzw. rdzeń hydrofobowy [50]. α_{S2} -CN zawiera dwie reszty cysteinowe i często występuje jako dimer lub tworzy wewnątrcząsteczkowe wiązania disiarczkowe [45]. β -CN jest znana jako najbardziej hydrofobową kazeina spośród wszystkich ze względu na bogatą zawartość proliny (35 aminokwasów) w łańcuchu polipeptydowym [51]. Z tego powodu w β -CN nie występują takie rodzaje struktur drugorzędowych jak α -helisa oraz β -harmonijka, a raczej nabywa struktur nieuporządkowaną (tzw. „random coil”) w roztworze wodnym [45]. Podobnie, α_{S1} -CN zawiera około 70% nieuporządkowanej struktury wraz z niewielkim udziałem α -helisy oraz β -struktury. Natomiast κ -CN charakteryzuje największa hydrofilowością ze względu na obecność łańcuchów glikanowych w jej strukturze. κ -CN jest typowo zlokalizowana na powierzchni miceli tworząc warstwę o grubości około 5-10 nm (tzw. „hairy layer”) i zapewnia jej stabilność poprzez odpychanie steryczne oraz elektrostatyczne [24]. Na przykład, zmierzony potencjał zeta dla κ -CN przy pH 7,4 był -50 mV, wówczas jak dla kazeiny on wyniósł -20 mV [52]. Oprócz stabilizacji sterycznej, κ -CN również zapewnia integralność struktury wewnętrznej miceli. Micele kazeinowe charakteryzują się stabilnością nawet przy wysokiej temperaturze (> 100 °C) oraz ciśnieniu (> 100 MPa), niemniej jednak są bardzo czułe na pH środowiska [53]. Na przykład, zakwaszenie do pH < 5 (punkt izoelektryczny kazeiny wynosi około 4,6) prowadzi do koagulacji białka, wówczas koloidalny fosforan wapnia się rozpuszcza. Równowaga mineralna odgrywa istotną rolę w stabilności układu micelnego kazeiny. Dodatek chlorku sodu (NaCl) może powodować częściowe zastępowanie wapnia przez sód w nanoklastrach, co

wiąże się z rozluźnieniem wewnętrznej struktury miceli oraz solubilizacją koloidalnego fosforanu wapnia (CCP) [46]. Jednocześnie wzrost stężenia elektrolitu prowadzi do zmniejszenia grubości podwójnej warstwy elektrycznej oraz obniżenia potencjału zeta cząstek, a w konsekwencji do osłabienia odpychania elektrostatycznego i zmniejszenia odległości między micelami.

Tabela 1. Właściwości fizykochemiczne poszczególnych frakcji kazeiny bydlęcej.

Rodzaj kazeiny	Masa cząsteczkowa (kDa)	Liczba aminokwasów w łańcuchu polipeptydowym	Liczba reszt fosfo-serynowych	Obecność łańcuchów glikanowych w strukturze	Ref.
α_{S1} -CN	24,5	214	8	–	[47–49]
α_{S2} -CN	26	222	11	–	
β -CN	25,1	224	5	–	
κ -CN	21,3	190	1	+	

Średnica hydrodynamiczna miceli kazeinowa może się wahać od 20 do 600 nm i średnio wynosi około 200 nm. Rozmiar miceli może być uzależniony od różnych czynników, takich jak skład poszczególnych frakcji kazeinowych, pH środowiska, temperatury oraz ciśnienia. Wykazano że wzrost zawartości κ -CN wraz ze spadkiem zawartości β -CN, sprzyjają tworzeniu miceli kazeinowych o mniejszych rozmiarach; wówczas ilość obecnej α_S -CN nie ma istotnego wpływu na jej wielkość [54]. Ponadto, istotny wpływ również miał wariant genetyczny obecnej κ -CN [55]. Procesy wysokociśnieniowe (> 200 MPa), na przykład homogenizacja, również negatywnie wpływają na stabilność miceli kazeinowych powodując ich dysocjację [56].

Ta podatność na reorganizację stała się podstawą najnowszych badań nad sztucznymi i reformowanymi micelami kazeinowymi. Współczesne strategie obejmują kontrolowaną remineralizację, cykliczne zmiany pH, wysokociśnieniowe przebudowywanie układu oraz projektowanie miceli zdolnych do inkorporacji związków hydrofobowych oraz jonowych. Kierunek ten ma zasadnicze znaczenie dla przemysłu mleczarskiego, ponieważ pozwala przejść od klasycznego wykorzystania kazeiny jako składnika receptury do jej świadomego użycia jako projektowanego nośnika o zaprogramowanej pojemności wiązania, określonej stabilności oraz sterowalnej kinetyce uwalniania [12].

4.2. Metody izolacji kazeiny

Jak wcześniej było wspomniano, kazeina reprezentuje dominującą frakcję białek mleka, której udział jest zakresie od 50 do 85%. Dotychczas są stosowane trzy metody ekstrakcji kazeiny:

fizykochemiczną, biologiczną oraz fizyczną [57]. Najstarszą z nich jest metoda fizykochemiczna, która polega na strąceniu kwasowym kazeiny w warunkach pH, które odpowiada jej punktu izoelektrycznemu (pH 4.6). Zakwaszenie przeprowadza się najczęściej poprzez dodanie kwasu solnego, siarkowego lub octowego, po czym uzyskany osad się oddziela poprzez sączenie lub wirowanie a następnie białko jest poddawane kilkakrotnemu przemyciu wodą oraz suszeniu. Metoda ta również się stosuje do otrzymywania kazeinatów. Kazeitaty to są rozpuszczalnymi solami kazeiny, otrzymywanymi w wyniku jej neutralizacji zasadami, takimi jak wodorotlenek sodu, potasu lub wapnia. Charakteryzują się one lepszą rozpuszczalnością w wodzie w porównaniu do kazeiny, dlatego są powszechnie użytkowane w przemyśle spożywczym jako dodatki poprawiające właściwości emulgujące, pianotwórcze i stabilizujące [58]. Biologiczna metoda ekstrakcji kazeiny polega na enzymatycznej koagulacji białka wywołanej dodatkiem enzymu podpuszczkowego. Po dodaniu chymozyny κ -kazeina, znajdująca się na powierzchni miceli, ulega hydrolizie, czemu towarzyszy uwolnienie glikomakropeptydu kazeinowego (GMP), co prowadzi do utraty stabilności miceli, ich agregacji z udziałem jonów wapnia oraz utworzenia skrzepu białkowego stanowiącego podstawę produkcji sera. Fizyczny sposób separacji kazeiny generalnie obejmuje stosowanie technik membranowych, takich jak mikro- oraz ultrafiltracja. Jest to perspektywne podejście które jednak wymaga istotnego postępu technologicznego. Wykazano że micidele kazeinowe ulegają wytrąceniu jedynie przy stosowaniu wysokiej siły osrodkowej ($>50000\times g$) co z praktycznego punktu widzenia jest słabo osiągalne w przypadku masowej produkcji przemysłowej [59]. Aby pokonać ten problem są pozyskiwane nowe materiały porowate lub kompozyty charakteryzujące wysoką wytrzymałością mechaniczną. Niedawno komercjalizacja ceramicznych membran mikrofiltracyjnych o jednorodnych porach na bazie ZrO_2 umożliwia przemysłowe rozdzielanie kazeiny i białek serwatkowych metodami fizycznymi [60]. Badania laboratoryjne pokazały że modyfikacja równowagi mineralnej mleka może wyraźnie zmieniać skuteczność rozdziału kazeiny i białek serwatkowych. Na przykład, dodanie chlorku wapnia ($CaCl_2$) miało najlepszy efekt na retencję kazeiny podczas mikrofiltracji z przepływem stycznym (tangencjalna), ale jednak nie pozwoliło to na całkowite oddzielenie kazeiny od białek serwatkowych. To wynika ze zdolności białek serwatkowych wiązać się z wapniem co z kolei utrudnia ich efektywne wypłukanie. Z innej strony, stosowanie wodorofosforanu sodu (Na_2HPO_4) pozwoliło na najlepsze oddzielenie kazeiny od białek serwatkowych, lecz kosztem niskiego uzysku. Niski odzysk kazeiny był związany z częściową utratą integralności miceli, co sprzyjało przechodzeniu kazeiny do fazy serum i zmniejszało jej retencję podczas mikrofiltracji, co sugeruje o konieczności dalszych badań w celu poprawy odzysku

otrzymywanego izolatu białka o odpowiednim stopniu czystości [61]. Warto również podkreślić że skład zarówno jak i funkcjonalność kazeiny będzie się różnić w zależności od stosowanej metody ekstrakcji.

4.3. Problem zasolenia białek

Stosowanie licznych buforów podczas izolacji może prowadzić do istotnego zasolenia produktu końcowego. Jak pokazano wcześniej, dodatek soli, takich jak cytrynian sodu, fosforan sodu czy chlorek sodu, może w określonych warunkach poprawiać rozpuszczalność i rehydratację proszków kazeinowych, co wiąże się z efektem salting-in [62]. Jednocześnie zbyt wysokie stężenie elektrolitów może prowadzić do spadku rozpuszczalności białka i jego wytrącania, czyli efektu salting-out. Wysoka zawartość soli może również indukować zmiany strukturalne białka, a w konsekwencji wpływać na jego właściwości funkcjonalne i biologiczne, co może obniżać powtarzalność wyników, zwłaszcza w testach biologicznych. Ponadto jony pochodzące z soli mogą zajmować potencjalne miejsca wiązania w cząsteczce białka, utrudniając jego dalszą modyfikację innymi związkami [46]. Nadmierne zasolenie próbek może również zakłócać wyniki analiz, szczególnie spektroskopowych (UV-Vis, spektroskopia fluorescencyjna), spektrometrii mas (MS) oraz elektroforezy (SDS-PAGE) [63]. Dlatego w celu uzyskania produktu o wyższym stopniu czystości konieczne jest zastosowanie odpowiednich metod odsalania i oczyszczania, takich jak ultrafiltracja [P1, P3].

4.4. Kazeina – idealny nośnik cząsteczek bioaktywnych

Unikatowa struktura miceli kazeinowej oraz właściwości fizykochemiczne znacznie zwiększa jej aplikacyjność szczególnie w układach dostarczenia leków oraz składników odżywczych. Kazeina może się wiązać z innymi cząsteczkami tworząc kompleksy lub koniugaty o ulepszonych właściwościach biologicznych [58]. Stosowania kazeiny jako nośnika w układach dostarczania związków bioaktywnych jest bardzo atrakcyjne ze względu na szereg zalet tego materiału. Z innej strony, warto wziąć pod uwagę że szerokie stosowanie kazeiny w medycynie może być ograniczone ze względu na jej immunogenne oraz alergenne właściwości. Kazeina jest białkiem nietoksycznym, biodegradowalnym oraz biokompatybilnym o wysokiej dostępności oraz niskiej cenie produkcji [49]. Charakteryzuje się ona wysoką odpornością na denaturację termiczną a także posiada doskonałe właściwości żelujące oraz powierzchniowo czynne. Białko to jest atrakcyjnym materiałem do funkcjonalizacji, ponieważ jej struktura zawiera liczne reaktywne grupy funkcyjne, takie jak grupy aminowe, karboksylowe i fosforanowe, które mogą być wykorzystywane do modyfikacji chemicznej. Kazeina znana jako biocząsteczka amfipatyczna może oddziaływać ze związkami hydrofobowymi zarówno jak i hydrofilowymi w istotny sposób zwiększając ich biodostępność w organizmie. Wcześniejsze

pracy pokazały że kazeina może być stosowana do wspomagania transportu jonów metali (Ca(II), Mg(II), Cu(II), Zn(II), Cr(III)), witamin (A, B12, D3, E), związków polifenolowych, polisacharydów oraz wielonienasyconych kwasów tłuszczowych [49,64]. Modyfikacja kazeiny różnymi cząsteczkami może prowadzić do istotnych zmian strukturalnych białka co w konsekwencji wpływa na jego aktywność biologiczną. Badanie mechanizmu oddziaływań w utworzonym układzie jest koniecznym etapem podczas opracowania kompleksów kazeiny o ulepszonej stabilności i funkcjonalności do dalszego zastosowania w przemyśle spożywczym oraz medycynie.

Z praktycznego punktu widzenia taka interpretacja otwiera dla kazeiny zupełnie nowy obszar zastosowań. Najnowsze prace wskazują na rozwój kazeinowych hydrożeli, włókien, filmów i opatrunków, w których białko to pełni jednocześnie funkcję strukturalną, sorpcyjną i biologiczną. Wartość tych materiałów wynika z połączenia cech istotnych dla przemysłu: szerokiej dostępności surowca, biodegradowalności, łatwości przetwarzania i możliwości funkcjonalizacji metalami lub innymi cząsteczkami aktywnymi. W tym sensie kazeina jawi się nie tylko jako komponent żywnościowy, lecz jako mleczarski biopolimer o rosnącym znaczeniu w technologiach biomateriałowych i aktywnych materiałów kontaktowych [13,14].

4.5. Rola jonów metali w funkcjonalności białek

Jony metali odgrywają ważną rolę w różnych procesach biologicznych, pełnią w organizmach funkcje katalityczne, strukturalne i regulacyjne. Uczestniczą w działaniu enzymów, transporcie tlenu, przewodzeniu impulsów nerwowych, skurczu mięśni, krzepnięciu krwi oraz utrzymaniu homeostazy organizmu. Około 30% białek należy do kategorii metaloprotein, więc wymagają kofaktora, na przykład, Mg(II), Zn(II), Fe(II/III), Cu(II), do prawidłowego fałdowania oraz spełnienia odpowiednich funkcji biologicznych. Spośród metali warto wyróżnić grupę metali szlachetnych do których należą, na przykład srebro (Ag), platyna (Pt), pallad (Pd), ruten (Ru), srebro (Ag), złoto (Au), osm (Os) oraz iryd (Ir) [65]. Chociaż metale te nie należą do pierwiastków niezbędnych dla prawidłowego funkcjonowania organizmu, są powszechnie stosowane w farmakologii jako środki o właściwościach przeciwbakteryjnych, przeciwwirusowych, przeciwzapalnych oraz przeciwnowotworowych.

4.5.1. Związki srebra: aktywność biologiczna, zastosowanie w medycynie oraz problem cytotoksyczności

Lecznicyz potencjał srebra został wykryty jeszcze w starożytności. Związki srebra, w szczególności azotan srebra miał zastosowanie w różnych gałęziach medycyny, takich jak dermatologia, oftalmologia, ginekologia oraz urologia. Srebro było powszechnie stosowane jako efektywny środek antybakteryjny aż do lat 1930-ch przed odkryciem antybiotyków.

Dotychczas wykazano że jony srebra Ag(I) posiadają działanie antyseptyczne, bakteriobójcze, przeciwzapalne oraz cytotoksyczne. Mechanizm bakteriobójczy związków srebra nie jest do końca wyjaśniony. Generalnie, naukowcy wyróżniają następujące etapy: 1) uszkodzenie ściany oraz błony komórkowej bakterii; 2) oddziaływanie Ag(I) z cząsteczkami wewnątrz komórki oraz 3) wywołanie stresu oksydacyjnego [66]. Jony srebra wiążą się z wysokim powinowactwem ze składnikami powierzchni bakterii, szczególnie z grupami tiolowymi białek i enzymów, zaburzając integralność błony komórkowej oraz zwiększając jej przepuszczalność. W efekcie komórka traci kontrolę nad transportem substancji i łatwiej ulega uszkodzeniu. Jony srebra szybko przenikają do wnętrza komórki bakteryjnej, gdzie bezpośrednio wiążą się z aminokwasami, rybosomami i DNA. W ten sposób inaktywują enzymy, hamują syntezę białek oraz replikację materiału genetycznego, co prowadzi do zahamowania podziału i namnażania bakterii. Ostatecznie jony srebra hamują działanie białek łańcucha oddechowego bakterii, prowadząc do zwiększonego wytwarzania reaktywnych form tlenu (ang. reactive oxygen species – ROS), co w konsekwencji wywołuje stres oksydacyjny, uszkodzenia białek i DNA oraz ostatecznie prowadzi do zahamowania wzrostu i śmierci bakterii. Obecność licznych celów molekularnych (ang. molecular targets) w komórkach bakteryjnych, w tym białek, enzymów, nukleozydów, nukleotydów, RNA, DNA, sprawia, że związki srebra stosowane w leczeniu ran mogą mieć przewagę nad antybiotykoterapią, ponieważ ryzyko rozwoju oporności na ich działanie jest mniejsze.

W niniejszych czasach srebro jest wykorzystane w medycynie do leczenia ran, dezynfekcji wody, powlekania sprzętu medycznego a także produktach higienicznych, domowych, rolniczych i przemysłowych [67]. Srebro jest stosowane do pokrycia implantów, igieł, cewników naczyniowych, stentów naczyniowych, a także niektórych powierzchni użytkowych. W preparatach miejscowych i opatrunkach srebro jest najczęściej stosowano w postaci azotanu srebra, sulfatiazolu srebra oraz sulfadiazyny srebra preparatach do leczenia ran oraz oparzeń [68]. Mimo że związki srebra wykazują silne działanie bakteriobójcze, ich stosowanie może prowadzić do działań toksycznych o charakterze ogólnoustrojowym lub miejscowym. Częściej obserwowana jest toksyczność miejscowa, związana z cytotoksycznym wpływem jonów srebra na komórki naskórka (keratynocyty) oraz skóry właściwej (fibroblasty). Skuteczne działanie przeciwdrobnoustrojowe osiągane jest przy stężeniu 30-40 ppm, podczas gdy działanie toksyczne wobec keratynocytów i fibroblastów odnotowano odpowiednio przy stężeniach powyżej 33 ppm i 50 ppm [69]. Ze względu na ograniczenia bezpieczeństwa oraz ryzyko działań niepożądanych, związki srebra są obecnie stosowane przede wszystkim zewnętrzn.

Jednak ich aktywność biologiczna i cytotoksyczność w dużym stopniu zależą od postaci chemicznej, stężenia oraz rodzaju zastosowanego ligandu.

Azotan srebra jest najbardziej stosowanym prekursorem do otrzymywania kompleksów Ag(I) z różnorodnymi ligandami. Należy podkreślić, że warunki syntezy kompleksów Ag(I) z udziałem AgNO_3 wymagają precyzyjnego doboru. Związki srebra(I) wykazują wysoką fotoczułość i w obecności światła mogą ulegać redukcji do srebra metalicznego [70], dlatego roztwory przygotowywano i przechowywano w ciemnych butelkach, aby ograniczyć dostęp światła i zwiększyć ich stabilność. Dodatkowo azotan srebra(I) w środowisku zasadowym może wytrącać się w postaci tlenku srebra(I) (Ag_2O), szczególnie przy $\text{pH} > 7$ [71]. W celu wyeliminowania tego problemu w niniejszej pracy zastosowano jako prekursor srebra(I) kompleks diaminasrebra(I) $[\text{Ag}(\text{NH}_3)_2]^+$, czyli odczynnik Tollensa [P1], którego trwałość wynika z wyższego powinowactwa amoniaku jako zasady Lewisa do jonu Ag(I) niż wody.

W przypadku kompleksu $[\text{Ag}(\text{NH}_3)_2]^+$ kluczowe znaczenie ma mechanizm substytucji ligandowej. Amoniak stabilizuje Ag(I) w roztworze alkalicznym, jednak w kontakcie z białkiem może zostać wyparty przez donorowe atomy tlenu, azotu i siarki obecne w karboksylanach Asp/Glu, grupach fosforanowych fosfoseryny oraz wybranych resztach histydyny i metioniny. Powstałe wiązania koordynacyjne nie mają wyłącznie charakteru statycznego; lokalne mikrośrodowisko białka może sprzyjać redystrybucji ładunku i częściowej redukcji Ag(I) do Ag(0), co inicjuje nukleację nanocząstek. Z punktu widzenia chemii koloidów kazeina pełni więc potrójną rolę: ligandu wiążącego metal, matrycy ograniczającej wzrost nanocząstek oraz stabilizatora powstającego układu hybrydowego [72,73].

4.5.2. Modyfikacja białek srebrem jako sposób otrzymywania biokompatybilnych środków antyseptycznych

Wiązanie srebra do innych biomolekuł, na przykład białek, może znacznie zredukować jego działanie cytotoksyczne. Do klasycznych przykładów takich preparatów, wprowadzonych do praktyki medycznej pod koniec XIX i na początku XX wieku, należą Collargol, Protargol oraz Argyrol [74]. Stosowano je jako środki przeciwbakteryjne i antyseptyczne, zwłaszcza w leczeniu ran, zakażeń błon śluzowych oraz zapalenia spojówek, ponieważ miały ograniczać drażniące działanie wolnych jonów srebra przy zachowaniu aktywności przeciwdrobnoustrojowej. Współcześnie idea wiązania srebra z nośnikami białkowymi jest nadal rozwijana w biomedycynie, gdzie białka pełnią funkcję stabilizatorów, nośników i modulatorów uwalniania srebra, co znajduje zastosowanie w projektowaniu materiałów przeciwbakteryjnych, opatrunków i innych wyrobów medycznych o poprawionej biokompatybilności. Zaprojektowanie kompleksu opartego na kazeinie i srebrze wykazuje

istotny potencjał aplikacyjny w medycynie, zwłaszcza jako środek antyseptyczny o wysokiej skuteczności przeciwdrobnoustrojowej oraz jednocześnie obniżonej cytotoksyczności. W pracy P1 przeprowadzono modyfikację kazeiny jonami Ag(I), wykorzystując dwie formy białka: kazeinę poddaną oraz niepoddaną procesowi odsalania metodą ultrafiltracji. Usunięcie jonów towarzyszących prowadziło do zaburzenia równowagi mineralnej układu, co mogło sprzyjać zwolnieniu dodatkowych miejsc w układzie micelarnym zdolnych do wiązania srebra. Weryfikację tej hipotezy przeprowadzono na podstawie analizy sorpcyjnej, która umożliwiła wyznaczenie maksymalnej pojemności sorpcyjnej ($q_{\max}$) oraz efektywności wiązania jonów Ag(I) przez kazeinę przed i po odsalaniu. Zgodnie z teorią twardych i miękkich kwasów i zasad (ang. Hard and Soft Acids and Bases – HSAB), jony Ag(I) zaliczane są do kwasów miękkich, dlatego wykazują największe powinowactwo do zasad miękkich, czyli ligandów zawierających atomy siarki, azotu oraz w mniejszym stopniu – tlenu. W kontekście białek istotną rolę w oddziaływaniu z Ag(I) mogą odgrywać takie aminokwasy, jak cysteina, histydyna, metionina, kwas asparaginowy oraz kwas glutaminowy [72]. Warto zaznaczyć, że wiązanie srebra może towarzyszyć redukcją Ag(I) oraz tworzeniu nanocząstek metalicznych. Wcześniejsze badania wykazały, że niektóre aminokwasy obecne w strukturze białek, takie jak lizyna, kwas asparaginowy, kwas glutaminowy, tyrozyna, tryptofan oraz cysteina, mogą uczestniczyć w redukcji prekursorów metali do postaci nanocząstek [73]. Zjawisko to ma szczególne znaczenie w przypadku srebra, ponieważ nanostruktury srebra odgrywają ważną rolę w opracowywaniu nowoczesnych preparatów przeciwbakteryjnych. Nanocząstki srebra (AgNPs), o rozmiarach w zakresie 1-100 nm, charakteryzują się dużym stosunkiem powierzchni do objętości, a więc wysoką powierzchnią właściwą, co przekłada się na ich wysoką reaktywność i znaczący potencjał terapeutyczny w zwalczaniu drobnoustrojów, w tym szczepów opornych na antybiotyki [65]. W konsekwencji otrzymane nanokompleksy kazeina-srebro mogą wykazywać korzystne właściwości biologiczne i stanowić obiecującą platformę do projektowania nowych materiałów biomedycznych o działaniu przeciwdrobnoustrojowym. Stosowanie szeregu odpowiednich technik instrumentalnych jest niezbędne do wiarygodnej charakterystyki otrzymanych kompleksów, gdyż pozwala precyzyjnie określić ich właściwości fizykochemiczne, strukturalne i funkcjonalne. Analiza dynamicznego rozpraszania światła (ang. dynamic light scattering – DLS), spektroskopii osłabionego całkowitego odbicia w podczerwieni z transformacją Fouriera (ang. attenuated total reflectance Fourier transform infrared spectroscopy – ATR-FTIR) oraz spektrofotometrii dostarcza informacji, czy wiązanie srebra prowadzi do zmian w organizacji miceli, konformacji białka oraz udziale określonych grup funkcyjnych w wiązaniu Ag(I). Techniki mikroskopowe, takie jak

skaningowa mikroskopia elektronowa z dyspersją energii promieniowania rentgenowskiego (ang. scanning electron microscopy with energy-dispersive X-ray spectroscopy – SEM-EDS) oraz skaningowa transmisyjna mikroskopia elektronowa (ang. scanning transmission electron microscopy – STEM) pozwalają na charakterystykę zmian morfologii powierzchni oraz potwierdzenie obecności nanostruktur, a tym samym formowania nanokompozytów białkowych. Elektroforeza SDS-PAGE oraz analiza trawienia pepsyną zostały zastosowane w celu sprawdzenia, czy modyfikacja wpływa na integralność frakcji białkowych, oraz określenia podatności układu Ag-CN na proteolizę. Badania desorpcji srebra są natomiast kluczowe dla określenia trwałości kompleksu w różnych warunkach pH. Charakterystykę aktywności biologicznej wybranych kompleksów kazeina-srebro przeprowadzono poprzez ocenę ich właściwości przeciwbakteryjnych oraz cytotoksycznych. Do badań aktywności przeciwbakteryjnej zostały wybrane bakterie Gram-dodatnie (*Staphylococcus aureus*) oraz Gram-ujemne (*Klebsiella pneumoniae*), w tym szczepy o istotnym znaczeniu klinicznym. Badano *Staphylococcus aureus* B38, szczep lekowrażliwy wyizolowany z rany stopy cukrzycowej, a także trzy szczepy *Klebsiella pneumoniae*: KP66 wrażliwy na karbapenemy oraz KP1 i KP2 posiadające geny NDM-1, odpowiadające za oporność karbapenemazową. Takie podejście pozwoliło ocenić, czy zsyntetyzowany kompleks zachowuje aktywność również wobec bakterii wielolekoopornych. Z kolei ocenę żywotności komórek przeprowadzono metodą kolorymetryczną MTT na mysich fibroblastach linii L929, powszechnie stosowanych do wstępnej oceny cytotoksyczności materiałów przeznaczonych do kontaktu ze skórą.

Takie rozumienie mechanizmu tworzenia układu Ag-CN dobrze wpisuje się w najnowsze badania nad mlecznymi biomateriałami przeciwbakteryjnymi. W literaturze pojawiają się już kazeinowe hydrożele i materiały opatrunkowe, w których biomineralizacja srebra zachodzi in situ, a uzyskany materiał łączy działanie przeciwdrobnoustrojowe z dobrą biogodnością. Wyniki uzyskane w niniejszej rozprawie mają wobec tego znaczenie wykraczające poza sam opis układu modelowego, gdyż wskazują drogę do projektowania wyrobów medycznych, powłok i aktywnych materiałów opakowaniowych opartych na surowcach mlecznych, w których funkcja antyseptyczna wynika z kontrolowanej chemii koordynacyjnej i nanostrukturyzacji białka [13,14].

5. Laktoferyna – białko o unikatowej strukturze oraz funkcjonalności

Laktoferyna bydlęca (ang. bovine lactoferrin – bLF) jest szeroko znana jako globularna glikoproteina wykazująca wysokie powinowactwo do wiązania jonów żelaza(III) ($K_D = 10^{-20}$

M) [75]. Warto podkreślić, że LF wyraźnie różni się od innych białek rodziny transferyn, przede wszystkim większą stabilnością strukturalną, szczególnie w warunkach kwaśnych. W porównaniu z TF uwalnianie żelaza z LF rozpoczyna się przy niższym pH, a sam proces przebiega około 100 razy wolniej [76,77]. LF zwykle występuje w granulach neutrofilów oraz wydzielinach gruczołów zewnętrznych, t.j. mleko, siara, ślina, śluz oraz łzy. Najbogatszymi źródłami LF są siara oraz mleko dojrzale, w których stężenie tego białka jest odpowiednio w zakresie 1-5 mg/mL oraz 0.02-0.2 mg/mL. bLF po raz pierwszy zidentyfikowano w 1939 roku, natomiast jej izolacji dokonano w 1960 roku [78]. Dotychczas określono sekwencje LF dla dziewięciu gatunków, w tym człowieka, bydła, bawołu, konia, kozy, owcy, wielbłąda, świni oraz myszy. Warto podkreślić, że struktura pierwszorzędowa LF jest wysoko konserwowana międzygatunkowo; na przykład, sekwencja laktoferyny bydlęcej jest w 69% identyczna z sekwencją laktoferyny ludzkiej. LF jest białkiem o dużej masie cząsteczkowej, mieszczącej się w zakresie od 77 do 88 kDa, zależnej od źródła pochodzenia, stopnia glikozylacji oraz zastosowanej metody izolacji [79]. Spektrometria mas z desorpcją/ionizacją laserową wspomaganą matrycą z analizatorem czasu przelotu (ang. matrix-assisted laser desorption/ionization with time-of-flight analyzer mass spectrometry – MALDI-TOF/MS) jest zaawansowaną techniką analityczną szeroko stosowaną w proteomice, szczególnie do analizy zarówno białek natywnych, jak i ich peptydów [80]. W metodzie tej matryca odgrywa kluczową rolę w procesie jonizacji analitu, ponieważ absorbuje energię impulsu laserowego i przekazuje ją do próbki, umożliwiając jej desorpcję oraz jonizację. Połączenie elektroforezy SDS-PAGE z analizą MALDI-TOF/MS stanowi użyteczne narzędzie do charakterystyki struktury pierwszorzędowej białek oraz identyfikacji modyfikacji potranslacyjnych (ang. post-translational modifications – PTM), w szczególności glikozylacji.

LF charakteryzuje się szerokim spektrum aktywności biologicznych, spośród których kluczową rolę odgrywa transport oraz regulacja wchłaniania żelaza. Oprócz tego wykazuje właściwości przeciwbakteryjne, przeciwwirusowe, przeciwgrzybicze, przeciwzapalne, immunomodulujące, antyoksydacyjne, prebiotyczne oraz przeciwnowotworowe [81]. W związku z tym, bLF jest uznawana za wartościowy składnik bioaktywny oraz jest szeroko wykorzystywana w produkcji preparatów dla niemowląt, suplementów diety, żywności funkcjonalnej oraz formulacjach terapeutycznych.

Z chemicznego punktu widzenia LF jest wyjątkowym białkiem koordynacyjnym, ponieważ łączy wysoką selektywność miejsc wiążących Fe(III) z dużą zdolnością do adaptacji konformacyjnej. Koordynacja żelaza przez dwie fenolanowe grupy tyrozyny, karboksylan asparagianinu, atom azotu imidazolowego histydyliny oraz anion węglanowy tworzy układ, w

którym zamknięcie domen białka stabilizuje kompleks i jednocześnie ogranicza przedwczesne uwalnianie metalu. Właśnie ta sprzężona zależność między koordynacją, ruchem domen a ładunkiem powierzchniowym sprawia, że LF może być traktowana jako naturalne rusztowanie koordynacyjne dla innych jonów metali. Znaczenie mają tu nie tylko klasyczne centra wiążące Fe(III), ale również powierzchniowe reszty donorowe oraz warstwa hydratacyjna modulowana przez glikany [15,82–88].

5.1. Struktura trójwymiarowa bLF

bLF posiada unikatową strukturę trójwymiarową. Jej łańcuch polipeptydowy składa się z 689 aminokwasów, które są przestrzennie zwinięte w dwa homologiczne płaty: N (1-341) oraz C (342-689), połączone krótką α -helisą (333-344). Stabilizacja wzajemnego ułożenia obu płatów białka jest zapewniana przez oddziaływania niekowalencyjne, głównie hydrofobowe [82]. Każdy z płatów dzieli się na dwie domeny, odpowiednio N1/N2 oraz C1/C2, i zawiera jedno specyficzne miejsce wiążące jon Fe(III). W związku z tym jedna cząsteczka LF jest zdolna do związania dwóch jonów żelaza, co odpowiada około 1,4 mg Fe na 1 g LF. Koordynacja żelaza zachodzi z udziałem czterech reszt aminokwasowych, w tym jednej reszty histydyny (His), dwóch reszt tyrozyny (Tyr) oraz jednej reszty kwasu asparaginowego (Asp), a także anionu węglanowego (CO_3^{2-}), w wyniku czego miejsce wiązania Fe(III) przyjmuje geometrię oktaedryczną [83]. W zależności od stopnia nasycenia żelazem LF może być klasyfikowana jako apo-LF (nienasycona żelazem: %Fe < 5%), holo-LF (nasycona żelazem: %Fe > 85%) oraz LF natywna (mieszanina form apo oraz holo: %Fe ~ 15-20%) [84].

5.2. Właściwości fizykochemiczne LF o różnym stopniu nasyceniu Fe(III)

Należy podkreślić, że poszczególne formy LF mogą różnić się stabilnością konformacyjną, właściwościami fizykochemicznymi, takimi jak właściwości elektrostatyczne powierzchni i odporność termiczna, oraz zdolnością do oddziaływania z innymi cząsteczkami [85]. Zgodnie z wynikami spektroskopii dichroizmu kołowego (CD), udział α -helisy, β -struktury oraz struktury nieuporządkowanej (random coil) w apo-LF wynosił odpowiednio 20,0/59,0/21,0, natomiast w holo-LF 11,5/70,0/18,5 [86]. Ponadto holo-LF wykazuje większą stabilność termiczną niż forma nienasycona, ponieważ jej denaturacja zachodzi dopiero w temperaturze około 90 °C, podczas gdy dla apo-LF następuje ona już przy 66 °C [85]. Różnice te wynikają bezpośrednio z odmienności konformacyjnych obu form białka. Badania krystalograficzne wykazały, że apo-LF przyjmuje bardziej otwartą strukturę, podczas gdy holo-LF charakteryzuje się konformacją bardziej zwartą i ściślej upakowaną [87]. Istotne jest również to, że bLF zachowuje stabilność strukturalną nawet po uwolnieniu jonów żelaza, co wiąże się ze swoistymi właściwościami konformacyjnymi miejsc wiążących ten pierwiastek. Po odłączeniu

żelaza płat N staje się bardziej elastyczny, podczas gdy płat C pozostaje w konformacji „zamkniętej”, co sprzyja utrzymaniu ogólnej stabilności apo-LF, nawet w warunkach kwaśnych, przy pH 4 [88]. Dodatkowo właściwości ładunku powierzchniowego bLF, zależne od dostępności jonizujących grup aminokwasowych, mogą zmieniać się wraz ze stopniem nasycenia białka żelazem. Wartości punktu izoelektrycznego dla apo-LF, holo-LF oraz LF natywnej wynoszą odpowiednio 6,4, 8,6 i 8,8, co może determinować zdolność tych form do wiązania oraz charakter ich oddziaływań z innymi cząsteczkami [84].

5.3. Rola glikozylacji w LF: aspekt strukturalny oraz biologiczny

Glikozylacja jest jednym z rodzajów modyfikacji postranslacyjnych występujących u białek, która odgrywa ważną rolę w stabilizacji ich struktury oraz może determinować właściwości biologiczne makrocząsteczki [89]. Glikozylacja białka polega ona na kowalencyjnym przyłączeniu reszt cukrowych do określonych aminokwasów; proces ten zwykle zachodzi w obrębie retikulum endoplazmatycznego oraz aparatu Golgiego. W przypadku bLF występuje przede wszystkim glikozylacja typu N, obejmująca przyłączanie łańcuchów cukrowych do reszt asparaginy (Asn). W strukturze bLF obecne są N-glikany typu wysokomannozowego, hybrydowego oraz złożonego. Białko to zawiera cztery stałe miejsca N-glikozylacji zlokalizowane przy resztach Asn233, Asn368, Asn476 i Asn545. Ponadto zaobserwowano, że dodatkowe miejsce glikozylacji może występować przy Asn281, szczególnie w bLF pochodzącej z siary [86]. Choć pełna rola glikozylacji bLF nie została jeszcze ostatecznie poznana, poprzednie prace wskazują na jej udział w ochronie białka przed proteolizą, modulacji aktywności przeciwbakteryjnej oraz przeciwwirusowej, a także wpływie na właściwości przeciwnowotworowe, w tym na zdolność wiązania oraz internalizację przez komórki nowotworowe [89–92]. Profil glikozylacji bLF może zależeć od wielu czynników biologicznych i środowiskowych, t.j. rasa, wiek, stadium laktacji, sposób żywienia, czynniki genetyczne organizmu oraz sezonowość [86]. Warto jednak zaznaczyć, że glikozylacja LF może ulegać zmianom również pod wpływem warunków technologicznych związanych z izolacją, oczyszczaniem oraz dalszym przetwarzaniem białka, choć aspekt ten nadal pozostaje słabo rozpoznany.

Z punktu widzenia zastosowań przemysłowych heterogeniczność glikozylacji nie jest jedynie ciekawostką strukturalną, lecz jednym z kluczowych atrybutów jakościowych preparatu LF. Profil N-glikanów wpływa na hydratację powierzchni, ekranowanie ładunku dodatniego, odporność na proteolizę, skłonność do agregacji oraz oddziaływanie z receptorami. Oznacza to, że proces izolacji, oczyszczania oraz przechowywania LF musi być postrzegany jako element kontroli jakości produktu o wysokiej wartości dodanej, a nie wyłącznie jako operacja

techniczna. Rosnąca komercjalizacja laktoferyny w żywności dla niemowląt, nutraceutykach i specjalistycznych formulacjach wymusza więc stosowanie metod, które zachowują integralność zarówno części peptydowej, jak i glikanowej białka [10,86,89–92].

5.4. Metody izolacji oraz oczyszczania LF

Zawartość LF w mleku krowim wynosi <1% całkowitej zawartości białek mleka, dlatego izolacja tego białka jest procesem złożonym i wieloetapowym [93]. Ekstrakcję LF z różnych surowców standardowo prowadzi się z zastosowaniem technik chromatograficznych, takich jak chromatografia jonowymienna i chromatografia powinowactwa, a także szeregu technik membranowych [86]. Dotychczas LF odzyskiwano z różnych surowców, m.in. z mleka pełnego, mleka odtuszczonego, serwatki słodkiej i kwaśnej oraz z siary. Chromatografia jonowymienna należy do najbardziej efektywnych oraz najczęściej stosowanych technik do produkcji LF na dużą skalę [94]. Mechanizm rozdziału opiera się na istotnych różnicach w punktach izoelektrycznych pomiędzy LF a innymi białkami serwatkowymi. W warunkach procesu LF wykazuje silne powinowactwo do fazy stacjonarnej, natomiast pozostałe białka serwatkowe ulegają łatwiejszej elucji z kolumny. Izolacja LF metodą chromatografii powinowactwa polega na specyficznym wiązaniu białka z ligandem (t.j. heparyna, jony metali, oligonukleotydy, inne białka serwatkowe) unieruchomionym na złożu, co umożliwia jego selektywne zatrzymanie i późniejszą elucję [95]. Z kolei techniki membranowe opierają się na rozdziale składników przez membranę według wielkości cząsteczek lub właściwości elektrostatycznych i są wykorzystywane głównie do wstępnego oczyszczania, zatężania oraz odsalania frakcji zawierającej LF. Należy podkreślić, że proces izolacji powinien być ściśle monitorowany pod względem stosowanych warunków, takich jak pH, siła jonowa buforu, temperatura i ciśnienie, ponieważ parametry te mogą w istotny sposób wpływać zarówno na skuteczność rozdzielania i czystość produktu, jak i na integralność struktury białka docelowego [86]. Jak pokazano w pracy [P3], właściwości elektrokinetyczne bLF są silnie uzależnione od warunków środowiska, zwłaszcza od stężenia elektrolitu, co wpływa na charakter ładunku powierzchniowego białka. Z kolei badania stabilności strukturalnej prowadzone w szerokim zakresie pH umożliwiły określenie warunków, w których LF jest najbardziej podatna na degradację.

Ultrafiltracja (UF) jest powszechnie stosowaną techniką membranową w mleczarstwie do zatężania oraz frakcjonowania białek serwatkowych o masie cząsteczkowej od 1 do 100 kDa. Po raz pierwszy UF była użyta w mleczarstwie w 1971 roku do oddzielenia frakcji białek serwatkowych od frakcji laktozy [96]. W przypadku laktoferyny bydlęcej pozwala ona uzyskać produkt o wyższym stopniu czystości dzięki usunięciu mniejszych cząstek, takich jak sole i

peptydy, zaadsorbowanych na powierzchni białka podczas kolejnych etapów izolacji. Wieloetapowemu procesowi izolacji LF zwykle towarzyszy stosowanie roztworów buforowych o wysokim stężeniu elektrolitów, w wyniku czego uzyskany produkt końcowy może zawierać znaczne ilości soli. Stopień zasolenia białka może być jednak uzależniony od rodzaju zastosowanego surowca. Przykładowo, zawartość składników mineralnych w mleku, serwatce słodkiej i serwatce kwaśnej wynosi odpowiednio 0,6-0,9%, 0,2-0,5% oraz 0,4-0,7% [97]. Chociaż niewielkie ilości soli mogą stabilizować strukturę białka, wyższe stężenia elektrolitów osłabiają oddziaływania elektrostatyczne odpowiedzialne za stabilność układu, co może prowadzić do agregacji i precypitacji białka. Ponadto w [P3] pokazano, że różnego rodzaju zanieczyszczenia próbki, w tym sole, mogą mieć negatywny wpływ podczas analizy spektrometrycznej powodując supresję sygnału białka [63]. Należy również podkreślić, że długotrwałe przechowywanie białka w nieodpowiednich warunkach może prowadzić do jego częściowej degradacji, a powstałe w wyniku tego procesu peptydy mogą stanowić istotne zanieczyszczenie próbki. Dotychczasowe badania wskazują, że przechowywanie LF w temperaturze -20 °C przez ponad 6 miesięcy może negatywnie wpływać na integralność strukturalną białka, a w konsekwencji znacząco ograniczyć jego potencjał biologiczny [98]. Oczyszczenie próbki po ultrafiltracji ma kluczowe znaczenie nie tylko dla poprawy jakości analizy spektrometrycznej, lecz także dla zapewnienia wysokiej odtwarzalności wyników oraz wiarygodności badań biologicznych. Eliminacja soli oraz innych niskocząsteczkowych zanieczyszczeń zmniejsza zmienność właściwości fizykochemicznych próbki, co sprzyja uzyskiwaniu bardziej spójnych oraz porównywalnych wyników. Jest to szczególnie istotne w badaniach aktywności biologicznej, zwłaszcza w testach komórkowych, gdzie obecność składników towarzyszących może pośrednio wpływać na konformację białka, jego właściwości powierzchniowe oraz zdolność do wiązania ze specyficznymi receptorami [99]. W pracy [P3] została przeprowadzona ocena wpływu dodatkowego etapu ultrafiltracji na właściwości fizykochemiczne bydlęcej laktoferyny, w szczególności na jej masę cząsteczkową, strukturę pierwszorzędową, morfologię powierzchni oraz skład pierwiastkowy.

Zagadnienie to ma wyraźny wymiar rynkowy, ponieważ laktoferyna należy obecnie do najbardziej wartościowych składników bioaktywnych pozyskiwanych z mleka oraz serwatki. Im wyższy stopień czystości preparatu, lepsze zachowanie natywnej struktury białka oraz większa powtarzalność jego właściwości, tym szersze są możliwości jego wykorzystania w recepturach o wysokim stopniu specjalizacji. Z tego powodu procesy membranowe, chromatograficzne i analityczne nie pełnią tu jedynie funkcji pomocniczej, lecz współdecydują o ekonomicznej opłacalności wytwarzania LF jako ingrediencji premium oraz o możliwości jej

wdrożenia do produktów, w których deklarowana aktywność biologiczna musi być ściśle związana z jakością molekularną preparatu [10,93].

5.5. Oddziaływanie LF z jonami metali

Choć LF wykazuje największe powinowactwo do jonów Fe(III), liczne prace wskazują, że białko to może również tworzyć kompleksy z innymi metalami, w tym Cu(II), Ca(II), Zn(II), Co(III), Mn(III), Cr(III), Al(III), Ag(I) oraz Au(I) [100]. Jony te mogą być wiązane zarówno w obrębie miejsc koordynacyjnych charakterystycznych dla Fe(III), jak i poprzez niespecyficzne oddziaływania z powierzchnią białka. Mechanizm ich wiązania zależy jednak od właściwości chemicznych danego metalu. Na przykład, w przypadku Mn(III) wykazano koordynację w specyficznym miejscu wiążącym apo-LF z udziałem dwóch reszt tyrozyny, jednej reszty kwasu asparaginowego, jednej reszty histydyny oraz anionu węglanowego [101]. Natomiast wiązanie Zn(II) przez natywną LF ma charakter bardziej niespecyficzny oraz zachodzi z udziałem powierzchniowych grup funkcyjnych białka [102]. Oddziaływania LF z jonami metali mają istotne znaczenie w projektowaniu nowych związków bioaktywnych. Koordynacja jonów metali przez LF może prowadzić do zmian konformacyjnych, modyfikacji stabilności oraz nadania białku nowych właściwości biologicznych lub wzmocnienia już istniejących funkcji. Wcześniej wykazano, że kompleksy LF-Fe, LF-Mn oraz LF-Zn charakteryzują się wyższą aktywnością przeciw ludzkiemu wirusowi niedoboru odporności (HIV) w porównaniu z apo-LF [103]. Sugeruje to możliwość wykorzystania tego typu układów w projektowaniu nowych preparatów o właściwościach przeciwwirusowych.

Z chemicznego punktu widzenia wielometaliczna reaktywność LF wynika z obecności kilku klas donorów elektronowych: fenolanowych grup tyrozyny, karboksylanów Asp/Glu, donorów azotowych histydyny i lizyny oraz, pośrednio, z anionów synergicznych stabilizujących określone geometrie koordynacyjne. O tym, czy metal zajmie klasyczne miejsce wiążące Fe(III), czy też utworzy bardziej powierzchniowy kompleks, decydują promień jonowy, preferowany stopień utlenienia, twardość lub miękkość zgodnie z teorią HSAB oraz podatność kompleksu prekursorowego na hydrolizę i akwapodstawienie. Właśnie dlatego LF może tworzyć zarówno kompleksy o wyraźnie zdefiniowanej koordynacji, jak i układy o charakterze mieszanym, w których wiązanie metalu sprzęga się ze zmianami ładunku powierzchniowego, agregacji i aktywności biologicznej [15,100–102,104,105].

5.5.1. Właściwości biologiczne kompleksów rutenu

Kompleksy rutenu (Ru) w ostatnich latach zyskują coraz większe znaczenie w projektowaniu nowych związków terapeutycznych ze względu na swoje zróżnicowane właściwości chemiczne

oraz biologiczne. Ruten posiada szeroki zakres stopni utlenienia (od $-II$ do $+VIII$), niemniej jednak w badaniach najczęściej są wykorzystywane związki $Ru(II)$ oraz $Ru(III)$ ze względu ich największą stabilność chemiczną [106]. Związki $Ru(II/III)$ posiadają szeroki spektrum aktywności biologicznej, wykazują przede wszystkim mają działanie przeciwnowotworowe, przeciwbakteryjne oraz antyoksydacyjne. Mechanizm przeciwbakteryjny kompleksów rutenu polega głównie na uszkodzeniu błony komórkowej bakterii oraz oddziaływaniu z wewnątrzkomórkowymi celami, zwłaszcza RNA, co prowadzi do zahamowania translacji i syntezy białka [107]. Obecnie kompleksy rutenu są coraz częściej rozpatrywane jako korzystniejsza alternatywa dla platynowych leków przeciwnowotworowych. Kompleksy rutenu wykazują aktywność przeciwnowotworową poprzez uszkodzenie DNA, wywoływanie apoptozy za pośrednictwem ROS oraz działanie przeciwpierzutowe. Wśród związków $Ru(III)$ o potencjale przeciwnowotworowym szczególne miejsce zajmują NAMI-A i KP1019, wykazujące mniejszą toksyczność względem komórek zdrowych oraz mniejszą skłonność do indukowania oporności na leczenie [108]. Niemniej jednak, nie wykazali one satysfakcjonującej efektywności w badaniach klinicznych. Wynikało to głównie z istotnych efektów ubocznych oraz ograniczonej biodostępności, co wymusza dalsze poszukiwanie bardziej optymalnych rozwiązań terapeutycznych.

Dla układów $Ru(III)$ szczególne znaczenie ma labilność sfery koordynacyjnej i możliwość przechodzenia pomiędzy formami $Ru(III)$ i $Ru(II)$ w środowisku biologicznym. Proces aquacji prekursorów chlorkowych zwiększa reaktywność kompleksu wobec donorów białkowych, natomiast lokalne warunki redoks mogą modulować trwałość utworzonego połączenia i jego aktywność biologiczną. W konsekwencji LF nie działa tutaj wyłącznie jako bierny nośnik, lecz jako aktywne rusztowanie koordynacyjne, które może wpływać na kinetykę wiązania, dystrybucję metalu i profil odpowiedzi komórkowej. Ujęcie to ma istotne znaczenie dla interpretacji otrzymanych kompleksów, ponieważ przesuwają analizę z poziomu prostego „załadunku” jonu na poziom projektowania funkcji całego układu metalobiałkowego [15,104–109].

5.5.2. Projektowanie kompleksów LF-Ru jako potencjalne podejście w terapii celowanej

Jedną z kluczowych funkcji biologicznych białek z rodziny transferryn, w tym i LF, jest utrzymanie homeostazy żelaza. Mechanizm komórkowego pobierania żelaza przebiega na drodze endocytozy receptorowej [110]. W pierwszym etapie dochodzi do związania białka z odpowiednimi receptorami zlokalizowanymi na powierzchni komórki, po czym kompleks receptor-ligand ulega internalizacji w wyniku endocytozy. Po wnikięciu do wnętrza komórki żelazo zostaje uwolnione w obrębie endosomu, natomiast forma niezwiązana z żelazem jest

ponownie jest uwalniana do krwiobiegu. Wykazano, że LF również może ulegać internalizacji przez komórki za pośrednictwem receptorów [111]. Receptory LF (ang. lactoferrin receptors – LFR) występują na powierzchni wielu typów komórek, między innymi komórek nabłonka jelitowego, hepatocytów, fibroblastów, neutrofilów, limfocytów oraz makrofagów [99]. Szczególnie wysoka ekspresja LFR obserwowana jest w nabłonku jelitowym, gdzie receptory te odgrywają istotną rolę w procesach transportu i absorpcji żelaza. Wykazano, że zarówno apo-LF, jak i holo-LF charakteryzują się porównywalnym powinowactwem do receptorów LFR obecnych w enterocytach Caco-2, przy czym wartości stałych asocjacji wynosiły odpowiednio $7 \times 10^4 \text{ M}^{-1}$ oraz $6 \times 10^4 \text{ M}^{-1}$ [111]. Układ LF-LFR może być również wykorzystywany jako narzędzie do celowanego transportu leków do komórek nowotworowych, które zwykle charakteryzują się podwyższoną ekspresją LFR na powierzchni [112]. Warto zauważyć, że ruten może naśladować żelazo w oddziaływaniach z białkami należącymi do rodziny transferyn, dlatego można przypuszczać, że jego pobieranie przez komórki odbywa się analogiczną drogą, tj. poprzez endocytozę receptorową [109]. Projektowanie kompleksów na bazie LF oraz związków Ru(III) może stanowić obiecującą strategię w terapii celowanej, pozwalającą na selektywne dostarczanie substancji aktywnej do komórek nowotworowych, zwiększenie efektywności leczenia oraz ograniczenie zjawiska lekooporności.

W pracy [P5] przedstawiono poznanie molekularnych mechanizmów koordynacji Ru(III) w obrębie bLF, a także określenie konsekwencji tego wiązania dla struktury, stabilności oraz aktywności biologicznej białka. Jako prekursor Ru(III) wybrano kompleks akwapentachlororutenianu(III) potasu ($\text{K}_2[\text{RuCl}_5(\text{H}_2\text{O})]$), który wykazuje dobrą rozpuszczalnością w wodzie. W porównaniu z powszechnie używanym hydratem chlorku rutenu(III) ($\text{RuCl}_3 \cdot x\text{H}_2\text{O}$), związek ten cechuje się określoną strukturą koordynacyjną, większą odpornością na hydrolizę oraz mniejszą skłonnością do niekontrolowanej polimeryzacji [104,105]. W konsekwencji wykorzystanie tego kompleksu do modyfikacji LF sprzyja uzyskaniu większej kontroli nad procesem oraz lepszej powtarzalności zarówno w badaniach mechanizmu wiązania, jak i podczas otrzymywania układów LF-Ru. Z kolei, obecność labilnej cząsteczki wody w sferze koordynacyjnej sprzyja jej wymianie na grupy funkcyjne LF, a zwarta budowa kompleksu wskazuje, że może on być efektywnie wiązany w obrębie biomolekuły. Praca obejmowała zarówno charakterystykę fizykochemiczną kompleksów LF-Ru, wykorzystanie metod obliczeniowych jak oraz określenie ich potencjału biologicznego oceniając właściwości antybakteryjne oraz cytotoksyczne. W pracy zostały przeprowadzone badania izotermiczne oraz kinetyczne w celu scharakteryzowania mechanizmu wiązania Ru(III) przez bLF. Do analizy izotermicznej najczęściej się stosują dwa klasyczne modele

adsorpcji: model Langmuira oraz model Freundlicha. Model Langmuira opisuje sorpcję monowarstwową na jednorodnych centrach wiążących o jednakowym powinowactwie, natomiast model Freundlicha odnosi się do adsorpcji na powierzchni heterogenicznej oraz dopuszcza możliwość tworzenia wielowarstw [113]. Na podstawie tych modeli można określić charakter oddziaływań, pojemność sorpcyjną, efektywność wiązania oraz spontaniczność tworzenia kompleksu LF-Ru. Badania kinetyczne przeprowadzono w celu określenia szybkości tworzenia kompleksu LF-Ru oraz identyfikacji etapów kontrolujących przebieg tego procesu. Do analizy danych zastosowano model kinetyki zerowego rzędu, zakładający stałą szybkość reakcji niezależną od stężenia substratów, co pozwoliło opisać zmiany stężenia Ru(III) w czasie. Dodatkowo wykorzystano model Webera-Morrisa, służący do oceny wpływu dyfuzji wewnątrzcząsteczkowej na przebieg wiązania [114]. W celu kompleksowej charakterystyki układów było wykorzystano techniki spektroskopowe, spektrometryczne, mikroskopowe, rozpraszania promieniowania, elektroforetyczne, metody modelowania molekularnego oraz testy biologiczne [86]. Wykorzystanie spektrofluorymetrii 3D służy do badania zmian w mikrośrodowisku reszt aromatycznych białka, zwłaszcza tryptofanu i tyrozyny. W praktyce pozwala wykryć, czy po związaniu metalu zmienia się konformacja białka, dostępność fluoroforów oraz lokalna polarność otoczenia. Analiza wygaszania fluorescencji poprzez sporządzenie krzywej Sterna-Volmera jest stosowana do oceny siły oraz mechanizmu oddziaływania między białkiem a ligandem/metalem. Pozwala wyznaczyć stałą wygaszania, stałą wiązania oraz oszacować, czy dominuje mechanizm statyczny, dynamiczny lub mieszany. Analizę dynamicznego rozpraszania światła (ang. dynamic light scattering – DLS) stosuje się do pomiaru rozmiaru hydrodynamicznego cząstek/agregatów w roztworze. Technika ta pozwala ocenić, czy po związaniu Ru(III) białko tworzy większe agregaty lub układ staje się bardziej stabilny. Pomiar potencjału zeta stosuje się do oceny stabilności koloidalnej cząstek. Technika ta pomaga określić, czy kompleks LF-Ru jest bardziej podatny na agregację oraz jak zmienił się ładunek powierzchniowy białka po związaniu metalu. Wykorzystanie analizy spektroskopii osłabionego całkowitego odbicia w podczerwieni z transformacją Fouriera (ang. attenuated total reflectance Fourier transform infrared spectroscopy – ATR-FTIR) pozwala na identyfikację grup funkcyjnych uczestniczących w wiązaniu metalu a także do oceny zmian w strukturze drugorzędowej LF. Zwłaszcza dokonanie dekonwolucji pasma Amidowego I pozwala monitorować zmiany w zawartości poszczególnych rodzajów struktur drugorzędowych, t.j. α -helisy, β -struktur oraz struktury nieuporządkowanej. Spektroskopia dichroizmu kołowego z wykorzystaniem promieniowania synchrotronowego (ang. synchrotron radiation circular dichroism – SRCD) jest metodą komplementarną do analizy zmian

zachodzących w strukturze drugorzędowej LF w wyniku modyfikacji Ru(III). Warto podkreślić że FTIR i SRCD mają różną czułość na poszczególne elementy struktury wtórnej, dlatego niewielkie rozbieżności między metodami są oczekiwane. Z kolei analiza małokątowego rozpraszania promieniowania rentgenowskiego (ang. small-angle X-ray scattering – SAXS) dostarczył informacji o organizacji przestrzennej LF-Ru w roztworze, w tym o promieniu bezwładności (R_g), maksymalnym wymiarze cząstki (D_{max}) oraz stopniu oligomeryzacji. Stosowanie kompleksowej analizy mikroskopowej, w tym mikroskopii świetlnej (light microscopy – LM), skaningowej mikroskopii elektronowej z dyspersją energii promieniowania rentgenowskiego (ang. scanning electron microscopy with energy-dispersive X-ray spectroscopy – SEM-EDS) oraz transmisyjnej mikroskopii elektronowej z dyspersją energii promieniowania rentgenowskiego (transmission electron microscopy with energy-dispersive X-ray spectroscopy – TEM-EDS) pozwoliły scharakteryzować morfologię powierzchni kompleksów LF-Ru oraz potwierdzić akumulację oraz dystrybucję rutenu w matrycy białkowej. Elektroforeza SDS-PAGE dostarczyła informację o masie cząsteczkowej, zachowaniu integralności struktury oraz możliwej degradacji. Przeprowadzenie trawienia kompleksów LF-Ru pepsyną umożliwia ocenić oporność kompleksu w warunkach zbliżonych do przewodu pokarmowego. Badanie desorpcji stosuje się do określenia stabilności kompleksu LF-Ru oraz stopnia uwalniania metalu z nośnika w symulowanym płynie żołądkowym oraz jelitowym. Uzyskane wyniki desorpcji oraz trawienia pepsyną mogą determinować o potencjalnej biodostępności związku. Zastosowanie metod obliczeniowych było niezbędne do uzupełnienia danych eksperymentalnych, ponieważ umożliwiło pełniejsze wyjaśnienie mechanizmów molekularnych odpowiedzialnych za wiązanie Ru(III) przez laktoferynę w układzie LF-Ru. Analiza dokowania molekularnego (ang. molecular docking) była stosowana do przewidywania najbardziej prawdopodobnych miejsc wiązania Ru(III) w strukturze LF, ze szczególnym uwzględnieniem specyficznych reszt aminokwasowych wiążących żelazo. Symulacje dynamiki molekularnej (ang. molecular dynamics – MD) pozwoliły na analizę stabilności kompleksu LF-Ru oraz ocenę zmian jego struktury konformacyjnej w czasie. Z kolei teoria funkcjonału gęstości (ang. Density Functional Theory – DFT) była wykorzystana do analizy natury elektronowej oddziaływań między Ru(III) a grupami donorowymi aminokwasów oraz do oceny ich preferencji koordynacyjnych. Badania przeciwbakteryjne z wykorzystaniem wybranych bakterii Gram-dodatnich (*Enterococcus faecalis*) oraz Gram-ujemnych (*Escherichia coli*, *Klebsiella pneumoniae*) pozwoliło oszacować wpływ związanego Ru(III) na pierwotne właściwości przeciwdrobnoustrojowe LF. Wpływ kompleksów LF-Ru na liniach komórkowych fibroblastów mysich (L929), nowotworu wątrobowokomórkowego

(HepG2) oraz gruczolakoraka jelita grubego (Caco-2) pozwoliło określić ich wpływ na aktywność metaboliczną, żywotność, cytotoksyczność oraz morfologię komórek.

W szerszej perspektywie badania te wpisują się w najnowszy nurt wykorzystania LF jako ligandu makromolekularnego i wektora ukierunkowanego transportu. Aktualne prace przeglądowe wskazują, że kompleksy oraz nanoformulacje oparte na LF są rozpatrywane nie tylko w kontekście żywności funkcjonalnej, lecz również precyzyjnej onkologii, systemów przekraczających bariery biologiczne i wielofunkcyjnych platform terapeutycznych. Oryginalność niniejszej rozprawy polega na tym, że punkt wyjścia dla takiej strategii stanowi surowiec mleczarski, a nie syntetyczny polimer czy nośnik niebiologiczny. Tym samym laktoferyna zostaje tutaj ukazana jako składnik, który może łączyć logikę technologii mleczarskiej z logiką chemii koordynacyjnej i nowoczesnych systemów dostarczania substancji aktywnych [15–17].

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Cele badawcze pracy

Głównym celem rozprawy było opracowanie kompleksowej metodologii badawczej przeznaczonej do wieloaspektowej analizy biokoloidów pochodzenia mleczarskiego, z uwzględnieniem ich właściwości fizykochemicznych, strukturalnych oraz funkcjonalnych. Istotnym elementem pracy była również ocena możliwości ukierunkowanej modyfikacji tych układów w celu poprawy lub rozwinięcia ich cech użytkowych oraz określenie potencjału ich wykorzystania w projektowaniu rozwiązań o znaczeniu aplikacyjnym i wdrożeniowym dla przemysłu mleczarskiego.

Cel był zrealizowany poprzez:

- przeprowadzenie wstępnej charakterystyki fizykochemicznej bLF, obejmującej ocenę jej stabilności koloidalnej w zależności od siły jonowej oraz określenie podatności na degradację w szerokim zakresie pH;
- zbadanie wpływu dodatkowego etapu oczyszczania białka metodą ultrafiltracji, mającego na celu eliminację zanieczyszczeń niskocząsteczkowych (soli, peptydów), na właściwości fizykochemiczne bLF, a także ocenę przydatności tego procesu w poprawie jakości analiz oraz reprezentatywności próbek na podstawie wyników identyfikacji białka metodą MALDI-TOF/MS;
- przeprowadzenie modyfikacji kazeiny jonami srebra w warunkach alkalicznych (pH 8) po raz pierwszy stosując kompleks diaminasrebra(I) ($[\text{Ag}(\text{NH}_3)_2]^+$) jako prekursor Ag(I) ; opracowanie metodologii do przygotowania kompleksów na bazie laktoferyny oraz rutenu z wykorzystaniem kompleksu akwapentachlororutenianu(III) potasu ($\text{K}_2[\text{RuCl}_5(\text{H}_2\text{O})]$) jako prekursora Ru(III) ;
- poddanie kazeiny ultrafiltracji oraz oszacowanie wpływu soli micelarnych na mechanizm oddziaływania kazeiny z jonami srebra oraz porównanie maksymalnej pojemności sorpcyjnej białka do wiązania Ag(I) przed i po odsalaniu;
- określenie przebiegu wiązania Ru(III) przez LF na podstawie badań izotermicznych oraz kinetycznych;
- wykorzystanie szeregu komplementarnych technik instrumentalnych do określenia stabilności oraz zmian strukturalnych zachodzących w wyniku wiązania kazeiny z Ag(I) oraz laktoferyny z Ru(III) ;
- zastosowanie metod obliczeniowych, w tym modelowania molekularnego oraz badań kwantowo-mechanicznych, w celu scharakteryzowania molekularnego mechanizmu

oddziaływań w układzie LF-Ru oraz porównania uzyskanych wyników z danymi eksperymentalnymi;

- określenie właściwości antybakteryjnych oraz działania cytotoksycznego uzyskanych kompleksów Ag-CN oraz LF-Ru;
- zbadanie możliwości wykorzystania bakterii kwasu mlekowego (*Lactiplantibacillus plantarum*) do kształtowania składu śmietanki w różnych warunkach inkubacyjnych uwzględniając dostęp/brak światła, mieszanie/brak mieszania, stosunek objętościowy śmietanka/bakteria oraz forma dodanego szczepu (biomasa, liofilizat) ze szczególnym uwzględnieniem zawartości witaminy D3 oraz zmian profilu kwasów tłuszczowych.

Do realizacji tych zadań wykorzystano następujące techniki:

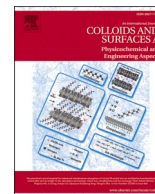
- wysokosprawną chromatografię ciekową w odwróconym układzie faz;
- chromatografię gazową sprzężoną ze spektrometrią mas;
- spektrometrię mas z laserową jonizacją/desorpcją próbki wspomaganą matrycą z analizatorem czasu przelotu;
- spektrometrię mas z plazmą wzbudzaną indukcyjnie oraz optyczną spektrometrię emisyjną z plazmą wzbudzaną indukcyjnie;
- spektroskopię w zakresie podczerwieni z transformacją Fouriera, spektroskopię w zakresie ultrafioletu i światła widzialnego, spektroskopię fluorescencyjną oraz spektroskopię dichroizmu kołowego z wykorzystaniem promieniowania synchrotronowego;
- technikę dynamicznego rozpraszania światła oraz pomiary potencjału zeta;
- małokątowe rozpraszanie promieniowania rentgenowskiego;
- elektroforezę w żelu poliakrylamidowym w warunkach denaturacyjnych;
- mikroskopię świetlną, skaningową mikroskopię elektronową z analizą dyspersji energii promieniowania rentgenowskiego, skaningową transmisyjną mikroskopię elektronową oraz transmisyjną mikroskopię elektronową z analizą dyspersji energii promieniowania rentgenowskiego;
- metody obliczeniowe, obejmujące dokowanie molekularne, symulacje dynamiki molekularnej oraz obliczenia kwantowo-mechaniczne.

Metodyki badań zostały szczegółowo opisane w pracach [P1], [P3], [P4] oraz [P5] stanowiących zwartą całość przekładanej dysertacji.

Pełne wersje publikacji

[P1] Immobilization of Silver Ions onto Casein

Tetiana Dyrda-Terniuk, Oleksandra Pryshchepa, Katarzyna Rafińska, Mateusz Kolankowski, Adrian Gołębiowski, Michał Gloc, Renata Dobrucka, Krzysztof Kurzydłowski, Paweł Pomastowski. Immobilization of Silver Ions onto Casein. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **2023**, 667, 131390. doi.org/10.1016/j.colsurfa.2023.131390



Immobilization of silver ions onto casein

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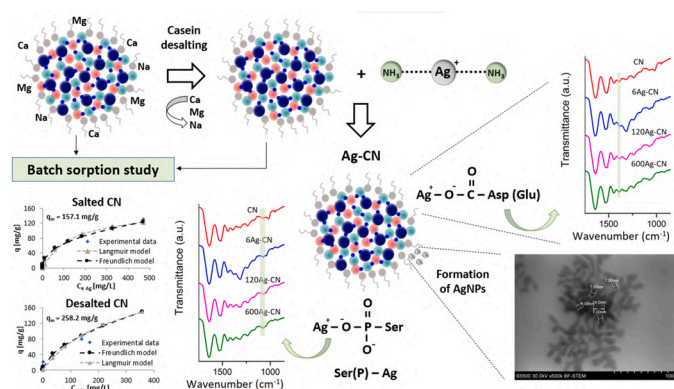
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HIGHLIGHTS

- Silver binding alters the structure of casein micelles.
- Silver dendrites can form upon silver reduction with caseins.
- Silver-casein complex is less toxic against eucaryotic cells than free silver ions.
- Different bacterial strains have different resistance against silver-casein complex.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Casein
Silver ions, antibacterial substance
Adsorption studies
Metal-protein complexes

ABSTRACT

In this study, the physicochemical and biological properties of silver-casein complexes were investigated. The casein modification was carried out by a novel method, i.e., at basic conditions (pH 8) using silver precursor in a form of ammonia complex $[\text{Ag}(\text{NH}_3)_2]^+$. The selected experimental conditions allowed us to obtain high colloidal stability of protein suspension and also minimized the risk of metal secondary reduction. Subsequently, the mechanism of silver immobilization was evaluated by using a batch sorption study (Freundlich, Langmuir isotherm modeling). Moreover, the influence of accompanying the protein ions on the silver binding process was studied (using ultrafiltration). Results revealed, that casein after the desalting step has much higher silver-binding capacity (258.2 mg/g) compared to untreated protein (157.1 mg/g). The nature of casein-silver interactions was comprehensively studied by multiple instrumental techniques, namely, spectrometric (ICP-MS), spectroscopic (DLS, ATR-FTIR, Fluorescence Spectroscopy), electron microscopy (STEM, SEM-EDX), and separation (SDS-PAGE). Results indicated, that silver sorption had a complex character. The heterogeneous character

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of metal binding processes was discovered. The DLS studies pointed out the great role of phosphoserine residues in silver-casein interactions and underlined their impact on the micelle size and structure. Moreover, STEM images confirmed the growth of silver nanoparticles upon metal binding to casein. According to FTIR analysis, carboxylic groups of aspartic and glutamic acid were identified as potential metal-binding sites which also could participate in silver reduction. Peptic digestion studies showed a relevant influence of silver incorporation on the structural stability of caseins. Finally, the biological activity of the 600Ag-CN complex was evaluated. Results indicated that the complex effectively inhibited the growth of both Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria. Still, the treatment of mouse fibroblast cell line L929 with analyzed complex (0.32 mg/mL of 600Ag-CN corresponding to 7.5 µg/mL of silver) decrease cell viability up to 60 %. The toxic activity of the complex may be connected to the Ag⁺ release. The silver desorption study showed that 600Ag-CN complex released 21.5 %, 49.3 %, and 68.7 % in pH 6.8, 4.5, and 1.2 respectively.

1. Introduction

Milk products represent a rich source of essential nutrients, including, proteins, peptides, fats, carbohydrates, vitamins, and minerals [1]. Among these components, proteins isolated from milk possess unique sets of health-beneficial effects, particularly antibacterial, antiviral, immunomodulatory, and anticancer properties [2]. Such a wide variety of biological activities induces an expanding trend in their supplementation [3]. Milk proteins are generally divided into two major groups: casein proteins (80 %) and whey proteins (20 %). Casein together with fat globules represents a non-soluble heterogeneous phase of milk emulsion. The biological importance of casein is ensured by the great content of nine dietary essential amino acids that promote muscle protein synthesis [4]. Casein calcium phosphate binding capacity plays an important role in the transport and bioavailability of these elements which are responsible for bone mineralization [5]. Besides, the technological process of casein isolation usually accompanies the distribution of milk minerals (in particular, calcium, magnesium, sodium, potassium, and zinc) between casein micelles and milk serum fractions [6]. For this reason, casein can act as a natural carrier of metal ions. The complex nature of the casein structure enables the distribution of multiple biologically active compounds, i.e. polysaccharides, fatty acids, polyphenols, vitamins, and drugs [7]. Hence, casein's multi-functionality and great delivery potential allow it to become a successful candidate for widespread implementation in the pharmaceutical, food, and cosmetic industries [8].

The exact structure of the casein protein remains undefined and sparks a huge interest among researchers since it doesn't consist of a single polypeptide chain but occurs as a heterogeneous supramolecular assembly. It is composed of four individual proteins, including α_{S1} -casein, α_{S2} -casein, β -casein, and κ -casein in the following mass ratio (w/w): 0.40:0.10:0.35:0.15 [7]. Phosphorylated serine residues of caseins are capable to bind calcium ions and associate forming globular aggregates that are commonly known as casein micelles. Each casein varies in its molecular and physicochemical properties, e.g. molecular weight, amino acids composition, the value of isoelectric point, hydrophilicity, surface potential, and degree of post-translation modifications. The number of phosphoserine residues reflects the information about the surface hydrophobicity of casein. Plenty of models of casein micelle have been previously described [9]. Nowadays, a submicelle model is considered the most accepted one. According to this model, casein micelle is built of 400–500 self-assembled structural subunits (submicelles) that are linked by colloidal calcium phosphate (CCP) [10]. The diameter of the submicelle is relatively small and is ranging 10–30 nm, while dispersed calcium phosphate clusters are approximately 2–3 nm in size [11]. Based on composition, submicelles are mainly divided into two groups: α_S - β , and α_S - κ type of submicelle. Submicelles of highly phosphorylated α_{S1} -casein, α_{S2} -casein, and β -casein reveal a great sensitivity towards calcium ions and tend to bury in the micelle core. However, submicelles rich in κ -caseins containing hydrophilic C-terminal chains form a hairy outer layer which contributes to maintaining and stabilization of casein micelle through electrostatic and steric repulsion [12]. The dual bonding model proposed by Horne in 1998 is known as

another progressive theory that describes the structure of the casein protein [13]. Based on this model, the micelle formation is driven by two major forces: hydrophobic interactions and electrostatic repulsion. The first one is responsible for the nucleation, cross-linking between hydrophobic regions (α_S - and β -casein), and further growth of polymers, while the negatively-charged moieties of κ -caseins inhibit casein self-association [12].

The size of casein micelles is highly dependent upon environmental factors, e.g. pH, temperature, and ionic strength. Extreme conditions lead to significant disruption in casein structural organization, e.g. dissociation, denaturation, and aggregation which provides to loss of protein functionality. Apart from physicochemical parameters, the size of casein micelle is affected by cow breed, lactation stage, feeding type, and season [14]. Some genetic factors, such as the presence of casein variants and posttranslational modifications of κ -casein play a significant role in casein particle size. It was found, that the non-homogeneity of β -casein and κ -casein as well as a higher number of glycosylated residues in κ -casein promote the formation of smaller micelles [15]. Typically, the diameter of casein particles is found in the wide range of 50–500 nm, and, the average size was reported at 200 nm [7]. pH is considered one of the most substantial parameters which allow controlling the size of dispersed proteins. Many studies have mentioned the instability of casein micelles at the pH of 5–6 and their tendency to create larger aggregates since this range is found close to the casein isoelectric point (pI 4.6) [16]. It was reported, that the hydrodynamic diameter of the micelle becomes smaller at pH $\geq$ 8. In turn, casein dispersions prepared at slightly alkaline conditions are characterized by less turbidity and higher colloidal stability. Heat treatment may have a significant effect on protein primary functionality. Casein heating at the relatively low ranges of temperatures from 6 °C to 50 °C showed the prevalence of hydrophobic interactions which induced the decrease in micelle size [17]. However, the application of extremely high temperatures (up to 150 °C) enhances casein susceptibility to aggregation and coagulation [18]. In addition, M. Glantz et. al. pointed out the great influence of mineral content in milk (in particular, Mg and Ca) on casein size [19]. Negatively charged carboxylic groups of aspartic and glutamic acids together with phosphoserine residues are mentioned as major functional groups involved in such type of casein-metal interaction. The excess of bounded metal is capable to reduce the net negative charge of individual caseins. Such effects cause the prevalence of hydrophobic interactions over electrostatic repulsions and facilitate protein precipitation [20]. Moreover, M. Grewal et. al. has revealed a notable modification in the secondary structure of individual caseins after the addition of metal ions, which emphasizes the need for the study of the metal impact on casein conformation as a preliminary step of synthesized complexes characterization [21].

Some transition metals belong to essential micronutrients. They act as essential mediators during many enzymatic and also play an important role in the functionality of the majority of membrane proteins [22]. Silver is a noble metal that is typically found in trace amounts (2.3 µg/L) in the human organism. Three possible ways of exposure to metallic silver are distinguished, e.g. absorption through the respiratory tract, skin contact, and ingestion (food, beverages, medicines) [23]. Due to its

strong antibacterial effect even at low concentrations, silver has a long-time history of successful utilization in antimicrobial therapy. Besides, the synthesis of silver nanoparticles became one of the fast-growing trends in nanotechnology because of their great range of biological activities, including, antibacterial, antiviral, antioxidant, immunomodulatory, and anticancer properties [24]. One of the leading trends is the production of nanoparticles and nanocomposites with the utilization of biological systems such as bacteria, plant extracts, proteins, etc. Previous studies have reported that silver ions are capable to interact with various proteins forming stable complexes. The extremely high binding affinity of silver ions is observed towards cysteine-rich proteins, such as metallothioneins. This class of proteins ensures the protection of human cells against high doses of absorbed metallic silver [25]. The trends of the last decades have been directed at searching for novel ingredients with diverse functionality based on natural compounds. Protein modification with small target molecules (ligands) provides changes in the structure and biological activity of the macromolecule. In the last few years, protein functionalization with metal ions is considered one of the modern approaches to modulate the primary properties of biomolecules [26,27]. It is worthy to mention, that deep characterization of silver-casein complexes is required to evaluate the potential application of novel complexes in different branches, particularly in biomedicine as an effective antiseptic agent.

The important step in the preparation of silver-modified casein protein is based on the selection of the appropriate form of silver (I) complex. Silver nitrate is the most commonly used reagent in nanocomposites synthesis. Our previous study on casein-silver interaction also was performed with the use of silver nitrate [27]. However, the aqua complex of silver nitrate exhibits a great tendency to photodegradation upon exposure to UV-VIS radiation [24]. Moreover, the complex is characterized by a pH-sensitivity and undergoes precipitation to silver (I) oxide Ag_2O at pH 7–8 [28]. Ammonia is known as a much stronger Lewis base than aqua molecules, thus ammonia water added in the excess to the aqua solution of silver nitrate promotes ligand substitution and formation of a more stable silver form $[\text{Ag}(\text{NH}_3)_2]^+$ (Tollens' reagent) [29]. Tollens reagent is rarely utilized for biomolecules modification with silver. In turn, it should ensure more effective sorption onto protein since such an approach could minimize the risk of metal secondary reduction [30].

The present work is focused on the studying of interactions between casein and silver ions. The casein modification was carried out by a novel method, i.e., at basic conditions (pH 8) using silver precursor in a form of ammonia complex $[\text{Ag}(\text{NH}_3)_2]^+$. The utilized conditions provided greater colloidal stability of protein dispersion and higher efficiency of the silver sorption process. It should be highlighted that the biological and physicochemical properties of the nanoparticles and nanocomposites are strictly dependent on the synthesis conditions. Thus, one of the aspects which were undertaken during the study was the investigation of the influence of different factors on the silver binding process, among others, silver ion concentration and protein purity. The respective factors usually are omitted by the researchers in their investigations. Utilization of batch sorption study, including Freundlich and Langmuir isotherm modeling provided comprehensive information about individual steps of silver sorption onto casein. The physicochemical characterization of casein-silver complexes was completed by using a series of spectrometric (ICP-MS), spectroscopic (DLS, ATR-FTIR, Spectrofluorimetry), electron microscopy (STEM, SEM-EDX) and separation (SDS-PAGE) techniques. Finally, the biological activity of complexes, e.g. antibacterial and cytotoxic properties, was studied.

2. Materials and methods

2.1. Materials

All chemicals used in this work were at the highest available purity.

Casein protein powder (99 % purity), silver nitrate, ammonia solution 25 % (w/w), nitric acid 65 % (w/w) were purchased from Sigma-Aldrich (Poland), Milli-Q water was prepared using a system from Millipore (Bedford, MA, USA).

2.2. Preparation of casein solution

Casein solution (pH 8) with a concentration of 10 mg/mL was prepared in a 50 mL Eppendorf tube. The appropriate amount of protein was suspended in 30 mL of deionized water. Casein suspension was dissolved by the gradual addition of 5 % ammonia solution. Then, the solution pH was dropped to 8 by acidification with 1–5 % nitric acid. Finally, casein solution was made up to 50 mL with water.

2.3. Casein desalination procedure

The initial desalination treatment allowed to evaluate the influence of salts on casein sorption capacity. Depletion of salts was performed by using Amicon® Ultra Centrifugal Filters cut-off 3 kDa. Firstly, casein dispersed in the water medium was centrifugated (8000 rpm; 15 min; 4 °C). Subsequently, the concentrated casein was washed two times with distilled water. The desalinated protein concentrate was transferred to Eppendorf tubes and freeze-dried for 24 h (Labconco FreeZone, Kansas City, MO, USA). The changes in salts concentration of casein, including sodium, calcium, and magnesium ions were determined in the first and in the last filtrate using Inductively Coupled Plasma-Mass Spectrometry ICP-MS (7900 ICP-MS, Agilent Technologies, Warsaw, Poland) analysis.

2.4. Preparation of silver solutions

The solution of silver ions (1200 mg/L) was prepared in a 50 mL flask at the basic conditions. For this purpose, the appropriate amount of silver nitrate salt was dissolved in 30 mL of deionized water. In the next step, the pH was lowered by adding 5 % ammonia solution till the complete dissolving of appeared black silver oxide Ag_2O . The obtained clear solution was adjusted to pH 8 by adding 1–5 % of nitric acid. The rest of the volume was filled up with water. Subsequent dilutions of silver solution were performed: 900, 600, 460 300, 120, 60, 45, 30, 15, and 6 mg/L. The accurate initial concentration of silver ions was determined by ICP-MS analysis.

2.5. Isotherm study of silver-casein interactions

Casein sorption capacity was examined by using silver solutions at particular metal ion concentrations. The reaction mixture was prepared in the following way: protein solution (0.25 mL) was mixed with silver ammonium solution (0.25 mL) in the black Eppendorf tube. Sample incubation (20 °C, 600 rpm) was carried out for 24 h on a thermomixer (Eppendorf Comfort, Hamburg, Germany). After the set time passed, analyzed solutions were transferred to Amicon® Ultra Centrifugal Filters cut-off 3 kDa and centrifugated (14,000 rpm; 10 min). Appropriate dilutions of supernatant were prepared in 1 % nitric acid. Silver concentration in the filtrate was determined by ICP-MS analysis. The same procedure was conducted for both, salted as well as desalted casein.

The sorption capacity of silver ions onto casein was calculated by the following equation:

$$q_e = \frac{(C_0 - C_e)V}{m} \quad (1)$$

where q_e – is the amount of bounded metal ions by protein (mg/g), C_0 – initial concentration of Ag^+ (mg/L), C_e – concentration of unbounded Ag^+ in the filtrate (mg/L), V – is the total volume of the protein-metal reaction mixture (L), m – the protein mass in the mixture (g).

Experimental data were fitted by using Freundlich (2) and Langmuir (3) adsorption models [27]:

$$q_e = K_F C_e^{1/n} \quad (2)$$

where K_F – Freundlich constant (L/mg), $1/n$ – adsorption intensity which implies the heterogeneity of binding sites on the protein surface.

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (3)$$

where K_L – Langmuir constant (L/mg), q_m – is the maximum amount of bounded silver ions as a monolayer (mg/g).

The efficiency of metal sorption (%E) onto casein is calculated:

$$\%E = \frac{(C_0 - C_e)}{C_0} * 100 \quad (4)$$

Thermodynamic characteristic of the silver-casein binding process will be expressed by calculation of the change of Gibbs free energy ΔG_{bind} , which allows us to determine the spontaneity of each step during the binding process [31]:

$$\Delta G_{\text{bind}} = -RT * \ln K_D \quad (5)$$

where ΔG_{bind} – Gibbs free energy of silver-casein binding (kJ/mol), R – gas constant, which is equal to 8.314 J/mol*K, T – the adsorption absolute temperature (294.15 K), K_D – distribution coefficient.

The distribution coefficient is an important parameter that informs about the metal binding affinity to protein:

$$KD = \frac{q_e}{C_e} \quad (6)$$

where q_e – is the amount of silver ions bounded by casein (mg/kg), C_e – the metal concentration in the remaining filtrate (mg/L).

2.6. Casein buffer capacity

Change in protein pH profile after the addition of silver ammonia complex was tested at the three different metal concentrations (6 mg/L, 120 mg/L, 600 mg/L). Casein (10 mg/mL) was mixed with a silver solution at the 1:1 (v/v) ratio. The pH of the prepared mixture was measured with a pH-meter (Mettler Toledo, Greifensee, Switzerland) at 20 °C, in the following time intervals: 1, 5, 10, 30, 60, 90, 120, 180, 240, 300, 360 min

2.7. Synthesis of casein-silver complexes

For the casein-silver complexes synthesis salinated CN was utilized. Synthesis was performed the same as in Section 2.5 with the utilization of initial silver solution concentrations of 6, 120, and 600 mg/L. After complexes separation from the reaction mixture with Amicon® Ultra Centrifugal Filters cut-off 3 kDa, they were washed twice with deionized water to remove excess silver ions. Finally, the casein-silver complexes concentrates were transferred to Eppendorf tubes and freeze-dried (Labconco FreeZone, Kansas City, MO, USA).

2.8. Determination of the casein micelles size by dynamic light scattering (DLS)

Particle size distribution of casein micelles was determined by DLS using a Zetasizer Nano-ZS (Malvern Instruments, UK). Firstly, freeze-dried powders of casein and 6Ag-CN, 120Ag-CN, and 600Ag-CN complexes were suspended in basic conditions (pH 8) and diluted to 0.5 mg/mL. Then, solutions were sonicated for 5 min and transferred to a sample cuvette for analysis. Casein micellar size was measured at a scattering angle of 90 °C and a wavelength of 633 nm.

2.9. Fluorescence spectroscopy

Changes in the molecular environment around protein fluorophores

were monitored by the application of fluorescence spectroscopy. Solutions of desalted casein and 600-AgCN complex at the concentration of 0.5 mg/mL (pH 8) were analyzed. Measurements were performed with FP-8300 Spectrofluorometer (Jasco, Pfungstadt, Germany) using a quartz cuvette with a 1-cm path length. Three-dimensional fluorescence spectra were recorded with 2 nm intervals at the following excitation and emission wavelength ranges: $\lambda_{\text{ex}} = 200\text{--}736$ nm and $\lambda_{\text{em}} = 210\text{--}750$ nm.

2.10. Attenuated total reflectance-fourier transform infrared spectroscopy

Utilization of Fourier Transform Infrared Spectroscopy (FTIR) enabled to distinguish functional groups which participate in metal complexation. Spectra of casein and Ag-CN complexes were collected in the MIR range (4000–900 cm^{-1}) by using an Alpha FTIR spectrometer (Bruker, Billerica, MA, USA) with diamond ATR (attenuated total reflection) crystal. Normalization and baseline correction of plotted spectra were performed by OriginPro 2016 software.

2.11. Electron microscopy analysis

Topology, surface morphology, and elemental distribution were analyzed in powdered samples of casein and silver-casein complex (600Ag-CN) by using of Scanning Electron Microscope (Hitachi S5500, Japan) combined with Energy Dispersive X-ray (EDX) detector. STEM image was made in bright-field (BF) mode on FEI Tecnai F20 X-Twin (Hillsboro, OR, USA). Firstly, casein and silver-casein powders were dispersed in distilled water and sonicated. Subsequently, solutions were dropped on the carbon-coated copper grid and dried until the solvent was removed.

2.12. SDS-PAGE analysis

SDS-PAGE electropherogram reflects the information about casein mass profiles under silver modification. Powders of CN and 600Ag-CN were dispersed in the distilled water in order to get the final protein concentration of 0.5 mg/mL. Sample separation was conducted in non-reduced and reduced modes by using 4–12 % Bis-Tris Plus polyacrylamide gel (Thermo Scientific, Waltham, MA, USA) and MES Running Buffer. Pattern visualization was carried out by gel staining in Coomassie Brilliant Blue (1 h) which was followed by destaining in deionized water (24 h).

2.13. Silver desorption study

The 600Ag-CN complex was subjected to the study. For the study HNO_3 solution pH 1.2, acetic buffer pH 4.5, and 0.1 M MOPS buffer pH 6.8 were utilized. The suspension of the 600Ag-CN complex with a concentration of 5 mg/mL was prepared in deionized water. 50 μL of as prepared suspension was added to 200 μL of buffers and transferred to Eppendorf tubes. The incubation was performed for 2 h at 37 °C with mixing at 300 rpm on a thermomixer (Eppendorf Comfort, Hamburg, Germany). Subsequently, the protein was separated with Amicon® Ultra Centrifugal Filters cut-off 3 kDa and the filtrate was subjected to silver quantification by ICP-MS. The dilutions for analysis were performed with 1 % HNO_3 solution.

The initial silver content in the complex was determined. For this purpose, 25 μL of 600Ag-CN suspension was mineralized with 100 μL of concentrated MS-Grade HNO_3 for 3 h at 80 °C. Before ICP-MS quantification first dilution was performed with deionized water and subsequent dilutions were made with 1 % HNO_3 solution.

2.14. Peptic digestion kinetics

The peptic digestion kinetics was performed according to the protocol described by O. Pryshchepa et al. with slight modifications [32].

Briefly, the digestion was performed in simulated gastric fluid free of pepsin. The 600Ag-CN and desalinated casein were subjected to the study. The pepsin and studied protein working solutions were 10 U/mL and 5 mg/mL respectively. The final reaction mixture comprised 1 mg/mL of protein and had the enzyme-to-substrate ratio of 0.2 U:100 µg. The digestion was performed at 37 °C for 5, 10, 20, and 30 min. The reaction was terminated by adding 35 % of the reaction volume of 0.7 M Na₂CO₃. The as-obtained samples were directly subjected to SDS-PAGE analysis in reduced mode. The shortened time (17 min) of electrophoretic separation was utilized in comparison to the standard procedure (22 min).

2.15. Study on antimicrobial properties

The antimicrobial activity was evaluated as minimum inhibitory concentration (MIC) against selected bacterial strains by broth micro-dilution method. The analysis was performed in 96-well plates and with the use of Mueller–Hinton broth (MHB) as a culture medium. The solutions of 600Ag-CN and CN with a concentration of ≈ 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, and 5.0 mg/mL were prepared in MHB. At the same time, the solutions with silver ions concentration of 1.88, 3.75, 7.5, 15.0, 30.0, 60.0, and 120 µg/mL were utilized. 24 h bacterial culture in MHB was diluted with the same culture media to the concentration of 0.5 McFarland and further diluted 100-fold to obtain working bacterial suspension. The working bacterial suspension was mixed with the solutions containing antibacterial agent at a ratio of 1:1. The incubation was performed at 37 °C for 18 h with mixing. The bacterial growth detection was evaluated based on the fluorescence measurement with the utilization of a microplate reader (Multiskan, ThermoFisher) and in vitro Toxicology Assay Kit, resazurin-based (Sigma-Aldrich, Poland) according to the protocol provided by the kit supplier. The study was performed on drug-sensitive *S. aureus* B38 strain from the deposit of Centre for Modern Interdisciplinary Technologies Nicolaus Copernicus University in Toruń isolated from a diabetic foot wound (permission of Ethic Committee of NCU in Toruń KB 68/20019). Moreover, it was tested the sensitivity to the as-synthesized complex of carbapenemase-sensitive *K. pneumoniae* strain KP66 and strains *K. pneumoniae* KP1 and KP2 which have NDM-1 coding genes. The *K. pneumoniae* strains were isolated from patients of the Dr. Władysław Biegański Regional Specialized Hospital in Grudziądz during the epidemiological survey. The presence of the resistance mechanisms was confirmed using PCR methods.

2.16. MTT cell viability test

For the MTT test cells were cultured on 96-well plates at 2×10^5 cells/mL and incubated under 5 % CO₂ at 37 °C for 24 h. When cells were adherent, Ag⁺, casein, and casein-Ag⁺ were added and incubated for another 24 h. The L929 cells without additional stressors were used as controls in all experiments. Then, 10 µL of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/mL in PBS) was added and incubated for 4 h at 37 °C. After incubation, the medium from the wells was discarded and the crystalline formazan was dissolved in DMSO. Absorbance was measured at $\lambda = 570$ nm and 650 nm using a Varioskan TM LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed as percentages relative to the negative control—cells in a standard medium without stressors that are considered 100 % viable. All experiments were performed in six independent replicates.

3. Results and discussion

3.1. Batch sorption study

A batch sorption study is an essential experimental tool widely applied to study sorption processes in water solutions. Our group is one

of the few that utilize the approach to study the interactions of metals with proteins. It is worth mentioning, that utilization of such an approach for lactoferrin showed the correlation between the shape of isotherm as well as derived sorption parameters with the properties of synthesized complexes [26,32,33]. The experimental data were fitted to popular and relatively simple Langmuir and Freundlich adsorption isotherm models which allowed us to describe processes that take place [34]. Langmuir model assumes the formation of an adsorbate monolayer, where all adsorbed particles interact with the same binding energies. In the Freundlich model, the adsorbent surface is characterized by heterogeneity of binding sites and the possibility of the creation of adsorbate multilayers [35]. The mechanism of casein-silver complex formation was investigated. Moreover, the influence of accompanying ions on the casein sorption capacity was evaluated. For this purpose, the preliminary purification of native casein was performed. To remove the excess micellar ions the ultrafiltration was utilized [36]. The concentration of the most abundant caseins cations, e.g., Ca²⁺, Mg²⁺, Na⁺ was determined by ICP-MS analysis in the first and last filtrate (Table 1).

Analyzed cations tend to interact with casein micelle. They predominantly bind to their phosphate and carboxylic groups at the following affinity order Mg²⁺ > Ca²⁺ > Na⁺ [37]. It is shown that after the second washing, cations are less willingly released from the micelle into the solution, which indicates that equilibrium concentration has been established.

It is worthwhile to mention, that selection of medium pH plays an important role in the protein's sorption properties, affecting their surface charge and affinity to the particular ligands. Zeta potential measurements over the pH range of 2–11 performed by A. Post et al. indicated a relatively high stability of casein micelle at pH > 7 ($\xi \sim -30$ mV) [38]. Highly negative casein surface at respective pH should facilitate the binding of silver ions to protein. The sorption properties of casein are poorly studied at alkaline conditions, thus pH 8 was chosen to conduct a batch sorption experiment of silver ions onto casein. However, the most popular silver precursor, i.e., silver nitrate undergoes hydrolysis in alkaline pH with subsequent formation of insoluble oxide. Thus, the novel method of casein modification with silver was evaluated which implies the utilization of [Ag(NH₃)₂]⁺. The respective sorption isotherms of silver onto salted casein and casein after desalting at pH 8 were compared in Fig. 1A and Fig. 1B respectively. In turn, Table 2 comprises the calculated sorption parameters.

Based on calculated values of determination coefficient R², experimental results of both analyzed caseins show a better fit to the Freundlich model which indicates the heterogeneous character of metal sorption and possible multi-layer formation [26]. The formation of multiple layers could be easily determined by a modified BET model in which equilibrium sorption q_e is expressed as a function of the C_e/C₀ ratio. Based on this model, we can distinguish three stages in the silver-casein sorption process (Fig. 1C and Fig. 1D). In the first step, the monolayer of silver ions is formed on the protein surface, which ends up at a silver initial concentration of 60 mg/L in the case of non-purified casein and at 120 mg/L for casein after purification. The second stage of metal adsorption begins with the application of silver with the initial concentration of 120 mg/L and 300 mg/L in the case of salted and desalted casein, respectively. Metal ions undergo adsorption to the previously created silver monolayer. The strength of protein-metal interactions decreases in second step [39]. Development of a third adsorption layer of silver ions on purified casein was favored at a metal

Table 1

Effect of casein preliminary purification on the cation concentrations in the remaining filtrate.

Ion concentration [µg/L]	First filtrate	Last filtrate
Ca ²⁺	58.4	22.3
Mg ²⁺	88.3	38.5
Na ⁺	696.0	428.0

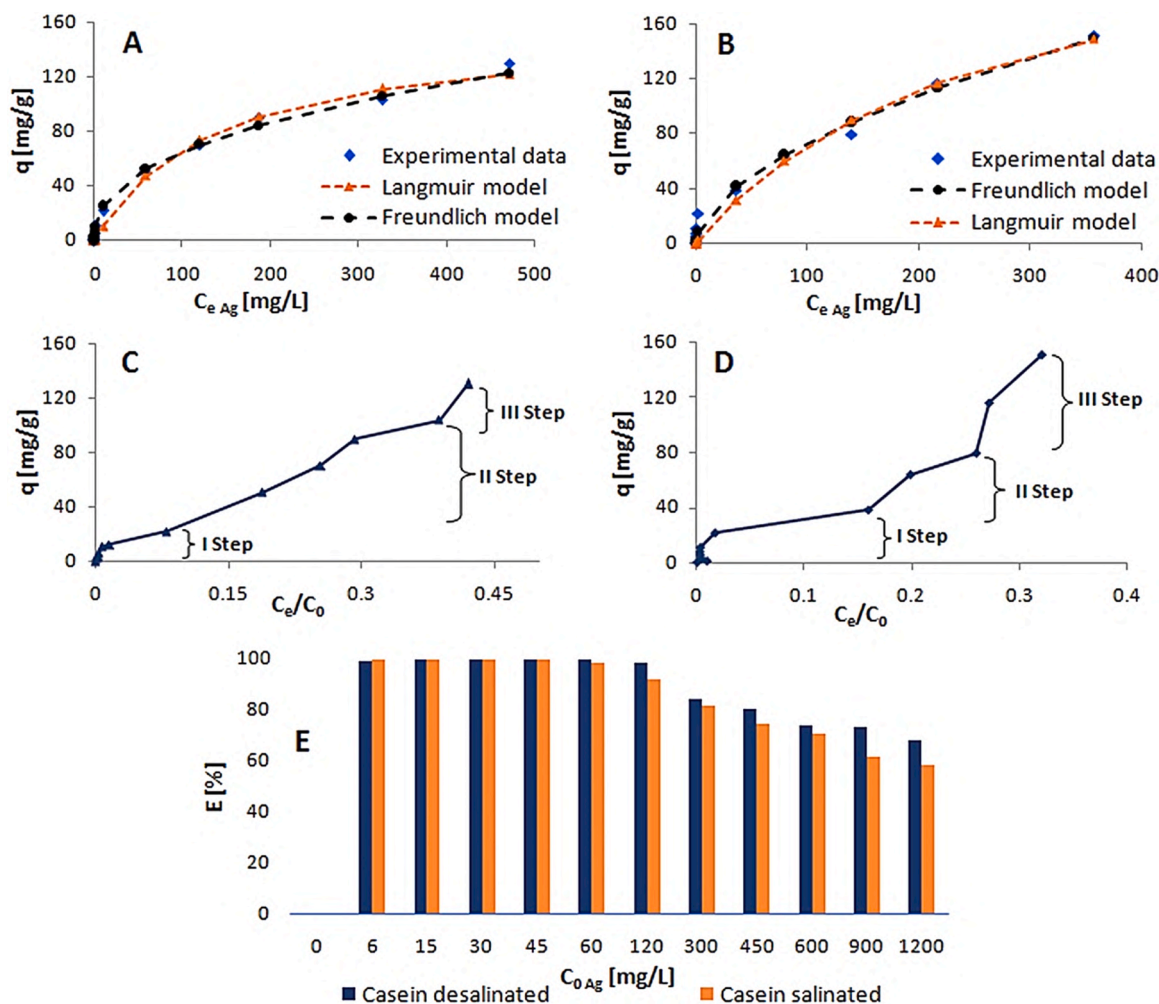


Fig. 1. Batch sorption approach on studying Ag-CN interactions: isotherm of silver ions sorption onto (A) salinated CN, and desalinated CN (B); modified BET isotherms reflect silver ions multilayer formation on the (C) salinated CN and (D) desalinated CN; the comparison of silver ions sorption efficiency on salinated and desalinated CN (E).

Table 2

Calculated parameters from Freundlich and Langmuir isotherm models.

Casein	Freundlich Isotherm			Langmuir Isotherm		
	n	K_F [mg/g]	R^2	q_m [mg/g]	K_L [L/mg]	R^2
Salted	2.47	10.18	0.997	157.1	0.0074	0.978
Desalted	1.79	5.57	0.985	258.2	0.0038	0.973

concentration of 600 mg/L while in the case of salinated protein it starts after utilization of silver initial concentration of 900 mg/L.

It was observed significant growth of metal sorption capacity after protein purification. The determined maximum sorption capacity (q_m) was 157.1 mg/g and 258.2 mg/g in the case of salted and desalted casein, respectively. The previous study on silver-casein interactions conducted in aqueous conditions by O. Pryshchepa et. al. showed that the calculated q_m value was 77.5 mg/g, indicating the significant role of metal precursor and reaction conditions on the sorption efficiency [27]. Still, the basic properties of utilized protein apparently have higher impact on the silver ion sorption capacity. The maximum sorption capacity of silver ions onto β -lactoglobulin in acidic conditions was 2.0- and 1.2-times higher compared to the sorption of salted and desalted casein in alkaline environment, respectively. The respective q_m for β -lactoglobulin was estimated as 317.26 mg/g [40]. In contrast, the q_m of bovine lactoferrin in acidic conditions was only 1.55 mg/g [32]. It

might be related to the difference in adsorbent surface nature and the presence of a higher number of potential binding sites. The sorption efficiency in dependence of silver initial concentration for both caseins is shown in the Fig. 1E. The chart reflects, that protein remained not fully saturated with metal ($E > 99\%$) until the addition of silver solutions at the concentration of 60 mg/L and 120 mg/L to salted and desalted casein, respectively. The following increase of initial concentrations accompanies by saturation of casein and reduction of sorption efficiency. The sorption efficiency of native casein was equal to 57.99 % at the highest silver initial concentration and it increased to 67.98 % after protein purification, indicating the relevant effect of the desalting procedure on the casein sorption capacity. The modification of salt content, probably, enabled the release of potential binding sites, thus, the higher amount of silver ions adsorbed by casein. Therefore, we can assume that an increase in casein sorption capacity is related to the ion-exchange mechanism in which Ca^{2+} , Mg^{2+} , and Na^+ ions were initially desorbed and replaced by silver ions [41]. It is worth noting, that association of these metals to casein micelle is mainly driven by phosphoserine residues, and the possible silver integration into protein internal structure also shouldn't be excluded [21]. In turn, the concept of Hard Soft Acid Base (HSAB) theory is helpful in the prediction of amino acids which will exhibit the strongest affinity to analyzed metal. According to the HSAB principle, silver ions belong to the group of soft acids while the coordinated ammonia ligand is known as a hard base. Thus, the formation of a more stable silver complex accompanies by an acid-base

exchange reaction and the creation of homogeneous pair of soft acid/soft base [42]. Based on this, the sorption of silver ions onto casein is mainly preferred by interactions with ligands containing S, N, and O atoms [43]. Silver ions reveal the highest binding affinity toward the deprotonated thiol group of cysteine or imidazole nitrogen of histidine at pH 8. In addition, a probability of complexation with the carboxylic group of glutamic and aspartic acid also exists [44]. Importantly, the binding-site geometry in silver complexes is highly influenced by the side-chain structure of chelating amino acids. The calculation studies performed by T. Shoeib et. al. indicated the predominance of silver bidentate complexes with aliphatic amino acids, while tri-coordinated complexes are abundant in the case of the rest amino acids [45].

Furthermore, the change of Gibbs free energy ΔG_{bind} of silver-casein binding was calculated for every sorption stage. The first step begins with ΔG_{bind} of -28.07 kJ/mol and -24.42 kJ/mol and ends up with ΔG_{bind} of -18.91 kJ/mol and -22.91 kJ/mol in the case of salted and desalted protein, respectively. Negative values of Gibbs free energy indicate that the sorption of silver ions onto casein occurs spontaneously [26]. The higher negativity of ΔG_{bind} informs us about stronger metal-protein interactions. Interestingly, the greatest metal binding affinity was noticed with the application of silver concentrations of 6 mg/L and 45 mg/L for salted and desalted protein, respectively. The difference might be caused by the potential barrier that silver ions should pass after micelle purification from salts [46]. In contrast, L. Canabady-Rochelle reported, that the calculated ΔG_{bind} of calcium binding to casein was equal to -17.32 kJ/mol, which in turn, is compatible with the suggested ion-exchange mechanism of silver sorption [47]. In the second step, ΔG_{bind} was ranging from -16.54 to -14.08 kJ/mol (casein salted) and -17.03 to -15.52 kJ/mol (casein desalted). The calculated values of ΔG_{bind} in the final step were equal to -13.75 kJ/mol in the case of salted casein, and -14.80 kJ/mol for desalted casein. The sharp decrease in silver binding affinity is directly associated with the multi-layer character of sorption and electrostatic repulsion between individual metal layers. Another possible reason for ΔG_{bind} rise might be the beginning of AgNP formation, which is considered a thermodynamically unfavored process [24].

3.2. Casein pH kinetics

Fig. 2 represents the pH kinetics of casein after the addition of the silver ammonia complex. It also demonstrated the protein buffer capacity upon the addition of a complexing agent at the different metal concentrations. The protein solution exhibited relative pH stability, varying from 7.7. to 8.3.

Gradual acidification was observed in the casein and casein mixture with 6 mg/L $[\text{Ag}(\text{NH}_3)_2]^+$ might be a result of carbon dioxide absorption. The pH of casein with 120 mg/L $[\text{Ag}(\text{NH}_3)_2]^+$ initially increased to

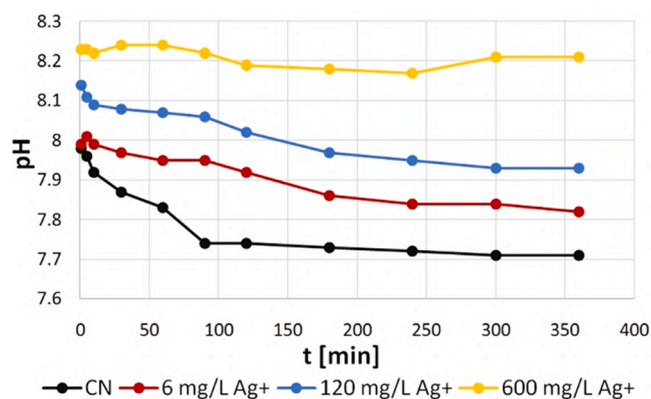


Fig. 2. pH kinetics of native CN (black), and mixtures of CN with silver ions (1:1 (v/v)) at the following metal concentrations: 6 mg/L (red), 120 mg/L (blue), 600 mg/L (yellow).

8.14, however, it started to reduce after 5 min of incubation. The pH profile of casein mixed with 600 mg/L $[\text{Ag}(\text{NH}_3)_2]^+$ was ranging between 8.17 and 8.24 during the whole time of incubation. Such pH rise might be explained by casein-silver interactions which led to the release of ammonia from the complex into solution as a proof of binding based on an ion-exchange mechanism.

3.3. Spectroscopic approaches

3.3.1. Dynamic light scattering analysis

Dynamic Light Scattering is an excellent technique that allows for rapid determination of the hydrodynamic diameter of the particles in dispersions. The modification of micelle size upon variable conditions, e. g. pH change, salt effect, heat treatment, and addition of other whey proteins, were comprehensively analyzed in the previous works [48–51]. Nevertheless, the study of the direct influence of silver on the casein micelle size hasn't been conducted yet. Instead, the metal ions has direct impact on the structure of casein micelles. Mean values of the hydrodynamic diameter obtained after triplicate measurements are filled in Table 3.

The obtained graph demonstrates a wide range of size distribution in the case of native casein as well as silver-modified micelles (Fig. 3). Each analyzed sample was characterized by polydispersity of casein micelles. The highest micellar size distribution was found at around 200–500 nm, which is in agreement with data from previous works [19]. Results indicated, that the lowest silver concentration does not cause significant changes in micelle size in this range. The gradual increase of hydrodynamic diameter begins with the application of silver concentration at 120 mg/L. Casein micelle growth observed in the 600Ag-CN complex might be a result of non-specific metal-protein interactions that caused some conformational changes, particularly in the protein's external structure. Such size modification could be associated with the multilayer metal sorption onto the protein surface [52]. Silver ions are capable to neutralize the protein surface charge, by interaction with negatively charged side-chains (Asp, Glu) of amino acids. In turn, such interactions provide to decrease in the electrostatic repulsions between micelles and increase their susceptibility to aggregate [53]. Apart from this, metal ions are capable to modify casein internal structure, in particular by interactions with hydrophobic regions of the micelle. Many studies mentioned, that divalent cations of calcium, magnesium, zinc, iron, and copper have a strong tendency to link with phosphoserine residues of casein at the basic pH [54,55]. Silver ions reveal a lower binding affinity toward phosphate groups in comparison to the above-mentioned metals since it is classified as soft acid according to HSAB principles (phosphate group PO_4^{3-} is known as hard base) [56]. Nevertheless, the formation of silver phosphate clusters AgP could lead to the disruption of protein structure and the following growth of micelle size. A similar effect was observed by Z. Zhao et. al. upon addition the high concentration of sodium chloride to milk. Authors concluded that the loosening of casein micelle structure was accompanied by replacement of calcium by sodium in nanoclusters [50]. In turn, micelles with lower size distribution were found at around 10–80 nm. These peaks could be assigned to smaller structural units (submicelles) that appeared as a result of partial casein micelle decomposition after salt removal [57].

Based on our results, a significant impact of silver concentration on the size of casein micelles was indicated. Application of higher metal concentrations (>120 mg/L) accompanied by a gradual increase of protein hydrodynamic diameter. The modification of casein size might

Table 3

Size distribution of native casein and silver-casein complexes.

Casein micelle size [nm]	Peak number	CN	6Ag- CN	120Ag- CN	600Ag- CN
	I	220	220	255	396
	II	24	21	24	79
	III	-	-	-	16

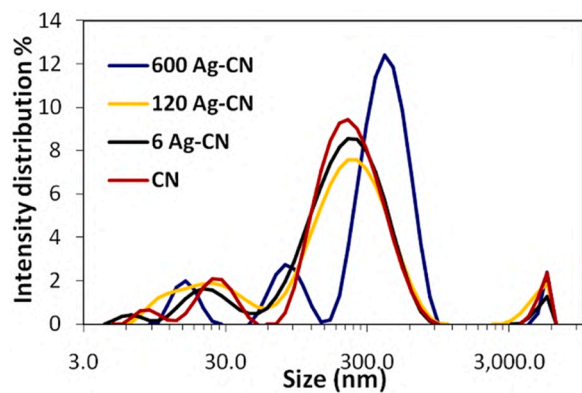


Fig. 3. Measured by DLS hydrodynamic radius distribution of native CN (red) and silver-casein complexes: 6Ag-CN (black), 120Ag-CN (yellow), 600Ag-CN (blue).

occur by metal-induced neutralization of protein surface charge as well as by silver integration into internal micelle structure.

3.3.2. Fluorescence spectroscopy

The conformational changes in casein structure upon binding with silver ions were monitored by using spectrofluorimetry. Fig. 4 shows three-dimensional spectra gained for CN and 600Ag-CN samples. Native casein exhibited a single emission peak at $\lambda_{ex}/\lambda_{em} = 278/342$ characteristic for aromatic amino acids, mainly, tryptophan and tyrosine residues [58]. After silver immobilization it was observed a blue shift of maximum fluorescence to 338 nm which implies that metal incorporation provides rearrangements of protein structure that ensure a less polar local microenvironment around protein fluorophores (in particular tryptophan) [59]. Remarkably, A. Sahu et. al. related a blue shift upon casein-curcumin formation to hydrophobic interactions between ligand and biomolecule [57]. The casein regions with the highest hydrophobicity are located in α_{S1} - and β -casein. The sequence of individual caseins includes two (Trp164, Trp199) and one tryptophan (Trp143), respectively, which potentially could mediate in interactions with silver ions [60]. The fluorescence intensity of casein was sensitive to the presence of silver ions and sharply decreased from 5219 a.u. to 4650 a.u. The reduction of fluorescence intensity with an increase of the metal/ligand concentration is widely named fluorescence quenching. The quenching is a complicated process that might be caused by different factors, e.g. energy transfer, collision, or formation of non-fluorescent ground-state complex [61]. In our case, the quenching phenomena was induced by interactions of silver ions with a more hydrophobic interior of casein micelle, probably by binding to

phosphoserine residues, which in turn, is in accordance in with our DLS results [62]. Therefore, we could assume, that silver ions are capable to penetrate to casein core, where they are complexed in the close accessibility to buried tryptophan residues.

3.3.3. ATR-FTIR spectral analysis

ATR-FTIR analysis was used for the identification of caseins' functional groups involved in interactions with metal ions. Derived spectra of native casein before and after desalting as well as silver-casein complexes at different metal concentrations are shown in Fig. 5. Casein modification with silver caused strong changes in the regions of amide bands which are highly sensitive to changes in protein secondary structure [63].

Both regions of the amide A band ($3300\text{--}3250\text{ cm}^{-1}$) and amide B ($3070\text{--}3040\text{ cm}^{-1}$) are assigned to N-H stretching vibrations in the polypeptide backbone [64]. Downshift of amide B band for AgCN complexes towards lower frequencies: 3075 cm^{-1} (non-purified CN), 3075 cm^{-1} (purified CN), 3057 cm^{-1} (6Ag-CN), 3049 cm^{-1} (120Ag-CN), 3067 cm^{-1} (600Ag-CN) indicate the strengthening of -NH intramolecular hydrogen bonding [64]. Peaks in the range $2965\text{--}2955\text{ cm}^{-1}$ and $2935\text{--}2920\text{ cm}^{-1}$ are related to $-\text{CH}_3$ and $-\text{CH}_2$ asymmetric stretching vibrations, respectively [65]. Amide I band ($1700\text{--}1600\text{ cm}^{-1}$) arises mostly from stretching vibrations of carbonyl

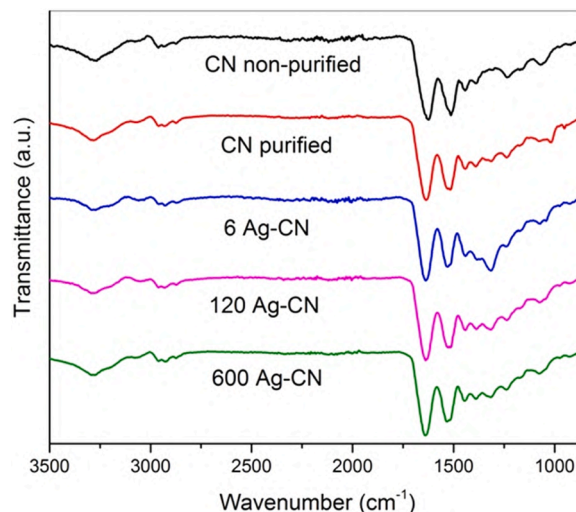


Fig. 5. ATR-FTIR spectra of native casein: salted CN (black), desalted CN (red), and silver-casein complexes: 6Ag-CN (blue), 120Ag-CN (pink), 600Ag-CN (green).

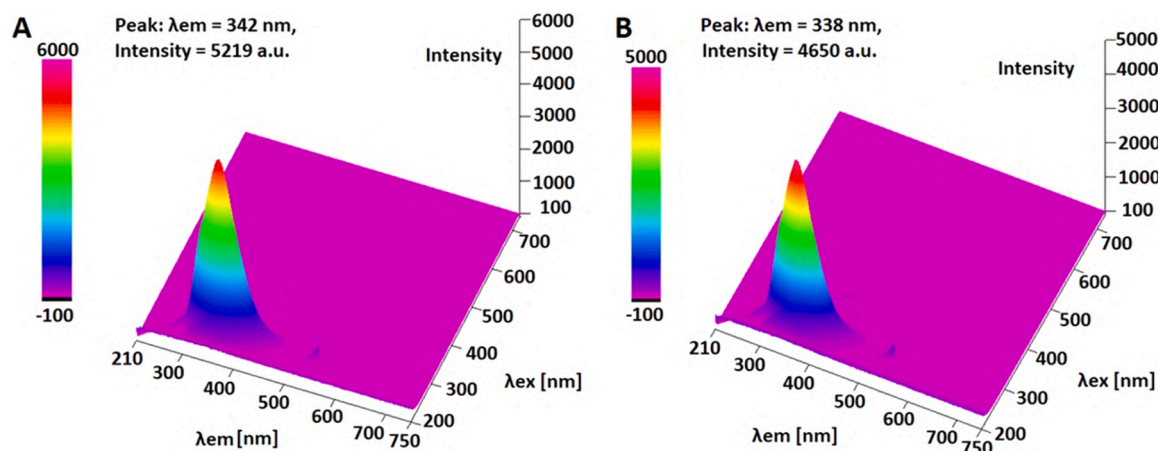


Fig. 4. Three-dimensional fluorescence spectra of: (A) native CN, (B) 600Ag-CN complex.

group C=O weakly coupled with N-H bending and C-N stretching vibrations [66]. Band assignment in this region allowed for the identification of the type of secondary structure present in the protein. Characteristic peaks at 1636 cm^{-1} (purified CN), 1638 cm^{-1} (6Ag-CN), 1638 cm^{-1} (120Ag-CN), and 1641 cm^{-1} (600Ag-CN) indicate the presence of parallel β -sheets in the secondary structure [64]. Interestingly, in the case of non-purified casein, the band corresponding to 1623 cm^{-1} may be attributed to cross- β -sheet which are typically found in amyloid fibers [67,68]. D. Thorn et. al. reported that α_{s2} - and κ -casein were highly susceptible to fibril formation under physiological conditions [69]. Nevertheless, our studies showed that protein desalting prevents this unfavorable process. In addition, S. Wu et. al. related a red shift of the Amide I band to possible interactions between the protein polypeptide chain and metal ions [70]. The Amide II band ($1600\text{--}1500\text{ cm}^{-1}$) originates from N-H bending and C-N stretching vibrations [71]. The region of the amide II band includes signals at 1513 cm^{-1} (non-purified CN), 1515 cm^{-1} (purified CN), 1530 cm^{-1} (6Ag-CN), 1528 cm^{-1} (120Ag-CN) which are characteristic of antiparallel β -sheet structure, while the peak at 1532 cm^{-1} (600Ag-CN) belongs to parallel β -sheet structure [72]. Shifting of the Amide II band towards higher frequencies is corresponding to the strengthening of hydrogen bonding within protein structure [73]. Signals around $1450\text{--}1440\text{ cm}^{-1}$ related to $-\text{CH}_2$ deformation vibrations. While, the range of $1400\text{--}1380\text{ cm}^{-1}$ could be assigned to COO^- symmetric stretching vibrations of aspartic and glutamic acids side chains [64]. Upon metal addition, the band shifting was noticed: 1387 cm^{-1} (non-purified CN), 1389 cm^{-1} (purified CN), 1383 cm^{-1} (6Ag-CN), 1387 cm^{-1} (120Ag-CN), 1391 cm^{-1} (600Ag-CN) which proves silver coordination by Asp and Glu residues [74,75]. The peak shifting towards greater frequencies observed in the complex with the highest metal concentration could be a result of silver nanoparticle formation. J. Li et. al. have noticed the a similar effect during the synthesis of golden nanoparticles by protein extracts where carboxylic groups were involved in metal bioreduction [76]. The Amide III band ($1350\text{--}1200\text{ cm}^{-1}$) is characterized by N-H in-plane bending coupled with C-N stretching vibrations [77]. Two peaks were noticed for purified casein and casein complexes in the Amide III band region which indicate the presence of two individual secondary structures of α -helix at 1313 cm^{-1} (purified CN), 1313 cm^{-1} (6Ag-CN), 1318 cm^{-1} (120Ag-CN), 1315 cm^{-1} (600Ag-CN) and β -sheet at 1238 cm^{-1} (purified CN), 1240 cm^{-1} (6Ag-CN), 1236 cm^{-1} (120Ag-CN), 1238 cm^{-1} (600Ag-CN) [78]. Nevertheless, the spectra of non-purified casein showed only one band in this region at 1238 cm^{-1} which originates from the β -sheet structure. Weak signals around $1180\text{--}1170\text{ cm}^{-1}$ arise from C-OH in-plane bending vibrations of the serine side chain [64]. Characteristic bands at 1113 cm^{-1} (non-purified CN), 1126 cm^{-1} (purified CN), 1132 cm^{-1} (6Ag-CN), 1126 cm^{-1} (120Ag-CN), 1126 cm^{-1} (600Ag-CN) arise from $-\text{CNH}_3^+$ rocking and $-\text{CH}_2$ wagging vibrations in protein polypeptide chain [79]. Changes in the peak position are probably induced by conformation changes in the casein polypeptide backbone structure. Signals at 1071 cm^{-1} (non-purified CN), 1073 cm^{-1} (purified CN), 1071 cm^{-1} (6Ag-CN), 1071 cm^{-1} (120Ag-CN), 1075 cm^{-1} (600Ag-CN) are corresponding to phosphate group O=P-O- stretching vibrations which traditionally occur in phosphorylated serine residues [80]. In turn, peak shifting in this region proves the interactions between silver ions and phosphoserine residues. Additional bands appeared in the range of $1040\text{--}1010\text{ cm}^{-1}$ at the spectra of purified CN and 6-AgCN complex. Peaks in this region arise from C-O stretching vibrations of serine and C-C stretching vibrations coupled with C-H in-plane bending vibrations of tryptophan [64]. Weak bands around $980\text{--}950\text{ cm}^{-1}$ characteristic for PO_3^{2-} stretching vibration allow to confirm casein phosphorylation [81].

FTIR spectroscopy has been extensively used for characterization of structural transformations in biomolecules. In our case, ATR-FTIR was an essential instrumental tool, which allowed to evaluate the importance of the casein desalting step on the protein stability. Thus, based on

the FTIR spectra, we can suppose that the preliminary procedure of casein desalting led to relevant conformational changes in the casein structure. The obvious shift of the Amide I band after the removal of micellar salts indicates the protein stabilization and conformational transition in secondary structure from less structural order of cross- β -sheet (fibril aggregates) to more organized β -sheet. Besides, it was observed the appearance of an additional peak attributed to the α -helix structure which implies the rearrangement in the secondary structure and strengthening of intramolecular hydrogen bonding. The position of amide bands has changed upon casein modification with silver. The greater peak shifting might be associated with pH changes which tend to increase at higher metal concentrations. Remarkably, H. Salaheldin et. al. have reported that the shifting of Amide bands towards higher frequencies was directly related to the formation of silver nanoparticles [82]. In addition, the peak assigned to Asp and Glu side chains was shifted after the metal binding. Based on this, we can assume, that these residues play a significant role in silver coordination and, probably, participate in the bioreduction and growth of AgNPs. In many previous works, glutamic and aspartic acids also have been mentioned as one of the most abundant mediators in the protein-induced synthesis of metal nanoparticles [83,84]. The characteristic peak shift in the range of $1080\text{--}1070\text{ cm}^{-1}$ also confirmed our suggestion on the participation of phosphoserine residues in the silver-casein interactions.

3.4. Electron microscopy imaging

Electron microscopy imaging is an important imaging tool used in our study for monitoring structural modification in casein micelles upon the binding with silver ions. STEM images of CN and 600Ag-CN complex are demonstrated in Fig. 6A, and B, respectively. The presence of dark regions after silver addition which indicates the formation of AgNPs. The size distribution of nanoparticles was found in the range of $6\text{--}16\text{ nm}$. As shown, AgNPs undergo the agglomeration process creating dendritic nanostructures (Fig. 6B) [85]. Silver ammonia complex is known as a silver precursor used in the one-step synthesis of high-quality AgNPs [30,86]. The selection of proper pH is important criterium that ensures the long-time stability of nanoparticles. Previous works reported a high susceptibility of AgNPs to agglomerate at the pH range of $3\text{--}7$ [87]. S. Ashraf et. al. has noticed, that AgNPs coated onto casein protein undergo a reversible aggregation at acidic conditions, starting from the pH of $7\text{--}8$ [88]. In addition, N. Wangoo underlined that protein concentration has a great impact on the monodispersity of AgNPs [89]. According to FTIR results, we can assume, that carboxylic groups of Asp and Glu mediate the bioreduction of silver ions. Casein is characterized by a heterogenic distribution of carboxylic groups. The majority of them are located in the internal micelle structure ($\alpha_{s2}\text{-CN} > \alpha_{s1}\text{-CN} > \beta\text{-CN} > \kappa\text{-CN}$), nevertheless, some of Glu and Asp residues are also present in the C-terminal part of κ -casein [20,90]. Carboxylic groups found on the protein surface reveal a higher accessibility to metal ions compared to those buried inside amino acids. However, such type of interactions will induce destabilization of casein structure by neutralization of negative surface charge and will increase susceptibility to aggregation [91]. I. Dickson et. al. have studied interactions of alkaline-earth metals with bovine caseins [92]. It was demonstrated that carboxyl and phosphoserine groups represent the potential metal binding sites. Thus, caseins with the highest number of Asp, Glu, and SerP moieties will be characterized with the greatest metal binding affinity. In our case immobilized silver ions might be distributed between the protein outer layer and internal structure. This mechanism could be described by Weber-Morris model which includes two steps: 1) initial sorption of metal ions to the protein external surface, 2) intra-particle diffusion of silver to the micellar interior binding sites [93].

SEM imaging enables monitoring the changes in the surface morphology of protein structure. It was reported a crystalline structure of casein before complexation with silver ions (Fig. 6C). Instead, silver treatment induced significant modifications in the caseins structure. It

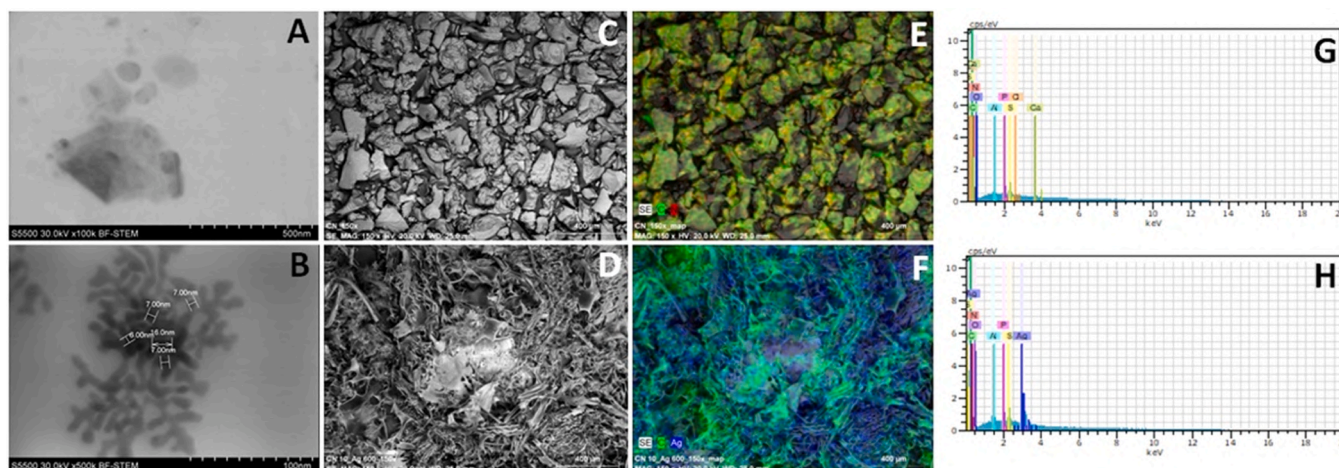


Fig. 6. Microscopy analysis: STEM images of (A) native CN, (B) 600Ag-CN complex; SEM micrographs of (C) native CN, (D) 600Ag-CN complex; SEM mapping of elemental distribution (E) native CN, (F) 600Ag-CN complex; EDX spectra of (G) native CN, (H) 600Ag-CN complex.

became fibrous and smoother, which also might be an effect of ultra-filtration (Fig. 6D). Results of SEM mapping analysis informed about silver integration into protein micelle and heterogeneous distribution of metal on the casein surface (Fig. 6E, F). EDX spectra reflected the chemical composition of pure casein and silver-casein complex (Fig. 5G, H). Obtained results were transferred into Table 4. Carbon, oxygen, nitrogen, and sulfur are known as key elements of each protein-based sample. The presence of phosphorus is attributed to casein post-translation modifications and derives from phosphate groups attached to serine residues [94]. Trace amounts of calcium and chloride found on the CN surface were removed upon protein modification. Chlorides might appear resulting in casein precipitation from milk by treatment with hydrochloric acid [95]. Slight impurities with aluminum probably derive from a spatula or aluminum foil which were used for sample preparation before microscopic analysis. According to the results, we can suppose that the lack of identified calcium in the formed Ag-CN complex might indicate its possible exchange by silver. The fact can be evidence for the previously predicted ion-exchange mechanism of complex formation.

3.5. SDS-PAGE study

Detection of casein fractions and their molecular mass distribution after protein modification by silver ions was investigated by SDS-PAGE analysis in both, reduced and non-reduced modes. Hence, in this study electrophoretic patterns of native casein and 600Ag-CN complex were examined, since we expect to observe the drastic conformational changes at the highest metal concentration (Fig. 7).

Sharp single bands between 25 and 35 kDa are related to α_s - and β -casein monomers, which constitute the major fractions of casein. Meanwhile, the characteristic pattern assigned to the κ -casein fraction was poorly visible on the electropherogram, except for native casein upon non-reducing conditions. The rare occurrence of monomeric κ -casein also has been reported by L. Rasmussen et. al. [96]. Electrophoretic patterns of casein oligomers were observed in the range of 50–140 kDa. Among all caseins, disulfide bridges are present exclusively in α_s - and κ -casein [97]. This feature indicates their ability to inter- and intramolecular interactions which are followed by aggregation processes. Patterns

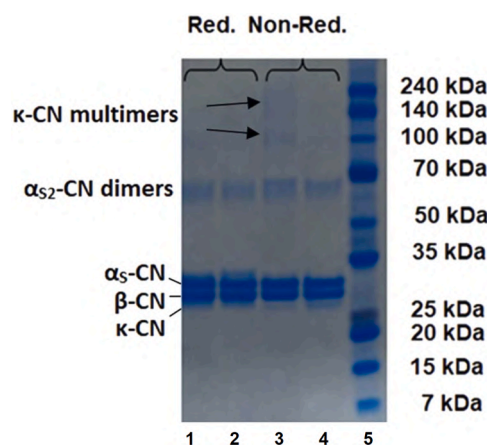


Fig. 7. SDS-PAGE profile of Ag-CN interactions: (1) native CN, (2) Ag-CN (reduced mode), (3) native CN, (4) Ag-CN (non-reduced mode), (5) protein marker.

observed in the range of 50–70 kDa for casein and silver-casein complex in both modes might be attributed to the intermolecularly linked dimer of α_s -casein which in turn, is in good agreement with results demonstrated by D. Thorn et. al. [98]. High-molecular weight complexes assigned to native casein appeared at non-reduced mode at 70–100 kDa and 100–140 kDa resulting in cross-linking of κ -casein and multimer formation [99]. The κ -casein exhibits the highest susceptibility to aggregate among the rest casein fractions. Multimerization of κ -casein is regulated by thiol groups of cysteine residues that might be bounded in three different combinations as Cys11-Cys11, Cys-11-Cys88, and Cys88-Cys88 [96]. Notably, J. Carver et. al. also mentioned the particular significance of proline and glycine residues in such type of casein-casein interaction [100]. Moreover, it was reported, that the presence of casein oligomers is highly affected by the applied method of protein isolation [101]. It was observed, that the addition of silver ions induced the cleavage of disulfide covalent bridges, thus high molecular patterns disappeared [75]. The κ -casein constitutes the outer side of the

Table 4

Elemental composition of native CN and 600Ag-CN complex according to EDX spectra.

m%	C	N	O	P	S	Al	Ca	Cl	Ag
CN	37.93	20.32	39.42	0.84	0.76	0.52	0.11	0.11	-
Ag-CN	33.89	22.29	36.85	0.76	0.75	1.28	-	-	4.18

casein micelle which prevents protein agglomeration through electrostatic repulsion. The functional role of κ -casein oligomers remains unknown. Some authors pointed out that the formation of disulfide-linked multimers contributes to the stabilization of casein micelle [99]. According to results, the high sensitivity of disulfide-linked multimers of κ -casein was observed in the presence of the reducing agent as well as silver ions, thus they dissociated into smaller monomers.

3.6. Silver ions desorption

Desorption study was performed to determine the strength of protein-metal interactions in the Ag-CN complex under different pH conditions. ICP-MS quantification revealed that the synthesized complex has 24.1 mg/g of silver. The q_m calculated from the Langmuir model was 6.5 times higher and accounts 157.1 mg/g. However, the complex preparation procedure includes the purification steps comprised of two ultrafiltration steps with deionized water. The respective treatment should be a reason for the elimination of less strong bonded silver ions from casein micelles. The fact also confirms the heterogeneous character of silver adsorption onto CN described with isotherm study. However, in our previous studies, the amount of remaining silver and iron in the complexes with bovine lactoferrin were much closer to the determined from Langmuir model q_m [26,32]. Such differences may be connected with the much more complicated structure of the CN which has a micellar structure.

It is well known that silver ions exhibit antibacterial properties. Plenty of studies point out that the ability of AgNPs to release silver ions is one of the most probable mechanisms of their antibacterial action which get the name “Trojan Horse” mechanism [24]. Thus, the knowledge about complex stability in different conditions is important for the prediction of its action in wound-healing preparations. Moreover, the issue is also important for the assessment of the complex safety in case of an undesirable entrance to the intestinal tract. The silver secondary oxidation from AgNPs with subsequent silver ions release to the solution is the subject of numerous research. It is shown to be dependent on different factors, i.e., the presence of oxygen and other oxidants as well as chelating agents in the reaction mixture, light irradiation, temperature, and pH. In the present study, three different buffers were utilized which imitate pH in different parts of the intestinal tract. Buffer's selection was performed considering the “Dissolution test for solid dosage form” of European Pharmacopoeia [102]. Moreover, the particular buffers were utilized taking into account that silver ions form insoluble salts with chlorides and phosphates. The ICP-MS quantification indicates that after 2 h of the desorption process the amount of released silver ions accounts 21.5 %, 49.3 %, and 68.7 % in pH 6.8, 4.5, and 1.2 respectively. The obtained results are constituent with previous works, where decreased pH has been shown to promote silver dissolution from AgNPs and their nanocomposites [32,103]. The respective concentrations of silver in solutions were 16.5, 11.8, and 5.2 ppm which indicates that utilization of the synthesized complexes should be strictly controlled as it was shown previously that silver even in the concentration of 0.5 ppm induces negative toxic effects in human cells [104].

3.7. Peptic digestion kinetics

Peptic digestion kinetics was conducted to evaluate the metal effect on the protein susceptibility to enzymatic hydrolysis. It was reported previously that protein digestion susceptibility is dependent on the structure of the protein, i.e., on the acceptability of the cleavage sites for the enzyme [105]. Moreover, the technique enables tracking changes in the protein structure upon metal binding [32,106]. Fig. 8 shows the resulting SDS-PAGE of casein peptic digest depending on the time of the reaction.

It can be seen slightly more intense bands of peptides at the time 5 and 10 min and the slightly less intense band of intact protein at the time of 20 min in digests of CN in comparison to digests of 600Ag-CN. The

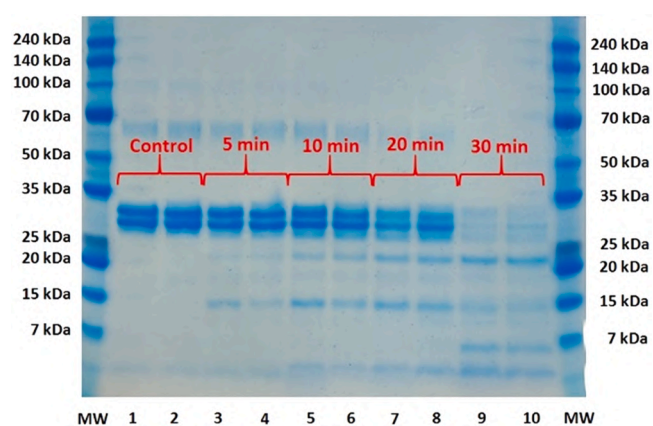


Fig. 8. SDS-PAGE of peptic digests of CN (1,3,5,7,9) and 600Ag-CN (2,4,6,8,10).

results indicate that CN digestion appears faster in comparison to the 600Ag-CN. In turn, in the case of 30 min of the reaction, it can be seen faster lysis of peptides of 600Ag-CN. The same pattern was observed for the complex of silver with bovine lactoferrin [32,106]. It was suggested that silver ions could influence the activity of the pepsin which leads to a decrease in the reaction rate. Moreover, the AgNPs' presence in the protein structure could impede access of pepsin to possible cleavage sites. Instead, the incorporation of silver in protein with subsequent changes in structure should be a reason for the faster digestion of smaller peptides. It is noteworthy to mention that in general CN has lower susceptibility to peptic digestion in comparison to bovine lactoferrin. Twice a higher amount of enzyme and a longer time was required to notice the formation of peptides in the solution [32,106] which should be connected to the more complex structure of the caseins [20]. It can be concluded that the produced complexes will have a longer shelf-life. Additionally, it could induce lower negative effects as smaller peptides can enter cells easier and thus promote the absorption of toxic silver.

3.8. Antimicrobial properties of synthesized complex

Determination of MIC values is a necessary step in the indication of the effectivity of the synthesized Ag-CN complex as a potential antimicrobial agent. In the study members of both Gram-positive and Gram-negative bacteria were examined, namely *S. aureus* and *K. pneumoniae*. Among all bacteria, *S. aureus* belongs to one of the most common causes of nosocomial bacteremia with a mortality rate reaching 20–30 % [107]. Infections caused by *K. pneumoniae* also have also been shown to be of high mortality rate even up to 48 %. The reason is connected with the development of specific enzymes – carbapenemases which enable penicillin's hydrolysis, all cephalosporins, monobactams, carbapenems, and even β -lactamase inhibitors [108]. Thus, the synthesis of new substances capable to inhibit such kinds of bacteria is of high interest among researchers. In the study drug-sensitive *S. aureus* from the depository of NCU, isolated from a diabetic foot wound was studied. Moreover, to evaluate the susceptibility of *K. pneumoniae* both drug-resistant and not to the preparations with silver three different strains were examined (KP1, KP2, and KP66). The *K. pneumoniae* strains KP1 and KP2 are characterized by the presence of NDM-1 carbapenemase coding genes while strain KP66 is sensitive to carbapenems.

The results indicate that in the case of silver ions for all three strains of *K. pneumoniae* MIC was established as 7.5 μ g/mL and for *S. aureus* 15 μ g/mL. The higher resistance of *S. aureus* against silver should be connected with the presence of a cell wall that prevents the silver entrance to the bacterial cell. In the case of the 600Ag-CN determined MIC for *S. aureus* was 0.63 mg/mL and for all three *K. pneumoniae* strains was 0.32 mg/mL which corresponds to 15 μ g/mL and 7.5 μ g/mL of silver respectively. Previous studies revealed that silver nanocomposite

based on bovine lactoferrin had much higher antibacterial properties in comparison to the corresponding amount of silver [32]. Several factors could be a reason for a such difference. Firstly, it was reported that only small nanoparticles had higher toxicity than silver ions [24]. In the present study, the nanoparticles in the complex formed dendrites, i.e., relatively big crystals comprised of multiple smaller nanoparticles. Secondly, the casein micelle's structure is much harder to disrupt which may impair the availability of silver ions for bacterial cells. Finally, lactoferrin is the protein that is reported to have antibacterial properties, and in combination with silver, the synergistic effect can be achieved leading to higher inhibitory activity [32]. However, it is noteworthy to mention that in the case of *K. pneumoniae* strain PK2 it was noticed that 600Ag-CN in the concentration of 0.16 mg/mL did not cause bacterial death but decreased their growth (see. Fig. S1). Thus, it indicates that 600Ag-CN complex has different action mechanism in comparison to free silver ions. Generally, results indicate that the synthesized complex has a high potential as an active substance of wound healing preparations.

3.9. MTT cell viability test

To examine the cytotoxicity effect of casein and silver-conjugated casein (600Ag-CN) in vitro, cell line—murine fibroblast L929 was chosen. The application of mouse fibroblast L929 cells is most frequently undertaken to evaluate cytotoxicity and may represent a sufficient in vitro screening model for skin formulations. Furthermore, the cytotoxicity assay with mouse fibroblasts L929 is in compliance with ISO 10993-5 standards and is often used for comparative studies of different types of nanoparticles. The antiproliferative effect was determined using colorimetric assay—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT test is based on the ability of the mitochondrial dehydrogenase enzyme to convert yellow tetrazolium dye into formazan crystal. The rate of formazan crystal formation is directly proportional to cell viability; the untreated (positive) control is set to 100 % viability.

In our experiment, both AgCN and Ag⁺ were used for comparison. The concentration of 600Ag-CN was in the range from 0.02 mg and up to 0.32 mg/mL which corresponds to Ag⁺ concentration from ~0.5–7.5 µg/mL respectively. As expected, silver ions at a concentration of about 1 µg/mL caused a significant decrease in cell viability to about 10 % (Fig. 9). However, the 600Ag-CN complex that corresponds to toxic levels of silver ions (~1–4 µg/mL) did not contribute to a decrease in cell viability. It can be suggested that silver ions are strongly associated with protein. This prevents the release of ions and their toxic effects on cells. Only at the highest complex concentration (0.32 mg/mL corresponding to 7.5 µg/mL of silver) decrease in the cell viability up to 60 % was observed. In turn, at the same concentration, complete suppression of

K. pneumoniae growth was inhibited while for *S. aureus* complete growth inhibition was observed at the complex concentration equal to 0.63 mg/mL (15 µg/mL of silver). The results indicate that the complex has comparable toxicity against both procaryotic and eucaryotic cells. Even though, for palliative wound healing any strategy can be utilized if it can decrease patients' suffering [108]. Still, more comprehensive studies should be performed before the introduction of the synthesized complex into wound-healing preparations.

4. Conclusions

This work aimed to describe the mechanism of casein-silver interactions. Silver ammonia complex [Ag(NH₃)₂]⁺ was selected as a metal precursor in complex synthesis. Application of this compound could be an interesting approach since it allows protein modification in alkaline conditions. The selected conditions should provide relatively high colloidal stability of the protein micelles. The study was performed on the mixture of different casein isoforms. Moreover, to examine the effect of micellar salts on silver-binding properties a desalting procedure was performed which, among others, ensured a more accurate determination of the silver-casein sorption mechanism. Obtained results indicated that both for salted and desalted casein, sorption isotherms showed more accurate fitting to the Freundlich isotherm model which is related to the heterogeneous character of silver binding to protein. Besides, it was reported, that casein desalting caused an increase in the protein sorption capacity from 157.1 to 258.2 mg/g. This effect might be associated with the ion-exchange mechanism of silver binding which is accompanied by a release of potential binding sites after salt removal. The notable increase of hydrodynamic diameter in silver-modified micelles might be described by two effects: 1) metal sorption to the external surface leading to neutralization of the protein surface charge and 2) the changes in the micelle structure (loosening) induced by silver interaction with phosphoserine nanoclusters. The peak blue shift as well as the quenching effect observed on fluorescence spectra confirmed the silver integration into the micelle core. Based on ATR-FTIR spectra, demonstrated, that casein desalting promoted conformational changes, such as cross-β-sheet → β-sheet transition and the appearance of an additional peak attributed to the α-helix structure. Besides, it was identified the importance of acidic amino acids (Asp, Glu) in metal chelation. Electrophoretic analysis showed the decrease in κ-casein oligomers content which should be connected with the cleavage of disulfide bonds by silver. Results of electron microscopy confirmed that the synthesis of the silver-casein complex under given conditions was accompanied by metal bioreduction and formation of silver nanostructures. Peptic digestion studies showed a relevant influence of silver incorporation on the structural stability of caseins. Finally, it was shown that synthesized 600Ag-CN complex inhibits the growth of *S. aureus* in the concentration of 0.63 mg/mL and *K. pneumoniae* in the concentration of 0.32 mg/mL. It also was reported that the synthesized complex was less toxic to mouse fibroblast cells in comparison to the corresponding amount of silver ions. Thus, it indicates the high potential of the complex as an antibacterial substance in wound-healing preparations.

CRediT authorship contribution statement

Tetiana Dyrda-Terniuk: methodology, investigation, data curation, formal analysis, validation, visualization, writing—original draft preparation, writing—review and editing; **Oleksandra Pryshchepa:** conceptualization, methodology, investigation, data curation, formal analysis, validation, visualization, writing—original draft preparation, writing—review and editing; **Katarzyna Rafińska:** methodology, investigation, data curation, formal analysis, validation, visualization, writing—original draft preparation; **Mateusz Kolankowski:** investigation, data curation, formal analysis, validation; **Adrian Gołębowski:** methodology, investigation, data curation, validation; **Michał Gloc:**

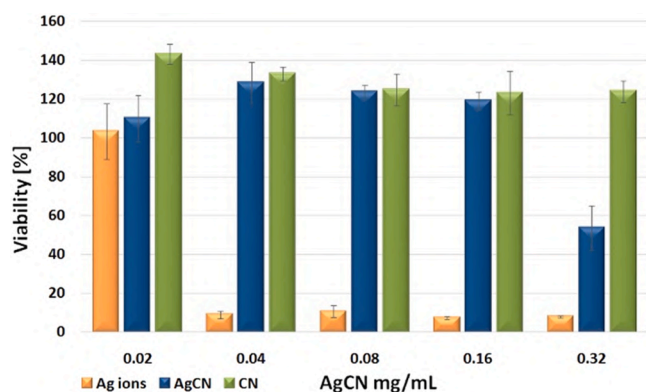


Fig. 9. Cell viability measured by MTT assay. Histograms shows the percentage, with respect to control cells (100 %), of viable cells after the exposure to AgCN, Ag⁺ and CN. Data shows the mean ± SE (n = 6).

methodology, investigation, data curation, validation; **Renata Dobrucka**: resources; **Krzysztof Kurzydłowski**: resources; **Paweł Pomastowski**: conceptualization, project administration, supervision, funding acquisition, writing—review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.colsurfa.2023.131390](https://doi.org/10.1016/j.colsurfa.2023.131390).

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Publikacja [P1]
Supplementary material

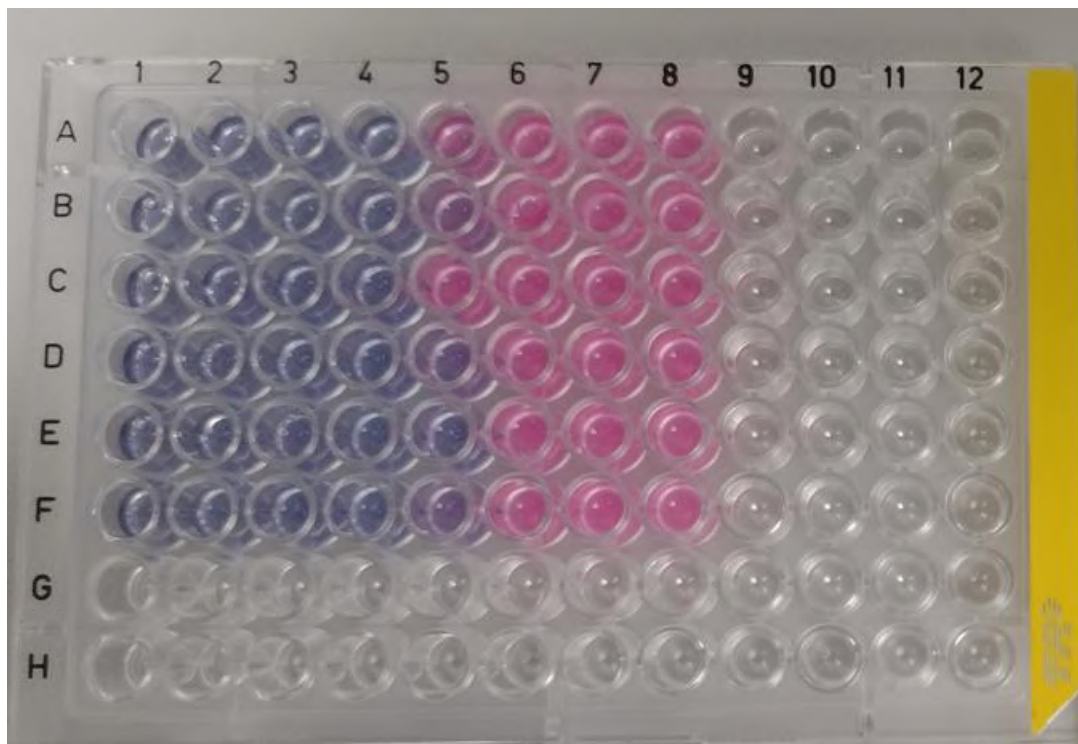


Figure S1. Photo presenting the viability of *K. pneumoniae* strain PK1 (**A-C**) and PK2 (**D-F**). The higher viability presented with more intense change in the resazurin colour from blue to red. The 600Ag-CN concentration in wells changes in the range 2.5 mg/mL (**1**), 1.25 mg/mL (**2**), 0.63 mg/mL (**3**), 0.32 mg/mL (**4**), 0.16 mg/mL (**5**), 0.08 mg/mL (**6**), 0.04 mg/mL (**7**), without complex (**8**).

[P2] The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties

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The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties

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ABSTRACT: Bovine lactoferrin (bLF) is widely known as an iron-binding glycoprotein from the transferrin family. The bLF molecule exhibits a broad spectrum of biological activity, including iron delivery, antimicrobial, antiviral, immunomodulatory, antioxidant, antitumor, and prebiotic functions, thereby making it one of the most valuable representatives for biomedical applications. Remarkably, LF functionality might completely differ in dependence on the iron saturation state and glycosylation patterns. Recently, a violently growing demand for bLF production has been observed, mostly for infant formulas, dietary supplements, and functional food formulations. Unfortunately, one of the reasons that inhibit the development of the bLF market and widespread protein implementation is related to its negligible amount in both major sources—colostrum and mature milk. This study provides a comprehensive overview of the significance of bLF research by delineating the key structural characteristics of the protein and elucidating their impact on its physicochemical and biological properties. Progress in the development of optimal isolation techniques for bLF is critically assessed, alongside the challenges that arise during its production. Furthermore, this paper presents a curated list of the most relevant instrumental techniques for the characterization of bLF. Lastly, it discusses the prospective applications and future directions for bLF-based formulations, highlighting their potential in various fields.

KEYWORDS: bovine lactoferrin, bLF, iron-binding affinity, functional food formulations, biomedical applications, biological activity

1. INTRODUCTION

Whey proteins (WPs) are recognized as one of the most valuable components in milk whose characteristic feature is a wide spectrum of nutritional and health promoting effects. WPs are represented by a mixture of globular proteins, e.g. β -lactoglobulin (β -LG), α -lactalbumin (α -LA), serum albumin (SA), immunoglobulins (Ig), lactoperoxidase (LPO), and lactoferrin (LF), dispersed in the continuous phase of the milk colloidal system which accounts for about 20% of the total protein fraction.¹ On the other hand, whey poses a serious group of waste products with extremely critical levels of BOD (biological oxygen demand) and COD (chemical oxygen demand).^{2,3} Direct recovery and further supplementation of WPs could be a prospective approach for improving the functionality range of dairy products as well as for environmental protection.

LF is known as globular iron-binding glycoprotein which belongs to the transferrin protein family.⁴ Chelated ferric ions inside the LF structure ensure the characteristic salmon-pink color of a protein.⁵ Bovine lactoferrin (bLF) is predominantly found in the granules of neutrophils and mammary gland secretions, such as colostrum, transitional milk, and mature milk. The lactation stage is considered one of the most reasonable factors which strongly affects the composition of the cow's milk. Significant changes of LF content are usually observed during the transition of colostrum (1.0–5.0 mg/mL) into mature milk (0.02–0.2 mg/mL).^{6–8} Remarkably, the amounts of lactoferrin and transferrin are relatively similar (LF,

0.83 mg/mL; TF, 1.07 mg/mL) in the colostrum phase; however, their concentrations sharply decrease in the lactation period (LF, 0.09 mg/mL; TF, 0.02 mg/mL).⁹ Since LF is known as a biomarker of inflammation processes, accurate protein detection is extremely important for the monitoring of cattle's health issues. For instance, the positive correlation between LF and immunoglobulin concentrations in serum is directly related to mastitis in cows.¹⁰

The molecular weight of LF might differ in dependence of its source of origin (Figure 1). It has been identified that LF mass from milk and colostrum was found in the range 83–87 kDa, while in neutrophils it was about 87–91 kDa. However, a single protein band appeared in both protein samples at 77 kDa resulting from the partial digestion of LF by N-glycanase.¹¹ Thus, based on the LF source, it might be present in different molecular forms whose size is highly influenced by the nature (heterogeneity) of glycosylation. The major factors that might induce variations in LF glycosylation are the stage of lactation, age, breed of cow, season, and feeding. For instance, Barboza reported that the degree of glycosylation in

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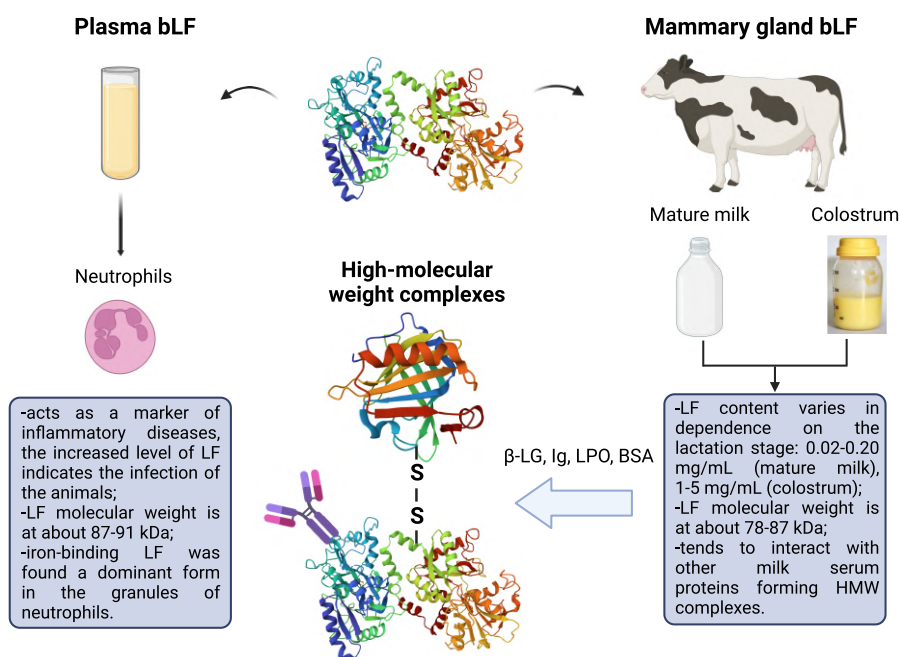


Figure 1. Comparison of bLF proteins from different sources of origin. Created with [BioRender.com](https://www.biorender.com).

human milk decreased after 2 weeks of lactation since colostrum was changed into mature milk.¹² In addition, Jia et al. observed the same tendency in the case of LF in bovine milk; thus the amount of N-glycans content decreased in the following order of lactation stages: colostrum > transitional milk > mature milk.¹³ Despite the monomeric form, bLF, the protein also may occur as high molecular weight complexes (HMW-LF). This phenomenon was especially abundant in nonlactating mammary secretions (Holstein cows).¹⁴ It was indicated that LF trimers ($M_w \sim 250$ Da) had much higher thermal stability as well as resistance to proteolysis than apo-LF and holo-LF.¹⁵ The structural stability of HMW-LF is probably caused by the structural integrity of larger oligomers. Besides, Ebrahim et al. showed that such HMW-LF complexes were sensitive to the addition of highly concentrated electrolyte solution (1 M NaCl) and dissociated into smaller structural units.¹⁵ LF is also capable of interacting with other proteins of milk, forming heterogenic protein complexes of LF-Ig, LF-CN, LF-SA, and LF- β -LG.^{14,16} Such bindings are characterized as noncovalent interactions which have a positive impact on the biological activity of the native LF molecule, enhancing some of its primary functions. Thus, Stephens et al. reported a higher antibacterial activity of LF-Ig complexes against *Escherichia coli* strains compared to LF alone.¹⁷

Until now, isolation of LF has been successfully performed from different mammals, including human, bovine, buffalo, goat, sheep, pig, horse, mouse, camel, and elephant. As it was reported, LF derived from human milk revealed the lowest similarity in sequence homology (>70%) in comparison to LF isolated from milk of other species.¹⁸ The difference in amino acid composition has a significant impact on the conformation stability of the biomolecule and other physicochemical properties (molecular mass, isoelectric point, thermal resistance).¹⁹ Currently, lactoferrin is a subject of great interest for researchers due to its high biological potential. LF is increasingly characterized as a multifunctional ingredient; it possesses antimicrobial, antiviral, immunomodulatory, and

anticancer and prebiotic activities, which make it one of the most attractive candidates in the biomedicine and biotechnology areas.²⁰ LF as an iron carrier avoids the formation of insoluble aggregates of ferric hydroxide; therefore, it might be safely applied in the treatment of anemia, especially in the case of pregnant woman.²¹ Previously, it was reported that 1.4 mg of iron might be maximum chelated per 1 g of protein, whereas the absorption of microelements by an organism is influenced by numerous factors, such as the daily diet, protein saturation state, pH conditions, human age, and physiological conditions.²² The additional advantage of LF's iron-binding capacity relies on its antioxidant effect through the prevention of lipid peroxidation and the potential formation of reactive oxygen species (ROS) as a result of the Fenton reaction.²³ The functionality of bLF can be developed as a result of interactions with other molecules (ligands), e.g. metals, vitamins, polyphenols, DNA, and lipopolysaccharides (LPS). Recently, interest in the synthesis of biologically active complexes based on bLF and metal ions has been growing. Such biologically active preparations allow for enhancement of beneficial and food values, especially in dairy products.²⁴ For example, Hinrichs et al. pointed out that cheese enrichment in whey proteins is a novel approach in the development of functional food.²⁵ Nevertheless, it is worth knowing that the lactoferrin incorporation will not only improve the nutritional quality and the functional properties of the final product; it will also require the reconstruction of the entire technological line.

Numerous health benefits and a wide spectrum of potential applications led to the significant growth of the demand for lactoferrin. The global production of bovine lactoferrin is expected to exceed a compound annual growth rate (CAGR) of 7.2% until 2028.²⁶ Currently, to the list of key LF manufacturers joined the following companies: Glanbia Nutritionals (Kilkenny, Ireland), Synlait Milk (Dunsandel, New Zealand), Fonterra (Auckland, New Zealand), Merck (Darmstadt, Germany), Milei GmbH (Leutkirch, Germany), and Tatura Milk Industries Ltd. (Tatura, Australia) as a part of the Bega Group (Bega, Australia). The market statistics

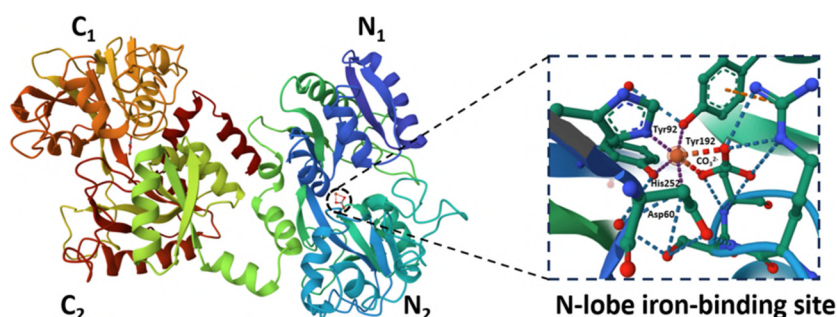


Figure 2. Three-dimensional structure of diferric bLF (Protein Data Bank code 1BLF).

accounts the biggest application share of lactoferrin to infant formula, followed by dietary supplements and pharmaceuticals. One of current problems relies on the finding of an efficient and selective isolation method which will enable large-scale production of biologically active lactoferrin. The amount of LF found in bovine milk is relatively low (about 1%) in comparison to other WPs. Hence, LF isolation might be a quite challenging and time-consuming process. The first successful purification of bovine LF was performed in 1960 by Groves using DEAE cellulose chromatography.²⁷ Comprehensive study of the nature of LF interactions has initiated the development of more advanced techniques. Traditional methods of ion-exchange chromatography, affinity chromatography, and membrane filtration have been improved by using more effective sorbents and modified membranes.^{1,28} In recent years, several complex techniques were investigated, including electrodialysis with a filtration membrane and magnetic affinity separation.²⁹

This review presents recent advances in the structural characteristics of the LF molecule, such as glycosylation and metal-binding properties, describes the novel methods of protein isolation, and clarifies the essential role of biomolecules in disease treatment.

2. LF STRUCTURE

The three-dimensional structure of bLF is generally described as a single polypeptide chain containing 689 amino acid residues folded into two symmetric homogeneous lobes (N-lobe 1–341 and C-lobe Tyr342–Arg689) which are linked by three-turn α -helix (12 residues long from 333 to 344).³⁰ According to crystallographic studies, each lobe is divided into two domains (N₁/N₂ and C₁/C₂). Although, the overall bLF structure has been known for a long time, it is worthwhile to mention LF genetic conservation/variation phenomena across different species. The LF gene is expressed in many species, e.g. human, bovine, buffalo, mouse, caprine, deer, equine, murine, and porcine. The protein genes from the transferrin family are typically composed of 17 exons (N-lobe, 2–8; C-lobe, 9–16, hinge region was at exon 9) whose lengths vary in the range 23–35 kb.³¹ Remarkably, it was found that 15 exons were identical between bovine and human lactoferrins, while the remaining two were responsible for gene differentiations that induced the variations in amino acid sequences.³² Seyfert et al. suggested that exons 4 and 12 play a significant role in the LF iron-binding properties and glycosylation.³¹ According to Wang et al.'s results, bovine and deer lactoferrins share 92% sequence homology.³³ The authors particularly highlighted the difference in the amino acid compositions of two major antibacterial peptides—lactoferricin and lactoferrampin. Nu-

merous molecular studies confirmed that genetic polymorphism is able to affect the protein conformational stability, the tendency to aggregate, and the biological activity.

The metal binding site is localized deeply inside the interdomain clefts of each lobe; thus one LF molecule is able to bind a maximum of two ferric ions. Interestingly, the binding cleft N-lobe reveals a more open conformation in comparison to the C-lobe.³⁴ A slight difference in their flexibility is caused by the presence of a disulfide bridge formed between residues 483 and 677 which restricts additional movements in the C-lobe.³⁴ Based on this, the metal binding capacity will differ between the N-lobe and the C-lobe. According to the iron saturation level, LF can be classified as apo-LF (saturation level <5%) and holo-LF (bound with two ferric ions). Native bLF is an intermediate state between apo-LF and holo-LF and accounts for approximately 15–20% of metal saturation.³⁵ Native bLF is naturally composed of apo-LF, holo-LF, and monoferric LF forms which are present in whole milk in different proportions.³⁶ The level of iron saturation has a great impact on the biological properties of a protein. For example, microbiological studies showed that apo-lactoferrin effectively inhibited the growth of pathogenic bacteria strains (*E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mannheimia haemolytica* A2), whereas the supplementation of holo-lactoferrin did not reveal any antibacterial effect.³⁷ In contrast, the supplementation of holo-LF ensured a more efficient cellular iron uptake, probably due to conformational specificity of the iron-saturated protein.³⁸ Traditional methods of protein detection (ELISA, HPLC) and iron determination (UV-vis, ICP-MS) do not solve the problem of distinction and quantification of each of the present forms.³⁹ Alternatively, Bokkhim et al. applied DSC studies to find the correlations between the iron saturation level and the thermodynamic parameters of protein denaturation.⁴⁰ However, the suggested approach had a limitation as the iron content exceeded 75% because of the similarity in thermal behaviors of mono- and diferric bLF. The results of cation-exchange chromatographic separation performed by Makino et al. showed the prevalence of apo-LF and monoferric LF fractions in native hLF.³⁶ Additionally, these forms might be distinguished based on their charge surface properties. For example, Kumar et al. observed three individual bands assigned to apo-LF, holo-LF, and monosubstituted LF which were present in buffalo LF samples. Results indicated a faster electrophoretic mobility as the level of iron saturation in protein increased.⁴¹

Specific amino acid sequences and their particular arrangement promote an LF high binding affinity toward ferric ions ($K_A = 10^{20}$).⁴² As a rule, iron complexation is accompanied by coordination of the bicarbonate ion, which plays a fundamental

role in holo-LF stabilization. The iron-binding site has an octahedral geometry in which the ferric ion is chelated by the imidazole ring of histidine (His253 in N-lobe, His595 in C-lobe), phenolate oxygens of two tyrosinases (Tyr92 and Tyr192 in N-lobe and Tyr433 and Tyr526 in C-lobe), the carboxylate of aspartate (Asp60 in N-lobe, Asp395 in C-lobe), and two atoms of oxygen from the bicarbonate ion (Figure 2). Remarkably, bicarbonate incorporation is mediated by interactions with the arginine side chain and hydrogen bonding with threonine and the N-terminus of helix 5 in order to maintain a higher protein conformational stability.^{43–45} In the absence of metal, the conformation of the LF binding site is retained by intramolecular interactions, including the formation of hydrogen bonding between the pair of side tyrosine chains and van der Waals forces between histidine and aspartic acid. Typically, the metal saturation is initiated by insertion of a bicarbonate ion and followed by the attachment of two tyrosine residues. In the final step, iron coordination is completed by chelation with histidine and aspartic acid residues.⁴⁶ The coordination bonding lengths range from 1.9 to 2.1 Å.⁴⁷ Nevertheless, the results of LF tryptic digestion performed by Rastogi et al. showed that the binding site located in the C-lobe fragment was slightly deformed from an ideal octahedron at pH below 6.8.⁴⁸ The geometry of the binding site is mainly influenced by the iron saturation level. The authors have demonstrated that gradually lowering the pH of the solution provides weakening of the bond strength between the ferric ion and the N^ε2 imidazole atom of histidine due to enhancing the repulsion forces between these atoms; thus the coordination distance between these atoms tends to be increased. Currently, in the PDB database there are published crystallized structures of apo-LF isolated from human, equine, and camel species, while holo-LF was analyzed from human, bovine, buffalo, and equine organisms. Remarkably, the crystallization of native LF (mixture of apo- and holo-LFs) has not been performed yet. As it was reported, the metal content has a great impact not only on the geometry binding site but also on the protein conformation in general. The iron-free human LF revealed a more open conformation in contrast to the iron-saturated form. What is important is that the N-lobe undergoes loosening in apo-LF, while the C-lobe constantly remains rigid even after the metal release.³⁴ The exact reason for that structural singularity of the C-lobe is still in question. Despite this, it would be interesting to compare the behaviors of both lobes in the LF native state. The conformational changes induced by iron binding might vary among LF different species. For example, camel apo-LF possessed both open-structured lobes, thereby showing a higher conformational similarity with apo-ovotransferrin than with other lactoferrins.⁴⁹ In contrast, the three-dimensional structure of mare apo-LF confirmed the presence of both N- and C-lobes in the closed state.⁵⁰ The authors explained that the phenomena might be influenced by a difference in the relative orientation between the N- and C-lobes and specific interdomain interactions within the protein structure; nevertheless, this issue requires a more detailed review in the future.

Although the classical assumption affirms the chelation of only two ferric ions per LF molecule, several studies confirmed that the presence of additional binding sites on the protein surface. As it was shown, the protein modification at appropriate conditions, e.g. the molar ratio of reagents (bLF:FeCl₃:NaHCO₃), pH, and temperature, enables the formation of a supersaturated Fe–LF complex.⁵¹ According

to results, a supramolecular structure of the synthesized complex was proposed in which LF molecules (15–16) were electrostatically linked through charged side chains of amino acids and Fe³⁺–HCO₃[–] ions. Alternatively, Nagasako et al. assumed that LF iron supersaturation might occur by electron donor–acceptor interactions with shares of cysteine, histidine, and tryptophan residues.⁵² Nonetheless, that question remains open for future research.

Iron exchange by other metals also may induce significant modification in the LF conformation. For example, replacement of ferric ions with copper in the N-lobe led to transformation of the binding site geometry to square pyramidal, resulting in the formation of hydrogen bonding between bicarbonate ion and tyrosine.⁵³ Smith et al.⁵⁴ studied lactoferrin complexes with different forms of vanadium (V³⁺, VO²⁺, VO₂⁺). According to the results, metal chelation was mediated by the same amino acid residues as in holo-LF; however, the structural importance of bicarbonate ion decreased with the increase of the vanadium oxidation state.⁵⁴ In contrast, the anion substitution of bicarbonate with the much bigger oxalate anion led only to slight changes of its arrangement in the binding site resulting in moving of the side chain of arginine away. Baker et al. also pointed out that the bigger size of substituted metal ions was characterized by lower affinity to LF.⁵⁵ Thus, a comprehensive study of the bLF structure is crucial for characterization of the protein conformational stability, detection of the potential binding sites, and indication of the type of intra- or intermolecular interactions. Besides, it provides critical insights into the establishment of the relationship between the structure and functionality of biomolecules which is highly important for designing new drug candidates.

3. FACTORS AFFECTING LF STABILITY

It is worthwhile to mention that LF conformation is characterized by high sensitivity to various physicochemical factors, such as pH, temperature, and ionic strength.⁵⁶ Therefore, careful planning of the experiment by the selection of appropriate conditions is a necessary step for the protection of the biomolecule's native structure and its primary biological function.

3.1. pH Conditions. LF stability and the level of iron saturation are tightly related to each other and are predominantly affected by the surrounding environment of the medium, especially pH. High iron-binding capacity is one of the fundamental features which contributes to conformational stability of the LF structure over a wide range of pH. The nonchelated form of LF is considered more sensitive to unfolding at extremely low values of pH. In contrast, holo-LF was able to maintain ferric ions in the structure even at pH 2.⁴⁸ LF desaturation is effectively carried out at pHs lower than 3.5, obtaining the protein with <2% metal.⁵⁷ As expected, that pH lowering provides the opening of the LF conformation. The metal release occurred much slower from C-lobe than N-lobe binding site with pH lowering, which might be related to the higher rigidity of the C-lobe and its greater binding affinity to ferric ion.⁴³ It has been demonstrated that iron dissociation begins at pH 6.7 in the N-lobe of bLF, while desaturation of the C-lobe was initiated at pH 5.6.⁵⁸ A similar character of desaturation was observed in the case of lactoferrin isolated from human milk and neutrophils.⁵⁹ Interestingly, in camel LF metal release in the C-lobe begins faster than in the N-lobe at pH 6.5 and 3.5, respectively.⁴⁹ The stability of LF complexes

Table 1. Isolation Approaches of LF from Different Sources

method of LF isolation	LF source	process	ref
cation-exchange chromatography by CM resin	bovine colostrum	First, milk must be defatted by centrifugation. Second, the casein fraction is removed from skimmed milk by acidic precipitation. Subsequently, the solution is neutralized by addition of NaOH. Some additional proteins were precipitated (salted out) by application of ammonium sulfate. Lactoferrin purification was carried out by the use of carboxymethyl Sephadex-C50 column and 0.2 M phosphate buffer (pH 7.7) as mobile phase.	102
combination of ultrafiltration with strong cation-exchange chromatography	bovine colostrum	A two-step ultrafiltration process was performed on a tangential flow filtration (TFF) model system with PLC membranes (nominal molecular weight cutoffs for each step were 100 and 10 kDa, respectively). The obtained retentate was adsorbed on a strong ion-exchange SP-Sepharose fast flow column and eluted with buffer solution composed of 0.05 M sodium phosphate with 0.1–1.0 M NaCl (pH 7.7).	100
ion-exchange chromatography on monolithic columns	acid whey	Preliminary microfiltration of whey was performed with ceramic alumina/zirconia asymmetric membranes (pore diameter < 0.8 μm). The obtained permeate was loaded on a CIM monolithic ion-exchange column. Selective LF isolation was related to its higher pI value in comparison to other whey proteins. Adsorbed LF was eluted with a relevant buffer solution. A TFF system was applied in order to desalt and concentrate the obtained LF fraction. Lastly, the protein was freeze-dried.	73
cross-flow microfiltration coupled with isolation on strong cation-exchange membranes	sweet cheese whey	Whey cheese was subjected to cross-flow microfiltration with use of a Microdyn tubular system (spun with polypropylene filter cartridge) in order to eliminate insoluble particles (lipids, casein). Subsequently, LF isolation from permeate was performed by application of the strong cationic membrane adsorber Sartobind S. The elution process was carried out in a three-step operating sodium chloride salt gradient. Finally, the obtained eluates were desalted using Sartocoon II with cellulose acetate membrane (area of 0.7 m ² and a cutoff of 30 kDa) and lyophilized.	103
electrodialysis with filtration membrane	whey protein isolate	LF undergoes aggregation in a salty SMUF buffer, such as that available naturally in whey. The solution containing the micelles of lactoferrin and other dairy proteins can then be separated by electrodialysis with a filtration membrane (PVA glutaraldehyde as a cross-linking agent, catalyzed by sulfuric acid), where proteins with smaller size will pass through, while the larger aggregates of will be retained.	104
separation on magnetic affinity adsorbents	acid whey	Preliminary, skimmed milk obtained by centrifugation was acidified in order to eliminate casein. Remained whey proteins were incubated together with PGMA-NH ₂ –heparin magnetic particles at 10 °C in an ice bath for 2 h. Subsequently, particles were removed from the reaction mixture by use of PBS buffer solution (pH 6) as eluent. The release of LF from magnetic affinity adsorbents was conducted by elution with 0.5 M NaCl.	29
affinity chromatography equipped with immobilized Cibacron Blue F3GA dye column	bovine colostrum	Colostrum milk was subjected to the traditional procedure of sample pretreatment, including defatting and casein removal. Subsequently, whey was slightly alkalinized to pH 6 by the addition of sodium hydroxide. The LF fraction was separated on a Cibacron Blue F3GA column using 0.1 M NaOH as eluent. The efficiency of the proposed methodology was related to strong electrostatic interactions between the anionic stationary phase and positively charged LF molecules.	105

might vary in dependence of the type of coordinated metal. For example, Jabeen et al. showed that Zn^{2+} dissociation from the C-lobe starts much lower (pH 4.6), in comparison to Fe^{3+} (pH 5.7).⁶⁰ Importantly, that pH-induced LF desaturation is easily reversible and does not lead to the loss of primary protein metal-binding properties.⁵⁷

3.2. Ionic Strength. Since LF dispersion represents a complex biocolloidal system, its stability is highly affected by ionic strength. The presence of co-ions induces some modification of the thickness of the electric double layer.⁶¹ Protein interactions with water molecules are strongly related to the type of present ions, which according to the Hofmeister effect might be classified as kosmotropic (cation, destabilizing; anion, stabilizing) and chaotropic (cation, stabilizing; anion, destabilizing). Sodium chloride is known, as a salt possessing a kosmotropic cation and a chaotropic anion, which tend to interact strongly with protein carboxylic and amino groups, respectively.⁶² Mela et al. reported that high electrolyte concentration (>30 mM NaCl) disrupts the balance between hydrophobic and electrostatic interactions in LF dispersion, leading to formation of negatively charged aggregates of apo-LF.⁶³ These aggregates are described as micelles with bulk positively charged groups inside aggregates and more exposed to environment negatively charged groups. Conformational rearrangements were corresponding to significant changes of charge density on the protein surface, leading to reduction of the value of the LF isoelectric point from 8.4 to 6 at ionic strengths above 150 mM. Nilsson mentioned that LF conformation undergoes destabilization by aggregation and formation of amyloid fibrils at a relatively high NaCl concentration (150 mM) and heat treatment to 65 °C.⁶⁴

3.3. Heat Treatment. Investigation of the heat stability of bLF is an essential step in technological processing which allows evaluation of the prospective of a macromolecule as a biologically active additive in new conceptions of food, cosmetic, and drug manufacturing processes. The treatment of extremely high temperatures provides significant conformational changes in the LF structure and subsequent loss of protein biological activity. It was shown that high temperatures are one of the driving forces that facilitate the release of bound iron ions by weakening their interactions with LF binding sites.⁶⁵ A previous study reported that LF thermal stability was positively correlated with the iron saturation level. Unsaturated bLF possess a greater heat sensitivity due to its less closed conformation, which makes its unfolding process more favored than that in holo-bLF. Bokkhim et al. has observed a tendency of apo-LF to denature at 70–71 °C, while the destabilization of holo-bLF occurred at 89–92 °C.⁴⁰ Besides, the pH of the medium is an important factor that might contribute to LF thermostability. Remarkably, the apo-LF form was resistant to the formation of aggregates during ultra-high-temperature (UHT) processing at low ionic strength and acidic conditions. It was pointed out that pH 4 was the most optimal one allowing LF to maintain its conformation and biological activity under high-temperature processing.⁶⁶ Heat treatment of bLF could induce a cleavage of disulfide bridges following by aggregation via thiol/disulfide interchange reactions. Many authors revealed that LF two-step aggregation corresponds to different thermal resistances of both LF lobes.⁶⁷ Large insoluble aggregates of apo-bLF solution start to appear at 60 °C.⁶⁸ Nevertheless, soluble disulfide-linked aggregates of holo-bLF (pH 6.6) were observed upon heating to 70–75 °C. These bands were more visible by application of SDS-PAGE in

nonreducing conditions, because disulfide bridges were not cleaved.¹⁶ Difference in aggregate solubility is caused by the presence of ferric ion in the protein structure. Destabilization of iron-free LF occurs too fast compared to iron-saturated LF, favoring the formation of insoluble aggregates. Modification in the tertiary structure of bLF as well as an increase of particle size restricts protein interactions with bacteria membrane, thus its bacteriostatic capacity was reduced.

The highest iron-binding capacity was characterized at lower ionic strength below 0.01 M.⁶⁹ Heating of LF above 80 °C at an ionic strength above 0.1 M decreased the level of iron saturation, precipitation, and partial unfolding. The authors reported that conformational changes involved breaking only noncovalent interactions (mainly hydrogen and electrostatic) resulting in releasing ferric ion. Heating over 72 °C provides only minor changes in the secondary structure of native LF (pH 7.5).⁷⁰ Simulation MD studies showed inconsiderable changes in the contents of different types of secondary structure in LF. These minor modifications mainly concerned a partial reduction of the number of hydrogen bonds resulting in partial unfolding of α -helix. The β -sheet structure was less sensitive; thus the content of this structure remained constant during thermal processing. However, the percentages of random coils and turns in the LF structure were slightly increased.⁴² On the contrary, Iafisco et al. showed that thermal denaturation of LF was accompanied by reduction of α -helix and a significant increase of β -sheet content.⁷¹ Goulding et al. showed a similar effect of the irreversible transition of α -helix into β -sheet structure by using a high temperature–short time (HTST) thermal process.⁷² Thus, the composition of the secondary structure of bLF may vary with regard to the applied method of heat treatment. The temperature of denaturation and enthalpy change of denaturation of holo-LF is much higher than that of apo-LF, which suggests greater thermal resistance of iron-saturated forms of protein.^{19,73} Application of high hydrostatic pressure as an alternative to heat treatment also confirmed much evident conformation stability of iron-saturated LF at extreme conditions compared to metal unsaturated LF.⁷⁴

Interestingly, relatively low temperatures are also capable of modifying protein biological functions. It was observed that LF storage of at 20 °C for 6 months significantly influenced the protein immunomodulatory function. The level of secreted tumor necrosis factor- α (TNF- α) decreased after hLF exposure.⁷⁵ TNF- α plays a crucial role in the regulation of pro-inflammatory cytokine secretion and macrophage differentiation providing more effective protection of the organism against infections.⁷⁶ Therefore, the consumption of fresh milk is extremely important in the case of infants since it allows preserving the beneficial properties of the product.

4. ISOLATION OF bLF

LF isolation at a high level of purity might be a challenging task since its total amount in milk is negligible compared to other whey proteins.⁷⁷ Currently, no standard procedure of lactoferrin isolation is established (Table 1). It is worth noticing that the selected conditions of LF extraction might affect the yield, purity level, and biological activity of the final product. Main factors that might induce the modifications in each of four levels of the protein structure are included: extremely low pH, high temperature, pressure, and salt concentrations, and addition of chemical denaturants. The mentioned factors could lead to breakdown of intermolecular forces (hydrophobic,

ionic, hydrogen, disulfide). For instance, Franco et al. reported that the thermal denaturation of lactoferrin occurs in the range from 60 to 90 °C in dependence on the saturation state.⁷⁸ Remarkably, thermal processing at high temperatures is frequently accompanied by irreversible denaturation and as a result the complete loss of the functionality of the biomolecule.⁷⁹ As it was previously reported, high pressure processing (>400 MPa) is capable of modifying the surface charge reducing the electrostatic repulsions between protein particles and facilitates the formation of aggregates.⁸⁰ A preliminary purification procedure involves a few pretreatment steps, the number of which depends on the type of raw material (whole milk, colostrum, sweet cheese whey, acid whey). Traditionally, whole milk is subjected to centrifugation to receive a skimmed milk. The whey fraction is obtained by removal of casein micelles from skim milk resulting in acidic precipitation at pH 4.6.⁸¹ Ion-exchange chromatography is considered one of the most integrated techniques of LF isolation. The major principle of protein fractionation is based on a significant difference of isoelectric points between LF (pI 7.8–8.8) and the rest of the whey proteins, including β -LG (pI 4.9–5.4), α -LA (pI 4.4–5.1), and BSA (pI 4.7–5.2).^{1,3} The separation process is accompanied by strong interactions of LF with the stationary phase, while other proteins are easily eluted from the column.

In neutral pH positively charged LF has a much higher affinity to a cation-exchange resin. Anionic sorbents containing strong sulfopropyl (SP) and weak carboxymethyl (CM) functional groups belong to the most commonly used resins in cation-exchange chromatography.⁸² The adsorption behavior and the strength of the interaction of the target protein with the stationary phase could be modified, controlling the surface charge of the biomolecule. Remarkably, Voswinkel et al. paid attention to that the binding affinity of LF to adsorbent is highly dependent on the protein saturation state.⁸³ According to results, apo-LF was more sensitive to applied separation conditions and was characterized by a lower adsorption capacity compared to holo-LF, especially at the low pH range. It might be related to the structural singularities of both LF forms and the difference in distribution of the surface charge. As previously mentioned, apo-LF is characterized by a more “open” conformation; therefore, it is possible to suggest that it has more accessible ionizable groups in contrast to holo-LF. The presence of minor amounts of lactoperoxidase (LPO) molecules (pI 9.3–9.6) and immunoglobulins (5.5–8.3) might become a serious hardship in the production of highly purified biologically active LF by ion-exchange chromatography.⁸⁴ LPO is known as a minor whey enzyme mainly involved in the immune defense function and whose content in bovine milk varies from 15 to 50 mg/L.³ The problem of LPO elimination could be solved by selection of the appropriate isolation conditions, e.g. flow rate of the mobile phase, buffer pH, and ionic strength.⁸⁵ Uchida pointed out that the efficiency of separation of LF from LPO might be improved by use of a buffer solution with pH >5 and ionic strength higher than 0.5.⁸⁶ For instance, Ng et al. applied two-step isocratic elution with 0.4 and 0.6 M sodium chloride buffer (pH 7) for fractionation of an LF–LPO mixture.⁸⁷ The detection of LPO is frequently performed with UV–vis analysis by measuring the maximum absorbance in the Soret region at λ_{ex} 412 nm.⁸² Negligible amounts of lysozyme (0.15–2.7 $\mu\text{g/mL}$) also might constitute an interfering factor (pI 11) during LF isolation.^{88,89} In this case, the protein separation was performed by use of UF

zirconia membranes, modified with charged cationic (ethylenediamine, EDA) groups.⁸⁹ Interestingly, Lech et al. has detected the presence of serum albumin after goat whey treatment on a CM-Sepharose column.⁹⁰ Thus, effective separation of both proteins was achieved by anion-exchange chromatography with a DEAE-Sepharose resin.

Affinity chromatography is another separation technique frequently used for the purification of whey proteins. The stationary phase is composed of immobilized antibodies (ligands) that selectively interact with the target molecule, while other components are easily eluted from the column. Sepharose modified with heparin, metal ions, oligonucleotides, monoclonal antibodies, organic dyes, and even other whey proteins was mentioned as an effective adsorbent for LF molecules.¹² Lampreave et al. showed that LF is favored to form noncovalent associations with whey proteins, in particular with β -LG.⁹¹ Therefore, the immobilization of insoluble β -LG particles might be a potential approach for the successful isolation of LF.⁹² Unfortunately, this method is insufficient for bovine milk matrix since it contains great amounts of β -LG which will also compete with LF for binding sites.

In recent years, membrane technology has been becoming more attractive and rapidly developed in food production, especially in the dairy sector. Membranes with specific pore sizes are widely used during diverse technological processes, e.g. microbial decontamination, defatting (microfiltration, MF), concentration and fractionation of whey proteins (ultrafiltration, UF), removal of low-molecular compounds, mainly salts, lactose, and peptides (nanofiltration, NF; reverse osmosis, RO).⁹³ LF purification by the membrane separation approach is not implemented to such an extent as chromatographic technologies, but it also has a great perspective of application in large-scale production. To the major advantages of membrane processing should be included less impact of adsorption and diffusion effects during mass transfer in comparison to chromatographic methods.⁹⁴ In addition, it enables high separation effectiveness and minimizes the risk of destruction, since samples are subjected to low-temperature conditions.⁹⁵ Unfortunately, membrane separation becomes challenging in the case of macromolecules with similar molecular weights (such as BSA, 65–69 kDa; LPO, 77–90 kDa; and LF, 78–84 kDa).⁹⁶ The selectivity of membrane adsorption technologies might be improved as a result of chemical modification of the membrane surface and attachment of specific functional groups. One such approach introduced the separation of milk proteins according to their electric properties with the use of charged membranes.⁹⁷ Basic principles assume that proteins with the same net charge as the filtration membrane are retained due to electrostatic repulsions, while components having neutral or opposite-charged surfaces easily pass through the membrane barrier.⁹⁸

Valiño et al.⁹⁷ showed that pH manipulation allowed for the effective separation of individual proteins in BSA and LF mixtures. In the experiment, the diafiltration was performed by using charge-modified membranes made of regenerated cellulose (100 kDa cutoff). The authors reported that the most optimal fractionation of BSA was performed at pH 5 with the use of a positively charged membrane; however, LF was successfully isolated by application of a negatively charged membrane at pH 9.⁹⁷ On the other hand, the separation at such conditions might be inefficient because it is known that the pI of LF varies in a wide range from 4 to 10 in dependence of the source of origin, level of glycosylation, the iron

saturation state, or instrumental approach.⁹⁹ Internalization of ion-exchange groups to the membrane surface, similar to ion-exchange columns, provides improved separation of LF from other milk proteins. Such an approach allows operation at high flow rates and minimal diffusion effects. For example, Plate et al. have demonstrated the separation of the LF–LPO mixture from sweet cheese whey by a membrane adsorber modified with sulfonic groups (Sartobind S).¹ It was revealed that LPO was gradually eluted by increasing the ionic strength from 0.1 to 0.2 M NaCl, while the LF fractionation required 1 M NaCl eluent. The obtained product was characterized by 95% purity. In contrast, Teepakorn et al. performed the purification of LF from BSA by use of monolithic columns equipped with cation-exchange membranes, the same as previously described.⁹⁵ In order to improve the LF recovery, the number of membranes equipped into the column was increased to 33. Elution with phosphate buffered saline (pH 6) provided a sufficient adsorption of positively charged LF on the stationary phase with the complete removal of negatively charged BSA molecules. On the other hand, the application of columns with monolithic material might be problematic due to poor reproducibility as a result of pore size heterogeneity. The combination of membrane and chromatographic techniques represents another progressive trend in the isolation of milk proteins. Lu et al. proposed an efficient two-step methodology aimed at large-scale LF isolation from bovine colostrum.¹⁰⁰ Primarily, colostrum whey was subjected to two-step UF on regenerated cellulose membranes with cutoffs of 100 and 10 kDa, respectively. In the second step, LF was eluted from the obtained filtrate using a cation-exchange SP-Sepharose column and phosphate buffer (pH 7.7). Remarkably, the combination of microfiltration with affinity chromatography enabled obtaining LF product with a final purity of 95%.¹⁰¹ A stable complex formed between LF and heparin Sepharose resin was subjected to microfiltration to remove the unbound protein fraction and other impurities. Finally, the target protein release was conducted by changing the buffer ionic strength. Thus, it is worthwhile to highlight that one of the most substantial advantages of combined isolation techniques relies on the possibility to obtain the product with higher recovery and at a higher purity grade.

The extracted LF fraction is typically subjected to dialysis (cutoff 12–14 kDa) for several days to remove salts and other contaminants. Alternatively, it might be concentrated by using the cross-flow filtration system Sartocore.¹ Drying is a key step in the final preparation of lactoferrin which relies on receiving the powdered product ready for packaging. Among the most popular drying methods widely utilized in lactoferrin preparation, freeze drying (lyophilization) and spray drying might be distinguished. Although previously it was mentioned that drying plays a negligible role in the structural stability and functionality of lactoferrin, the recent studies showed by Morel et al. proved the opposite effect.¹⁰⁶ The authors noticed that the spray-drying approach was more destructive in the case of bLF samples inducing the denaturation of 14–17% of the structure, while freeze drying caused only 7% of damage. Such a difference might be related to the extremely high temperature applied during spray drying (>140 °C) in contrast to freeze drying (≤40 °C). What is important is that the selected method had no visible impact on the protein bacteriostatic activity. According to results obtained by Wang et al., the freeze-drying technique was much more effective in water removal; thus the determined moisture content was only 2.7%

(w/w) while it was about 5–9% (w/w) in the protein after spray drying.¹⁰⁷ Remarkably, the degradation effects in the lactoferrin structure were not much evident as in the previously mentioned study, and they varied in the range 0.9–2.0%.

5. RECOVERY OF WASTE PRODUCTS

As previously mentioned, the total amount of LF in cow milk is trace and varies at about 0.01–0.05% (w/w).¹⁰⁸ Waste products derived as a result of LF production constitute the additional milestone in the industrial sector. The main goal of this issue involves the maximum engagement of byproducts obtained at every single stage in order to reduce the effects of environmental pollution and prevent economic losses. Generally, dairy waste might be divided into two categories: effluent and sludge.¹⁰⁹ Milk fat, separated during milk microfiltration, might be utilized as a crucial element in the formulation of various dairy products, e.g. cream, cheese, cream cheese, butter, and anhydrous milk fat (AMF).¹¹⁰ Interestingly, the milk fat content plays a significant role in the creation of unique organoleptic features (taste, texture, color) in the final product.¹¹¹ The recovered casein is characterized by a high nutritional value, which enhances its attractiveness in food manufacturing. Casein produced by acid precipitation is insoluble in water; thus it is preferably dissolved by the addition of a strong alkali, forming caseinates.¹¹² Due to its unique structure, casein is widely utilized for the delivery of numerous bioactive compounds, stimulating the development of novel dietary supplements or pharmaceuticals.¹¹³ The whey fraction remaining after LF isolation represents a rich source of high-value proteins, e.g. α -LA, β -LG, SA, and Ig. Whey proteins are characterized by rich contents of essential amino acids which are easily absorbed by organisms and stimulate muscle protein synthesis.¹¹⁴ Recently, the production of functional beverages fortified with whey proteins for athletes is gaining in popularity.¹¹⁵ The major challenge in the spreading of these beverages is focused on the selection of the appropriate packaging and storage conditions to ensure the maximum stability of whey proteins and to prevent their denaturation and the loss of biological activity.

6. THE ROLE OF LF–METAL INTERACTIONS

Shortly after the isolation, the native protein is subjected to some chemical modifications in order to obtain an LF form with a desired level of iron saturation. LF desaturation is traditionally performed by dialysis against citrate buffer at pH <3.5 and then against deionized water. The described process is known as reversible; thus the protein resaturation might be carried out by mixing of apo-LF with ferric nitriloacetate (Fe-NTA) and then against deionized water in order to remove unbound ions.³⁵ As it was shown in previous studies, LF is characterized by the greatest thermal and conformational stabilities among the rest of the proteins from the transferrin family.¹¹⁶ Besides, the native structure of bLF exhibits a relatively high resistance in the presence of proteolytic enzymes.¹¹⁷ Many authors directly associated that phenomenon with the conformational specificity of binding sites between LF and TF. The release of iron ions occurs 100 times slower in LF compared to TF. Binding clefts of apo-TF, as a rule, possess a more wide-open conformation being exposed to the aqueous environment. On the contrary, the binding site located in the C-lobe of LF favors maintaining the closed conformation even during desaturation; therefore, the protein contact with the

polar medium is always partly restricted.³⁴ LF saturation accompanies the closure of binding clefts which occurs in both lobes.¹¹⁸ The greater percentage of bounded metal provides the growth of surface tension of LF; thus the holo form of the protein achieves a more compact structure.¹¹⁹ In contrast, Bluard-Deconinck et al. did not notice the obvious difference in iron-binding capacity between released N- and C-fragments of LF.¹²⁰ Indeed, Lin et al. have related the changes in metal complexation between C- and N-sites of apo-hTF with different rates of bicarbonate insertion to each lobe.¹²¹ Ferric ions exhibited a more visible binding preference to the C-lobe compared with the N-lobe as the concentration of bicarbonate in the solution decreased. Baker also pointed out the importance of hydrogen interactions in the dilysine pair (Lys206–Lys296) in the TF structure and its susceptibility to iron release.¹²² Remarkably, based on previous studies, the presence of a more compact conformation of the N-lobe in rTF and hTF than in bLF was reported.⁴⁷ In this case, the conformational variations between LFs from other species were related to the specificity of amino acid composition and lobe orientation, but not to iron-binding affinity.⁴⁷ In addition to this, SDS-PAGE analysis confirmed a higher susceptibility of the bLF structure to trypsin digestion than bTF, showing a greater degradation effect after enzyme treatment.¹²³ Comparing both iron-saturated proteins, 41% of TF remained undigested after 24 h, while in the case of LF it was only 6%. Regarding this, TF could be a more compatible candidate for cellular iron uptake in contrast to LF.

The metal incorporation mostly affects the LF tertiary structure; however, a slight alteration in the secondary structure is also present.¹²⁴ Based on CD studies, the determined ratios of α/β structures at pH 7 were equal to 20.0/59.0 and 11.5/70.0 for apo-LF and holo-LF, respectively.⁵⁶ Indeed, Xia et al. reported that the percentage contents of the individual secondary structures in apo-LF and holo-LF were relatively similar and ranged about 18–20% of α -helix and 53–55% of β -structure.¹²⁵

A characteristic feature of LF among other whey proteins is a comparatively high isoelectric point. Based on the UniProt database, the percentage content of basic amino acids (14.66%) in the bLF polypeptide chain is higher than that of acidic amino acids (11.03%). The protein surface contains regions with high concentrations of positive charge, such as the N-terminal region where the majority of basic amino acids are located.¹²⁶ Although the protein isoelectric point is directly related to the amino acid composition, the level of iron saturation also has a great impact on the physicochemical nature of the protein surface and the charge distribution.¹²⁷ As a result, the isoelectric points of apo-LF and holo-LF can vary due to differing availability of charged amino acids.³⁰ For example, Lys637 located in the C₁ domain and the Arg463 and Lys544 residues of the C₂ domain are deeply buried in holo-LF, although they became more exposed in the cleft of the apo form.⁴⁶ Isoelectric points of native LF and holo-LF determined by potentiometric titration were found in the range 8.0–9.0 and dropped to 5.7, in the case of apo-LF.¹¹⁹ The major reason for such a broad pI range for LF protein might also be related to the type of applied instrumental method, e.g. isoelectric focusing (IEF), two-dimensional gel electrophoresis (2D-PAGE), capillary isoelectric focusing (cIEF), and potentiometric titration. For example, IEF performed by Voswinkel et al. showed a negligible difference between the pI values of apo-bLF and holo-bLF, which were in the ranges 9.3–9.4 and 9.4–

9.5, respectively.⁸³ However, the study of Yoshida et al. carried out with the same technique reported the cationic character of bLF, whose pI ranged from 4.8 to 5.3.¹²⁸ Potentiometric titration is another popular technique widely used for characterization of electrochemical properties of biomolecules which is based on the measurements of zeta potential in the wide pH range by gradual addition of strong acid/basis. Many aspects might be important during the analysis, predominantly the type of dispersion medium, ionic strength and the protein concentration.¹²⁹ For example, Pryshchepa et al. determined that the pI of bLF dispersed in 0.09% (w/w) sodium chloride solution at the concentration of 1 mg/mL was at about 7.4.¹³⁰ However, Valiño reported that the pI of bLF suspension (1 mg/mL) in 0.01 M NaCl medium (which is equal to 0.06% (w/w)) rose to 9.2.¹²⁹

Despite iron, several studies have confirmed the ability of LF to interact with other metal ions, including Cu(II), Zn(II), Mn(III), Co(III), Ti(IV), and Ag(I).^{131,132} It has been reported that iron substitution with other metals induced exclusively minor changes in the hLF structure; thus their binding mechanisms are considered to be similar. Nevertheless, among all of them, iron was characterized with the highest binding affinity, indicating that the formation of LF–Fe complex is the most favorable.¹³² Indeed, even inconsiderable conformational modifications of LF in protein structure might cause fundamental changes of its biological activity. The previously mentioned metals belong to d-block elements (transition metals) which are frequently described as essential mediators of various biological processes in the human organism. Iron, copper, manganese, zinc, and copper act as protein inorganic cofactors required for activation of enzymatic reactions and for maintaining the structural stabilities of biomolecules.¹³³ Besides, many metal–protein complexes mediate in the regulation of cellular metabolism, oxygen transport, and prevention of DNA damage.¹³⁴ Importantly, metals are also capable of promoting the process of protein glycosylation.¹³⁵ For example, Prabhu et al.¹³⁵ pointed out that manganese and iron ions might be involved in the regulation of glycosylation pathways since they induce the activation of glycosylation enzymes.¹³⁴ However, the direct impact of these metals on the glycosylation profile of LF has not been discovered until now.

7. LF GLYCOSYLATION

Protein glycosylation is a post-translation modification which has a fundamental influence on the biological activity of a macromolecule in different aspects, including structural stabilization, folding process, proteolytic resistance, immune response, signal recognition, bacterial adhesion, and antiviral activity.⁸¹ Glycans are considered the markers of evolution, and their structures and diversity at each position are specific to each species and are influenced by different factors. Milk contains such glycosylated components as LF, κ -CN, immunoglobulins (IgG, IgA, IgM), α -LA, and LPO.^{136,137} Glycosylation plays a crucial role in the physicochemical characteristics of bLF, particularly the molecular weight and ionic charge. The molecular mass of bLF is variable throughout seasonality, the lactation period, and the cow's feeding. All of the mentioned factors contribute to the difference in carbohydrate composition and glycosylation level of a biomolecule. For instance, the overall number of N-glycans has decreased from 41 to 22 after the transition of the colostrum into mature milk.¹³ Besides, the structural diversity

Table 2. Specification of the Relevant Instrumental Techniques for LF Characterization

protein characteris- tic	instrumental techni- que	classification of the instrumental tech- nique	specific details	applied conditions for LF detection	ref
molecular mass	SDS-PAGE	electromigration	The key principle of the separation relies on the different mobility of the charged particles under an electric field that appear as a result of the difference in their molecular sizes. The molecular mass of bLF varies in the range 78–91 kDa in dependence of the source of isolation and glycosylation state.	protein separation in 4–12% polyacrylamide gel; reducing agent, 2-mercaptoethanol; running buffer, 1× MES; voltage, 200 V; time of separation, 22 min; gel staining, Coomassie Blue R-350	11, 131
	MALDI-TOF/MS	spectrometric	MALDI is a soft ionization technique. The appropriate chemical substance is the matrix, which mediates in the energy transfer from laser beam to analyte and then enables its desorption and ionization. LF mass was found at about 82–84 kDa in dependence on the stage of lactation.	MALDI-TOF/TOF mass spectrometer UltrafleXtreme (Bruker Daltonics); laser, Nd:YAG (355 nm); laser frequency, 2 Hz; matrix solution, sinapic acid dissolved in 30:70 (v/v) mixture of acetonitrile and 0.1% trifluoroacetic acid (TFA)—TA30; <i>m/z</i> range, 10–100 kDa	13, 130
iron saturation level	UV-vis	spectroscopic	The total LF concentration is recorded at 280 nm. The characteristic band at 465 nm indicates the iron(III) coordination by LF. The calculation of absorbance ratio A_{465}/A_{280} is relevant for determination of the iron saturation level of LF.	UV300 spectrophotometer (Thermo Scientific, USA); path length of quartz cuvette, 1 cm	153
	ICP-MS	spectrometric	In contrast to UV-vis, ICP-MS analysis is considered more accurate for determination of the negligible amount of bounded iron in LF (<2%).	samples of LF in mineralized HNO_3 analyzed by ICP-MS ELAN spectrometer (PerkinElmer, USA); plasma gas, Ar; gas flow, 15 L/min	57
particle charge properties	zeta potential measurements	spectroscopic	Electrophoretic mobility was determined based on the Henry equation. The zeta potential was calculated according to the Smolouchowski approximation. The measured zeta potential is highly influenced by the type of electrolyte (NaCl, KCl) used and ionic strength.	Malvern Nano-ZS ZetaSizer (Malvern Instru ments, U.K.); temperature, 25 °C; cuvette, DTS1070; equilibration time, 2 min	129, 154
	IEF	electromigration	Different pI values determined by IEF also might be a matter of the selected separation conditions, e.g. the type of carrier ampholyte (CA), pI marker, buffer composition, and separation time. The possibility of distinction of several LF fractions is based on their pI values.	Multiphor II apparatus (Amersham, Freiburg, Germany); precast gels Servalyt and Blank precotes (Serva, Germany); gel parameters, $126 \times 125 \times 0.3$ mm; temperature, 5–8 °C; voltage, 2000 V; current, 6 mA; power, 12 W	59, 83
three-dimensional molecular structure	XRD	crystallographic	XRD provides a detailed insight into the protein's three-dimensional structure. It indicates the possible metal-binding sites and their geometry, and it is essential for understanding the structural difference between apo- and holo-LF. XRD enables the verification of bLF stability under various environmental conditions. The technique is considered a basis of drug design and development.	synchrotron beamline BM14 at European Synchrotron Radiation Facility (Grenoble, France); space group, $P2_1$; cell dimensions, $a = 75.8$ Å, $b = 49.4$ Å, $c = 97.9$ Å; resolution range, 2.42 – 38.7 Å	46, 60
secondary structure	CD	spectroscopic	The assignments of individual secondary structures are found in the following ranges: (1) 190–195 nm (max), 208 nm (min), and 220–222 nm (min) for α -helix; 195–200 nm (min) and 215–218 nm (max) for β -sheet. The monitoring of ellipticity at 220 nm is frequently used to estimate the protein denaturation.	spectropolarimeter J-720 (JASCO, Tokyo, Japan); path length of quartz cell, 10 mm; UV region, 190–260 nm; scan speed, 50 nm/min; temperature, 25 °C	155
	FTIR spectroscopy	spectroscopic	Amide I is considered the most relevant for secondary structure profiling: 1660–1650 cm^{-1} (α -helix), 1650–1640 cm^{-1} (random coils), 1680–1670 cm^{-1} (β -turn), 1690–1680 cm^{-1} and 1640–1630 cm^{-1} (β -sheet). Different deconvolution approaches, including Gaussian and Lorentzian fitting, second derivatives with appropriate software program (OMNIC, OriginPro, PeakFit) are frequently used in calculations of the contributions of individual structures in proteins.	FTIR spectrometer (NEXUS Nicolet, USA) in attenuated total reflectance (ATR) mode; spectral range, 4000–650 cm^{-1}	116, 125
	Raman spectroscopy	spectroscopic	The region of 1700–1620 cm^{-1} was selected for distinction of each type of secondary structure. In contrast to the FTIR technique, Raman spectroscopy is more sensitive to samples containing nonpolar functional groups and is less influenced by interferences caused by hydrogen bonding between analyte and water molecules.	JASCO NRS-2000C; excitation, Ar ⁺ laser (514 nm); detector, charge-coupled device (160 K); spectral resolution, 4 cm^{-1}	71
changes in the tertiary structure	fluorescence spectroscopy	spectroscopic	Aromatic amino acids constitute about 9% of the total sequence in the bLF polypeptide chain. The measurement of the fluorescence intensity ratio F_{330}/F_{350} is related to protein destabilization, unfolding, and the exposure of aromatic amino acids to a polar microenvironment.	Cary Eclipse spectrofluorimeter (Varian, Middelburg, The Netherlands); emission range, 300–400 nm; path length of quartz cuvette, 1 cm	73, 156
aggregation state	DLS	spectroscopic	Destabilization processes such as protein unfolding and formation of larger aggregates accompany a considerable increase of single particle size up to 30 nm. The protein hydrodynamic size was calculated according to the Stokes–Einstein equation.	Malvern Nano-ZS ZetaSizer (Malvern Instru ments, U.K.); temperature, 25 °C; backscatter angle, 173°; cuvette, rectangular polystyrene cell (10 mm path length); equilibration time, 2 min	72, 157
	SEC	chromatographic	SEC is the appropriate technique for the evaluation of sample purity. The appearance of the additional fractions might indicate the tendency of LF to self-associate or the ability to form complexes with other milk proteins.	SEC-HPLC system (Agilent Technologies, USA); detector, diode array detector; column, TSKgel G3000SW _{XL} (7.8×300 mm, 5 μm , Tosoh Biosciences LLC, USA); flow rate, 0.5 mL/min; temperature, 22 °C; elution type, isocratic	72

Table 2. continued

protein characteris- tic	instrumental techni- que	classification of the instrumental tech- nique	specific details	applied conditions for LF detection	ref
glycosylation pat- terns	TEM	microscopic	TEM determines the morphologies of protein aggregates (amyloid fibrils, amorphous aggregates). It provides the insight into the mechanism of protein aggregation.	isocratic; mobile phase composition, 30% (v/v) acetonitrile and 0.1% (v/v) JEOL JEM 1010 electron microscope (Tokyo, Japan); accelerating voltage, 80 keV; sample placement, Formvar carbon-coated nickel grid (200 mesh)	64
	MALDI-TOF/MS	spectrometric	The protein sequence analysis as well as its structural modifications might be determined based on the characteristic peptide mass fingerprint (PMF) signal list obtained by the appropriate software (Mascot, Sequest). MALDI tends to generate singly charged ions and better tolerates salts and other impurities. The amount of <i>N</i> -glycans was explored by deglycosylation.	MALDI-TOF/TOF MS spectrometer (Bruker Daltonics GmbH, Germany); laser, nitrogen (337 nm); matrix solution, 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL) in 50:50 (v/v) mixture of acetonitrile and water for glycopeptide analysis; mode, reflector positive ion (neutral <i>N</i> -glycans), linear positive ion (glycopeptide), linear negative ion (isolated <i>N</i> -glycans)	158
	LC-ESI-MS	chromatography coupled with mass spectrometry	The liquid samples subjected to the ESI process aimed to generate gas phase ions typically undergo ionization under high voltage in the metal capillary which is followed by solvent evaporation. ESI favors the formation of large hydrophobic peptides with charge states ranging from +2 to +4, providing more detailed fragmentation patterns. A salted sample might provide evident signal suppression. ESI-MS characterizes by exceptional sensitivity to slight impurities, for example salts, that is expressed by a higher tendency of formation of charged adducts which might also suppress the signal intensity from analyte.	HPLC system (Datsystem Millennium, HPLC pumps Waters 510, detector Waters 486); column, C18 Vydac (250 × 10 mm, 5 μm); gradient elution, 0.01% TFA (solvent A) and 0.07% TFA in 95% acetonitrile (solvent B); flow rate, 3.5 mL/min; single quadrupole mass spectrometer (Micromass); scanning range (<i>m/z</i>), 300–1600	151
thermal stability	DSC	calorimetric	The significant difference in thermal resistance of LF is in dependence on iron saturation. The different ratio of apo- and holo-LFs is capable of modifying the thermal stability of native protein.	device, differential scanning calorimetry (DSC) STARE System, METTLER TOLEDO, Schwerzenbach, Switzerland; heating range, 25–100 °C; scanning rate, 10 °C/min (nitrogen flow)	119
LF–ligand interactions	ITC	calorimetric	ITC is used for the investigation of the mechanism of interactions (affinity, stoichiometry, thermodynamics) between LF and other molecules, especially metals. Based on the determined thermodynamic parameters, the character of LF–ligand interactions (electrostatic, hydrophobic, hydrogen bonding, van der Waals forces) is evaluated.	VPITC microcalorimeter (MicroCal VP-ITC, Malvern Panalytical, Malvern, U.K.); sample cell, LF (1.425 mL); reference cell, MES buffer (pH 5.5); total number of injections, 58 (5 μL each); injection timing, 10 s; interval, 200 s	157, 159

of the detected glycans might be affected by the type of applied mass spectrometry technique (MALDI-TOF/MS, ESI-MS).

Glycans in proteins are composed of *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), sialic acid (NeuAc), mannose (Man), galactose (Gal), and fucose (Fuc) monosaccharide residues. Monosaccharide units linked in a particular order represent a glycan chain. Generally, the *N*-type of glycosylation is only present in the case of the LF molecule, which implies that glycan chains are covalently attached to the nitrogen atom of the asparagine side chain via GlcNAc.¹³⁶ The glycan core consists of two GlcNAc and three Man residues. Three types of *N*-glycosylation are distinguished, e.g. complex, hybrid, and high mannose. This classification is based on the carbohydrate moieties associated with the glycan core. The heterogeneity of glycan chains is predominantly affected by enzyme expressions in the bovine mammary gland (mainly, glycosyltransferases and glycosidases), lactation period, and availability of protein substrate.^{12,138,139} Complex and high mannose are the dominating types of glycosylation in the cow's mature milk.¹⁴⁰

Glycan content in the C-lobe was higher than that in the N-lobe, including three glycan chains.⁴⁷ Asn368 is linked with one unit of GlcNAc and Asn476 is bounded to one unit of α -1,4-Man and two units of GlcNAc; however, the most glycosylated residue was Asn545, which is connected to four units of α -1,4-mannose and two units of GlcNAc.⁵⁸ Based on the number of glycosylation sites, LF can be classified into LF-a and LF-b. Four completely glycosylated distinct *N*-linked glycosites (Asn233 is located in the *N*-terminal while Asn368, Asn476, and Asn545 are found in the C-terminal) are present in LF-b.¹⁴¹ Despite all mentioned, LF-a has a fifth potential glycosite which is found at the Asn281 position. The LF-a form is more abundant in bovine colostrum than in mature milk (glycosylation levels are 30 and 15%, respectively).¹⁴² Both molecular forms might be separated by use of cation-exchange chromatography with carboxymethyl resin. SDS-PAGE electrophoresis verified a greater size of LF-a (84 kDa) in comparison to LF-b (80 kDa) as a result of additional glycan units at the Asn281 residue.¹⁴³ The structure of the glycan chain linked to Asn281 was found to be heterogeneous involving GlcNAc, GalNAc, Man, Gal, and Fuc moieties.¹⁴² bLF-b was about 10 times less resistant toward trypsin digestion than bLF-a. Thus, the glycan chain at the Asn281 position may actively participate in the stabilization of the protein structure by limiting the availability of basic amino acid residues to enzymatic action.

Despite *N*-glycan attached at the Asn281 position, other chains also may contribute to the stability of the protein's three-dimensional structure. Indeed, the glycan chain at Asn545 is arranged between two domains; thus it can easily participate in interactions with protein amino acids.⁶⁰ It has been observed that this type of "protein–glycan" interactions prevents the release of metal ion at lower values of pH and protect bLF against proteolysis, enhancing the rigidity of the C-lobe conformation. On the other hand, Van Berkel et al. showed no difference in iron-binding capacity between glycosylated and nonglycosylated forms of hLF.¹⁴⁴

The origin of LF is a crucial factor which affects the composition and heterogeneity of glycan chains. It was reported that bLF contained a high amount of sialylated *N*-glycans, while fucose residues occurred predominantly in hLF.¹⁴⁵ The content of sialylated *N*-glycans was higher in bovine milk compared to human and goat milk.⁹³ Sialic acid in

the glycan chain might be responsible for the maintenance of metal in the protein structure. Desialation of human LF by neuraminidase treatment induced the alteration in the iron-binding capacity of bLF; thus the LF saturation level was 3-fold reduced.¹⁴⁶ Interestingly, that resialation provided partial restoring of the initial amount of bounded metal. Besides, sialic acid is known as the only charged saccharide present in LF, which is traditionally linked in the terminal position of complex or hybrid *N*-glycans. Consequently, it can be assumed that these carbohydrate residues are capable of modifying the surface charge in glycosylated proteins. For example, Shimazaki et al. related the difference in pI values of bLF isolated from mature milk (pI 8.94) and colostrum (pI 8.3–8.52 and pI 8.18–8.32) with different contents of sialic acid in the protein.⁹⁹ The phenomenon of charge heterogeneity observed in the pI of colostrum LF also could be associated with some variations in the glycosylation profile. Importantly, the distribution of negative charge in bLF molecules was increased after the sugar release (deglycosylation). Analogically, Barrabés et al. noticed that a higher content of sialic acid has a tendency to shift the protein isoelectric point to lower values.¹⁴⁷

The role of glycosylation in the protein structure is not completely understood until now. The monosaccharide diversity of LFs from different species may contribute to protein biological activity. In particular, this feature can influence the antibacterial potential of bLF. For example, Karav reported that the complete deglycosylation of LF significantly reduced its antimicrobial action against *E. coli* DH5a strains.¹⁴⁸ It was shown that apo-LF-a had higher antibacterial activity compared to apo-LF-b, indicating the importance of additional glycan at Asn281.¹⁴³ Various studies have noticed that sialic acid is capable of indirectly interacting with the outer membrane of Gram-negative bacteria. The highly acidic character of sialic acid ensures an effective chelating of calcium ions from lipopolysaccharides leading to destabilization of the bacterial membrane.¹⁴⁹ On the other hand, it was shown that in the presence of diasialated hLF the adhesion of *Salmonella enterica* significantly increased.¹² Desialated bLF exhibits higher antiviral activity against rotavirus infection than native bLF. Treatment by deglycosylated and desialated bLF showed a more effective inhibition of influenza virus infection, especially at the initial step of viral invasion.¹⁵⁰ It was suggested that desialation facilitates the interaction between anionic cellular membrane and bLF; thus the protein becomes more competitive for host cells binding. Furthermore, sialic acid removal enhances the bLF–virus interactions which is leading to prevention of the binding of viral particle to cellular receptor.¹⁵¹ It was reported that the highly fucosylated *N*-glycan core in human milk positively influences the gut microbiota of infants, reduces the number of pathogen infections, and promotes the growth of beneficial *Bifidobacterium* and *Lactobacillus* strains.¹⁵²

8. MULTI-INSTRUMENTAL APPROACH FOR LF CHARACTERIZATION

The application of a series of instrumental techniques enables the comprehensive characterization of native LF as well as its complexes, including the molecular mass, isoelectric point, metal saturation level, unfolding degree, secondary structure composition, and conformational stability (Table 2). Besides, the combination of the appropriate analytical techniques will provide the information on the most unknown aspect of LF structure—glycosylation. To the most relevant tools com-

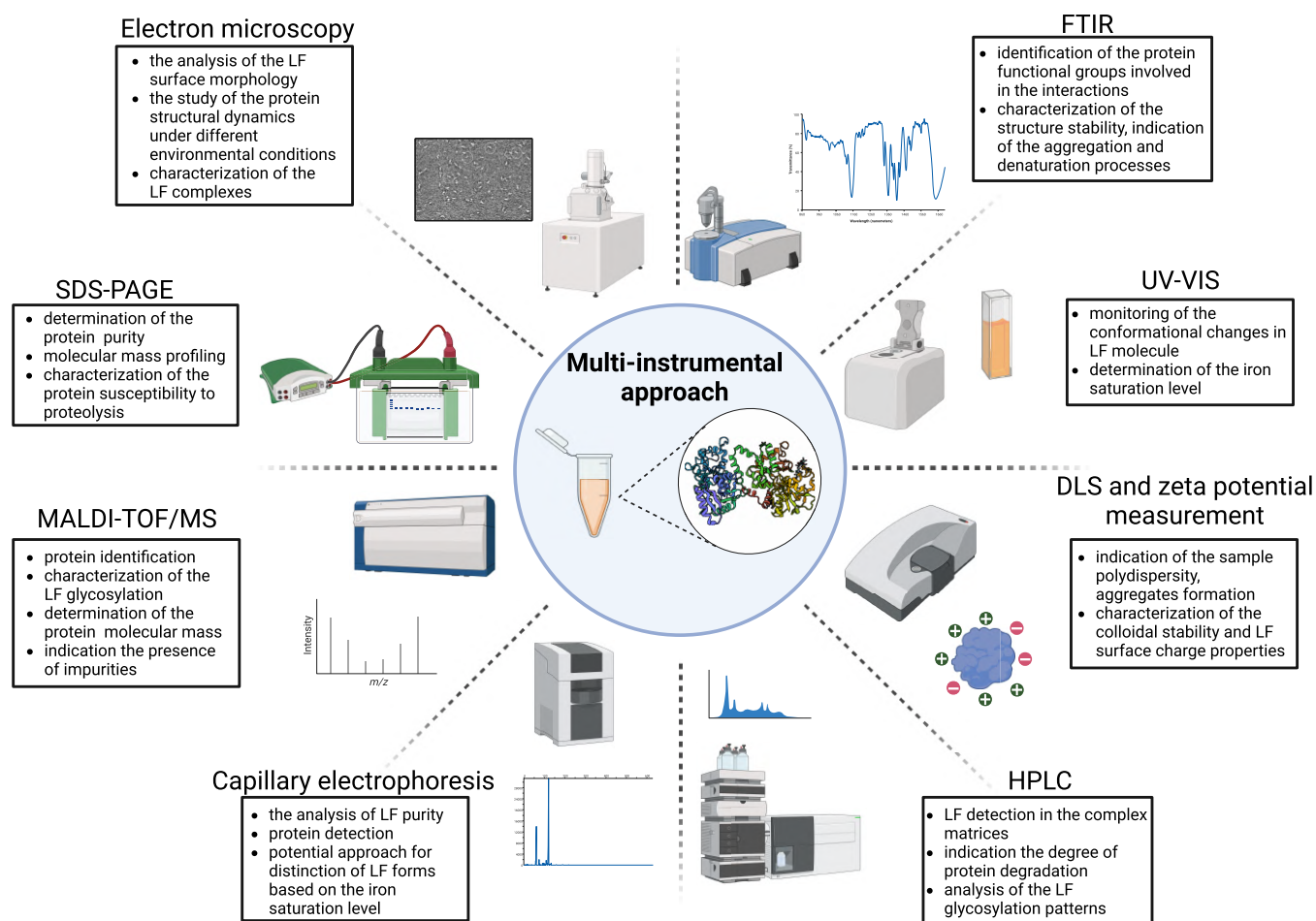


Figure 3. Multi-instrumental approach for studying lactoferrin protein. Created with BioRender.com.

monly used in the context of LF research are included spectroscopic, electromigration, crystallographic, chromatographic, microscopic, calorimetric, and spectrometric techniques (Figure 3). The advanced instrumental studies on lactoferrin are necessary for the establishment of the relationship between protein structure and its functionality. The present section aims to display the specific details of the methods and the possible issues and challenges during LF detection.

8.1. Spectroscopic Techniques. 8.1.1. Ultraviolet–Visible (UV–vis) Spectroscopy. UV–vis spectroscopy is known as one of the most popular methods for studying the iron-binding properties of lactoferrin. The measurements of the absorbance relationship A_{465}/A_{280} can be helpful in the distinction of the degree of iron saturation in bLF.¹⁵³ Generally, the decrease of the absorption intensity ratio indicates the release of ferric ions from the complex. Remarkably, different glycosylation levels of hLF do not contribute significant changes in UV–vis spectra.¹⁶⁰ The analogical mechanism of cation– π interactions induced the rise of the absorption peak in the case of lactoferrin complexes with copper(II), manganese(III), and cobalt(III) ions at the following wavelengths: Cu–LF 438 nm ($\epsilon = 4800 \text{ mol}^{-1} \text{ L cm}^{-1}$), Mn–LF 435 nm ($\epsilon = 9620 \text{ mol}^{-1} \text{ L cm}^{-1}$), and Co–LF 405 nm ($\epsilon = 10\,340 \text{ mol}^{-1} \text{ L cm}^{-1}$).^{161,162} Remarkably, that characteristic LMCT band was not present after the addition of Mn(II) and Co(II) ions, which might be related to the coordination of these metals to other binding sites in the LF molecule, not including deprotonated tyrosine

residues.¹⁶⁰ Lucas studied protein spectral properties in the presence of Li^+ , Na^+ , and Cs^+ ions. The authors reported that the change of the absorption peak position was mainly influenced by hydrogen interactions and proton transfer between metal cations and the tyrosyl group.¹⁶³ Indeed, UV–vis spectroscopy is characterized by the much lower sensitivity of iron quantification in comparison to the ICP-OES and ICP-MS techniques. Bokkhim et al. reported that the absorption peak at 465 nm was not visible during the detection of apo-LF.¹⁶⁴ Despite this, the application of the UV–vis approach is limited in the case of supersaturated LF complexes. According to a basic assumption, holo-LF is capable of binding a maximum of two ferric ions (q_m 1.4 mg/g); however, Pryshchepa et al. found the 54 total iron-binding sites (q_m 36.3 mg/g) in one bLF molecule.¹⁶⁵

8.1.2. Fluorescence Spectroscopy. Fluorescence spectroscopy allows the determination of the stability of LF conformation and indicates unfolding processes. Destabilization effects related to protein interactions with metals might be confirmed by the red shift of fluorescence emission maxima from 330–340 nm to 350–355 nm.¹⁶⁶ The pH-induced exposure of aromatic amino acids is commonly corresponding to the denaturation of the polypeptide chain. For example, the emission maxima of apo- and holo-LFs at pH 2 were detected at 353 and 348 nm, respectively.⁵⁶ Measurements of the fluorescence intensity ratio F_{330}/F_{350} allow indication of the degree of protein unfolding upon different physicochemical conditions.⁷³ For instance, the intrinsic fluorescence might be

helpful for monitoring the conformational alterations in the protein structure during iron chelation. As it was previously reported, the level of metal saturation is another factor which affects the position of the LF emission peak. Thus, Wang et al. noticed the characteristic band of apo-LF at 336 nm, while in the case of holo-LF it was shifted to 343 nm, indicating the exposure of tryptophan to a more polar environment.¹⁵⁶ Similar results were reported previously by Sreedhara et al. that indicated the maximum emissions at 337.07 and 342.96 nm, for apo-LF and holo-LF, respectively.⁵⁶ Remarkably, Moshtaghie et al. did not observe any difference in the position of the emission band (λ_{em} 355 nm) between apo- and holo-LFs; nevertheless, the fluorescence intensity decreased (quenched) to 35% after the addition of metal.¹⁶⁷ The presence of metals has a tendency to reduce the fluorescence (quenching) of the LF molecule. Previously, Harrington et al. related the fluorescence quenching induced by Fe(III), Mn(III), and Co(III) ions to intermolecular energy transfer which corresponds with specific interactions of metal ions with tyrosine residues and conformational changes around LF binding sites.¹³² Ainscough et al. showed that among all of cations, ferric ions were the most effective quenchers, probably because of their the highest binding capacity to LF.¹⁶⁸ The analysis of the protein fluorescence quenching is made with the Stern–Volmer relationship, which is expressed by eq 1:

$$\frac{F_0}{F} = 1 + K_q[Q] \quad (1)$$

where F_0 is the protein fluorescence intensity in the absence of quencher, F is the protein fluorescence after the addition of quencher, K_q is the Stern–Volmer quenching constant [M^{-1}], and $[Q]$ is the molar concentration of the quencher [M].

The mentioned model is capable of delivering the essential information on the binding affinity and stoichiometry of complex formation. The major problem that arises in studying LF conformational changes upon iron saturation is based on the nonlinear behavior of the Stern–Volmer plot.¹⁶⁸ This phenomenon might be explained by the accessibility of fluorophores located in two potential binding sites and their different abilities to interact with metal ions.¹⁶⁹ Therefore, the results obtained by spectrofluorimetry seem to be complicated for interpretation of the mechanism of Fe–LF complex formation. In this case, experimental conditions, e.g. temperature, solvent type, pH, and concentrations of protein and ligand, should be carefully controlled, since all of these factors might affect the plot linearity.

8.1.3. Fourier-Transform Infrared (FTIR) Spectroscopy. FTIR spectroscopy is an essential instrumental tool which delivers necessary information on the stability of protein conformation and ensures the identification of active functional groups involved in protein–metal binding. Positions of characteristic amide bands at the spectral ranges of 1700–1600 cm^{-1} (amide I), 1600–1500 cm^{-1} (amide II), and 1350–1200 cm^{-1} (amide III) allow distinguishing the type of secondary structure (α -helix, β -sheet, turn, loop, random coil, unordered structure) and determining the strength of interchain hydrogen bonding.¹⁷⁰ Asp and Glu residues are commonly found in the ranges of 1430–1380 cm^{-1} and 1600–1550 cm^{-1} (amide II), respectively. The frequency of these peaks might be considerably shifted upon interactions with metal ions.¹⁷¹ The difference $\Delta(\nu_a - \nu_s)$ indicates the form of metal chelation by carboxylate (unidentate, >260 cm^{-1} ; bridging, <200 cm^{-1} ; pseudobridging, <150 cm^{-1} ; bidentate, <105

cm^{-1}).¹⁷² These calculations might be an helpful in verifying the geometry of LF binding sites upon metal binding. The combination of FTIR spectroscopy with thermoanalytical techniques, especially DSC, might provide necessary information on the stability of the LF tertiary structure. Recently it was discovered that absorption peaks at 1690 and 1615 cm^{-1} frequently appear upon protein denaturation, while the band at 1627 cm^{-1} reflects the formation of amyloid aggregates.^{64,116} FTIR spectroscopic analysis study of the protein–ligand molecular interactions might provide the important information on the iron role in the LF conformational stability. For example, FTIR spectrum obtained by Pryshchepa et al. confirmed the contribution of tyrosine residues and deprotonated aspartate and glutamate side chains in the chelation of ferric ions.¹⁶⁵ What is important is that the performed deconvolution analysis by Hadden et al. did not detect any visible difference in the secondary structure content of LF after the removal of iron.¹¹⁶ So it was in later research.¹⁷³ Thus, it is worthwhile to conclude that FTIR spectroscopic analysis might not be a suitable approach for distinction of LF forms at the different saturation states.

8.1.4. Raman Spectroscopy. Raman spectroscopy is a fundamental instrumental technique which is relevant for the study of structural stability and detection of inter- and intramolecular processes within a molecule such as folding, defolding, and aggregation.¹⁷⁴ In addition, it enables monitoring of the conformation changes in the protein structure upon modification with different ligands. Similarly, as in the case of FTIR spectroscopy, Raman spectroscopy might be combined with DSC studies to monitor the heat-induced changes in the secondary structures of apo- and holo-LFs. As a result, Iafisco et al. has pointed to a positive correlation between the intensity of bands assigned to tyrosine at 1520 and 1170 cm^{-1} and the metal saturation level.⁷¹ In contrast, the study on iron-binding proteins (ferritin, transferrin) indicated the appearance of new peaks related to Fe–O and Fe–NO coordination bonds in the range 460–420 cm^{-1} .¹⁷⁵ The direct application of Raman spectroscopy in the study of protein glycosylation might be challenging due to possible signal overlapping that causes some difficulties in spectra interpretation. Previously, it was found that LF deglycosylation is typically accompanied by visible conformational changes and polarity modifications, especially around aromatic amino acids. Consequently, the band shifts might be observed in a few spectral regions: 1610–1600 cm^{-1} (Tyr), 1360–1340 cm^{-1} (Trp), 1280–1210 cm^{-1} (Tyr), 1010–1000 cm^{-1} (Phe), and 880–870 cm^{-1} (Trp). Unfortunately, the glycosylation profiles in bLF were poorly studied with Raman spectroscopy until this time. Ainscough et al. studied the spectral properties of metal-saturated hLF complexes substituted with Fe(III), Cu(II), Mn(III), Co(III), and Cr(III) ions.¹⁶⁸ On the basis of the obtained Raman spectra, the authors assumed similar binding sites for all of the synthesized complexes. This indicated the presence of intensive signals attributed to tyrosine residues in the frequency ranges 1660–1650 cm^{-1} , 1510–1490 cm^{-1} , 1280–1250 cm^{-1} , and 1170–1160 cm^{-1} , which confirmed their strong coordination with metals. One of the meaningful disadvantages of Raman spectroscopy is related to fluorescence interferences that are induced by the operating laser excitation wavelength (mostly 532 nm).¹⁷⁶ As a result, it leads to signal suppression and the lowering of the method sensitivity. Surface enhanced Raman spectroscopy (SERS) is an advanced approach of Raman

spectroscopy in which the analyte excitation is assisted by metal nanostructures (AgNPs, AuNPs). In contrast to traditional Raman spectroscopy, it characterizes by improved sensitivity and ensures the reduction of the fluorescence background. Despite this, SERS analysis might demonstrate a low reproducibility due to size heterogeneity of nanoparticles. In addition, it was revealed that the LF concentration had an impact on the spectral characteristics; thus the application of the SERS approach requires a detailed optimization of the conditions.¹⁷⁷

8.1.5. Dynamic Light Scattering (DLS) and Zeta Potential Measurements. DLS is a relatively simple technique which allows for rapid determination of the hydrodynamic diameter of dispersed biomolecules. The size of LF particles is relatively small and ranges between 2 and 13 nm.⁶³ DLS is considered an ideal technique for monitoring the sample polydispersity, as it is known that LF not only occurs in monomeric form but also might undergo self-association. As it was reported, the formation of LF aggregates was regulated by intramolecular electrostatic interactions, thereby forming a spherical micelle structure. What is interesting is that the direct role of chelated iron in the LF hydrodynamic size has not been extensively studied. According to crystallographic studies apo-LF showed a more open structure, while holo-LF was characterized by a more compact conformation.⁴⁷ Thus, it might be assumed that the protein hydrodynamic size will decrease as the iron saturation increases. Valiño et al.¹²⁹ reported that the diameter of apo-LF varied from 8 to 13 nm in the pH range 4–9. Unfortunately, information on the size changes of the saturated LF form is limited. Jabeen showed that the hydrodynamic diameter of the diferric LF dissolved in Tris buffer at pH 8 was equal to 6.94 nm.⁶⁰ In addition, the determined size of the iron-saturated C-lobe was 3.83 nm (pH 6.5), while the same fragment complexed with zinc ions was about 4.21 nm (pH 3.8). It is worth noting that the values of hydrodynamic size of apo- and holo-LFs could be barely comparable, since the measurements should be repeated at the same conditions. The results might differ in dependence on the pH, the temperature, the protein concentration, the type of used electrolyte, and the ionic strength. Remarkably, Mela et al. showed that the salt-induced aggregation with 100 mM NaCl solution promoted the growth of the LF diameter even to 100 nm.⁶³ Besides, protein functionalization with other ligands frequently leads to modification of the lactoferrin surface and structural rearrangements. The typical increase of LF hydrodynamic diameter might be related to the reduction of conformational rigidity as well as to the formation of additional coating layers.

Interestingly, the way of protein drying also has a valuable impact on the particle size.¹⁰⁷ The measurement of the electrokinetic potential (zeta potential, ζ) is an essential step in the designation of protein surface electrical properties and colloidal stability. Values higher than $\zeta \pm 25$ mV inform about the prevalence of electrostatic repulsions over attraction forces in analyzed dispersions.^{178,179} Pryshchepa et al. showed that the LF charge is highly dependent on sample conditions, and values of ζ gradually changed from +20 to –6 mV over the pH range 4–9.¹⁸⁰ Previous results indicate that the LF surface remains positively charged over the wide pH range. The reduction of the zeta potential to 0 mV occurs around pHs close to the protein isoelectric point. According to the majority of works, it was revealed that the LF isoelectric point is 8–9, which implies the prevalence of basic-character amino acids in the protein sequence. On the contrary, Yoshida reported that

the LF surface charge was less positive and the pI value was found around 4.8–5.3.¹⁸¹ As it was previously mentioned, the pI of bLF is highly dependent on the iron saturation level. Significant conformational modifications induced by iron saturation provide valid changes in the bioavailability of amino acids, especially with charged side chains. According to Bokkhim et al., the LF desaturation accompanies the exposure of negatively charged regions; thereby the pI was indicated at about 6.3.¹¹⁹ In contrast, the ionizable protein groups tend to be bulked inside the compact structure of holo-LF; thus the determined pI increased to 8.6. Besides, the negatively charged surface might be neutralized as a result of interactions with ferric ions. The measurement of the zeta potential is widely used for characterization of the colloidal stability and surface charge properties of LF, but this approach is not relevant for the quantitative analysis of the iron content. As in the DLS studies, the obtained zeta potential results are strongly affected by the measurement conditions. Functionalization of proteins usually accompanies evident changes of their physicochemical features. Immobilized metal ions tend to electrostatically interact with protein functional groups (in particular, with Asp and Glu) inducing neutralization of the surface charge or inversion to more positive.¹⁸² Besides, the study of AgNPs coated by bLF at pH 7 showed that the protein has a tendency to reduce the negative surface charge of metal nanoparticles from –28 to –6 mV.¹⁸³ Destabilization of AgNPs was predominantly induced by their interactions with basic side chains of bLF. The opposite effect was observed in LF complexes with hyaluronic acid (HA).¹⁸⁴ As a result, the zeta potential of pure LF has changed from +9.6 to –30 mV in LF–HA systems.

8.2. Electromigration Techniques. **8.2.1. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE analysis has been widely used for the verification of molecular profiles in biomolecules. The key principle of the separation relies on the different mobilities of charged particles under an electric field that appear as a result of the difference of their molecular sizes (2):

$$\mu_e = \frac{q}{6\pi\eta r} \quad (2)$$

where μ_e is the particle electrophoretic mobility [$\text{m}^2 \text{s}^{-1} \text{V}^{-1}$]; q is the particle charge [C]; η is the dynamic viscosity of the medium [$\text{Pa}\cdot\text{s}$]; r is the size of the particle [m].

A single bLF band is traditionally expected in the range 78–80 kDa; nevertheless, small portions of high molecular weight aggregates also might be present. What is important is to select the proper LF concentration since the small protein amount might undetectable on the electropherogram. Gel permeation chromatography (GPC) might be alternatively utilized instead of SDS-PAGE. In this approach, the molecules are separated based on their hydrodynamic sizes. The basic principle of GPC fractionation assumes that the larger molecules are eluted first from the column, while the smaller components tend to interact with column porous beads; thus they are eluted last. GPC is considered a relevant technique for the detection of high molecular weight (HMW) LF associates. As it was mentioned previously, LF tends to interact with other whey proteins and also could undergo self-aggregation. LF tetramers belong to one of the most abundant multimeric fractions whose bands might be observed at about 300–350 kDa.¹⁸⁵ Wang et al. indicated several LF fractions at about 150, 250, 300, and 800 kDa as a result of interactions with other milk

components, e.g. caseins, immunoglobulins, serum albumin, and lysozyme.¹⁴ The LF size might be affected by various factors, e.g. degree of glycosylation, source of isolation, way of storage, and type of bounded ligand. It has been reported that the molecular weight of unsaturated hLF after the complete removal of carbohydrate chains decreased from 80 to 75 kDa.¹⁸⁶ Generally, it is expected that the molecular weight of LF is not strongly affected by the degree of metal saturation. Nevertheless, Pryshchepa et al. observed a slight difference in mobility between native bLF and supersaturated Fe–bLF.¹⁸⁷ Nagasako et al. explained this phenomenon by the presence of additional metal binding sites which contribute to the modification of the protein surface charge.⁵² Ying et al. confirmed the difference in electrophoretic mobility between both saturated and unsaturated forms of LF.¹⁸⁸

Iron saturation is accompanied by visible conformational changes in the LF structure; however, the authors pointed the impossibility of the distinction of mono- and diferric LF forms by SDS-PAGE analysis. In addition, it was found that iron chelation first occurs in the N-lobe and then in the C-lobe. SDS-PAGE analysis is widely applied during enzymatic digestion in order to estimate the susceptibility of protein structure under different proteolytic conditions. Sharma et al. determined that the size of the bLF C-lobe generated by hydrolysis with proteinase K was slightly bigger than the N-fragment, as their bands appeared at 38.6 and 38.4 kDa, respectively.⁵⁸ Rastogi et al. also did not notice any significant difference in the size between of both lobes of bLF obtained by tryptic digestion, where each was at about 38 kDa.⁴⁶ The gradual dropping of pH to 2–3 creates more favorable conditions for the acidic hydrolysis of LF and provides cleavage of the polypeptide chain and formation of smaller biologically active protein fragments.¹⁸⁹ On the basis of *in vivo* studies Furlund et al. observed the greatest degradation level in the LF molecule after treatment with gastrointestinal juice (pH 2.5) after 30 min.¹⁹⁰ Besides, the stability of LF might vary depending on the protein origin. According to Ma et al.'s studies, it was detected that bLF exhibited much lower resistance to pepsin digestion in comparison to hLF.¹⁹¹ The difference may be a result of glycosylation heterogeneity in proteins from other species. SDS-PAGE is an essential tool used in LF detection and the examination of its purity in the individual fractions during the isolation process. For example, α -lactalbumin as one of the most abundant milk proteins is commonly observed at 15 kDa on LF electropherograms.⁷² Therefore, the presence of the additional patterns will indicate the low purity grade of the achieved product as a result of inappropriate separation conditions.

8.2.2. Capillary Electrophoresis (CE). Capillary electrophoresis is regarded as a more economically friendly separation method in comparison to chromatographic techniques. Similarly to SDS-PAGE, the molecule separation is based on the difference in electrophoretic mobilities. The major problem that arises in CE analysis is during the protein detection related to interactions between analyte and coated functional groups of the capillary wall, inducing the electroosmotic flow (EOF) problem. As it was shown, the LF detection might be performed in positive voltage (pH 4) and in negative voltage (pH 10) as well. Nevertheless, it was confirmed that the LF analysis at pH 10 provided the improved sensitivity and the peak shape.¹⁹² Accordingly, it could be assumed that the optimal analysis of LF molecules by CE is more favorable at the basic conditions above the determined isoelectric point,

since the protein as well as silanol groups of the capillary wall both remain negatively charged. Recently, Li et al. performed an effective separation of LF by modification of running buffer with the nonionic surfactant polyethylene glycol dodecylether (Brij 35) using acetic acid as the sample buffer.¹⁹³ It was observed that the addition of Brij 35 had a positive effect on the specificity and selectivity of the analysis in the context of LF study. Affinity capillary electrophoresis (ACE) is one of the developed approaches of the traditional CE which is based on the addition of some modifiers to running buffer which characterize a high binding affinity to analyte and aim to improve the separation efficiency. Consequently, the formed protein–ligand complex minimizes the risk of interactions with the capillary wall and reduces EOF. For example, Heegaard et al. showed that no peak of LF was observed by using unmodified sodium phosphate buffer (pH 6–8); however, LF was detectable after the addition of heparin to the buffer solution (pH 8).¹⁹⁴ The identification of apo- and holo-LF forms by CE was not comprehensively investigated until now. Nowak et al. studied the separation of apo- and holo-LFs testing fused-silica and neutral capillaries.¹⁹⁵ The results did not show significant changes in the migration time of both forms; nevertheless, their peak positions were exchanged after the inversion of polarity in the capillary. Thus, it might be assumed that the CE approach is not selective for proteins with different iron saturation degrees since the electrophoretic mobilities of apo- and holo-LFs do not differ significantly between each other.

8.3. Crystallographic Studies. **8.3.1. X-ray Diffraction Analysis (XRD).** Crystallographic analysis provides detailed information about the organization of the protein structure on the atomic level. Moreover, the visualization based on electron density maps is a powerful tool for monitoring conformational changes in complex-organized structures, like metalloproteins. It gives an opportunity to determine the number of potential binding sites, their geometry, localization inside crystallized biomolecule, and coordination distances.¹⁹⁶ Shongwe et al. reported the geometric rearrangements in the N-lobe of hLF upon iron(III) substitution by copper(II) ions.¹⁹⁷ According to the gained results, incorporation of Cu^{2+} ions was accompanied by the formation of a monodentate complex (instead of the observed bidentate carbonate site, as in Fe–LF) with carbonate anions which led to the transition of the coordination geometry from octahedral to square pyramidal. Indeed, XRD studies clarify the nature of protein–metal interactions and predict the biological properties of created complexes. Smith et al. mentioned the importance of interactions between ruthenium(III) and hLF in the development of novel antitumor agents.¹⁹⁸ Crystallographic research proved the octahedral geometry of the Ru–LF complex was maintained by two histidine residues and four water molecules.¹⁹⁹

8.4. Chromatographic Techniques. **8.4.1. High-Performance Liquid Chromatography (HPLC).** High-performance liquid chromatography (HPLC) is known as a fundamental analytical tool widely employed for sensitive detection and accurate quantification of biologically active components in the complex matrix. In the case of LF analysis, an extensive pretreatment procedure of milk samples is required. It normally obligates defatting, the precipitation of caseins, and centrifugation.²⁰⁰ It is worth noting that HPLC is relevant for the quantification of intact LF but not protein hydrolysates.²⁰¹ HPLC is applied during the optimization of

protein isolation to determine the method's effectiveness, the denaturation level, and the content of biologically active LF. Parra-Saavedra et al. successfully performed a separation and determination of LF concentration in human milk.²⁰² The authors selected Kinetex XB C18 as a stationary phase and subjected a sample to gradient elution with a mixture of A and B phases as 0.1% (v/v) trifluoroacetic acid in water and acetonitrile, respectively. On the other hand, Zhang et al.²⁰³ reported that C4 was more optimal for bLF quantification in contrast to a C18 column. The authors pointed out that the protein separation by a column with a shorter carbon chain (less hydrophobic) improved the overall peak shape, reduced the baseline drift, and provided a better repeatability.²⁰³ Despite the high efficiency and short time of analysis, the separation by C4 columns might induce worse peak resolution in the case of complex samples. Remarkably, a C8 column might be an alternative variant instead of C4 and C18. The LF concentration in the goat milk was quantified using a Poroshell 300SB-C8 column and linear gradient elution with water/acetonitrile/trifluoroacetic acid at the following volume ratios (v/v) of phase A (95:5:0.1) and phase B (5:95:0.1).²⁰⁴ Tsakali et al. applied similar conditions for the determination of bLF content in feta cheese whey utilizing a Zorbax SB 300-C8 column and gradient elution with an acetonitrile, water, and trifluoroacetic acid mixture at the proportions of 50:950:1 v/v (solvent A) and 950:50:1 v/v (solvent B).²⁰⁵ While HPLC is considered a great technique for the analysis of native proteins, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is suitable for overall bLF quantification as well as for the identification of characteristic peptides. The determination of bLF in complex samples is typically performed in multiple reaction monitoring (MRM) mode. The major task during the MRM approach is based on the selection of the appropriate precursor ion and then to optimize the collision energy. For example, Yuan et al. chose the ETTVFENLPEK (230–240) fragment for further investigations due to the highest mass response among other peptides.²⁰⁶ As it was described in an earlier section, the major advantages of the LC-MS/MS approach is related to a capability of monitoring protein post-translational modifications. In addition, LC-MS/MS was also utilized for evaluation of the effect of protease action on the level of bLF degradation. The peptide separation was performed using a C18 column as the stationary phase and a mixture of 70% acetonitrile and 0.1% formic acid (v/v) as the mobile phase. The number of identified peptides during *in vitro* and *in vivo* conditions was variable and depended on a number of factors, e.g. pH, time, and protease composition (gastric and duodenal juices).¹⁹⁰

8.5. Electron Microscopy Techniques. Electron microscopy is considered a powerful tool which is frequently applied in the estimation of the quality of milk products by characterization of their microstructures.²⁰⁷ Imaging techniques also are suitable for an effective monitoring of structural changes in samples under different environmental factors, e.g. temperature, pH, and ionic strength, and the detection of precipitates. Rodzik et al. have noticed apparent changes in the morphology of individual caseins upon their modification with Zn²⁺ ions.²⁰⁸ It has been reported that the formation of protein aggregates was preferred at the highest metal concentration (600 mg/L). Imaging techniques are important in verifying the decomposition heterogeneity of adsorbed metal. In addition, metal immobilization onto the protein surface represents a novel approach of nanocomposite synthesis, which in turn

corresponds with expanded biological activity of biomolecules. Obtained micrographs by Król-Górniak et al. demonstrated that incorporation of zinc ions into the ovalbumin structure was followed by the formation of ZnO nanostructures.²⁰⁹

8.6. Calorimetric Techniques. **8.6.1. Isothermal Titration Calorimetry (ITC).** ITC is a popular nondestructive calorimetric technique widely used for the biophysical study of protein–ligand interactions. Various parameters characterizing the metal binding to the biomolecule, including enthalpic (ΔH) and entropic (ΔS) changes as well as stoichiometry (N), might be determined by ITC analysis. In turn, ΔH and ΔS values will provide the information concerning the nature of binding forces between the protein and metal ions and indicate the spontaneity (3) of protein–ligand complex formation:¹⁸⁴

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

where the following imply, for $\Delta G < 0$, a spontaneous process; for $\Delta G = 0$, binding equilibrium; and, for $\Delta G > 0$, a nonspontaneous process.

Thus, the following relationships are attributed to (1) for $\Delta H > 0$ and $\Delta S > 0$, hydrophobic interactions; (2) for $H < 0$ and $\Delta S < 0$, hydrogen bonding and van der Waals forces; and (3) for $H < 0$ and $\Delta S > 0$, electrostatic interactions.¹⁸³ Greater values of the association constant (K_A) indicate higher stability of the Me–LF complex. Tang et al. reported the highest binding affinity of zinc ions to LF ($K_A = 2.7 \times 10^5$ L/mol) in contrast to the rest of whey proteins.²¹⁰ Accordingly, for calculated thermodynamic parameters ($\Delta H = -100$ kJ/mol; $\Delta S = -250.5$ kJ/mol·K), the prevalence of hydrogen and van der Waals interactions in the Zn–LF binding mechanism can be assumed. In contrast, Bou-Abdallah et al. reported that hTF binding with Fe³⁺, Ti⁴⁺, VO²⁺, and VO₃[−] is a spontaneous process predominantly driven by electrostatic interactions.²¹¹

8.6.2. Differential Scanning Calorimetry (DSC). DSC is a popular calorimetric technique which allows determination of thermostability and monitoring of the phase transitions of LF and LF complexes under heating conditions. Protein denaturation is an endothermic process whose value of enthalpy change (ΔH_d) is correlated with the thermal resistance of the biomolecule.²¹² It has been reported below that iron saturation positively influences LF thermal properties. Thermograms obtained by Bokkhim et al. showed that the chelation of two ferric ions per LF molecule corresponded to a significant shift of the denaturation peak (T_d) from 70–71 °C to 90–92 °C.⁴⁰ Remarkably, DSC might potentially be applied for distinction of LF forms based on their iron saturation states. As it was reported, native alpaca LF exhibited three peaks on the thermogram at 66, 77, and 89 °C that are probably related to apo, monoferric, and diferric forms, respectively.¹⁹ Other authors highlighted that the thermostability of native LFs among the species might vary in dependence of contributions of these LF forms.¹²⁵ In turn, two denaturation peaks observed at 61 and 90 °C for bLF might be explained by the different thermostabilities of the N- and C-lobes and their abilities to release iron.^{119,213} As it was mentioned before, the content of bounded iron in LF is strictly dependent on pH; thus the selected medium conditions also have a considerable effect on the protein stability under heating. The denaturation temperatures of apo- and holo-bLFs dissolved at pH 3 were registered at 36 and 49 °C, respectively.⁵⁶ Nevertheless, they increased to 66 °C (apo-LF) and 90 °C (holo-LF) at pH 7. Importantly, the applied experimental conditions had an impact on the reversibility of

Table 3. Biological Potential of LF and Related LF Complexes

biological activity	LF form	information	ref
antioxidant	apo-LF	relatively high binding affinity of apo-LF to Fe ²⁺ and Cu ²⁺ allows reduction of the prooxidant effect of these ions (Fenton reaction)	217, 218
	holo-LF	activation of the gene expression of antioxidant markers; overexpression of antioxidant enzymes	219
	LF–Se	enhanced activity of antioxidant enzymes (GPx, GR, GST) within cells and tissues with deficiency of selenium; selenium is capable of interacting with glutathione and enables maintaining equilibrium between the oxidant/antioxidant systems	220
anticancer	holo-LF	potential activator of natural killer cells inducing apoptosis cancer cells (MDA-MB-231, MCF-7); modulation and decrease the expression of inhibitors of apoptotic proteins (surviving); overexpression of Bcl-2 pro-apoptotic proteins (Bax, Bak), mediated in the mitochondrial pathway of apoptosis	35, 221
	CGA–LF	LF complex with chlorogenic acid (CGA) inhibited the proliferation of human colon cancer cells (SW480) in a dose dependent manner (the most optimal mixture consists of 100 μM CGA and 200 μM LF); treatment of cancer cells with CGA–LF complexes promote their apoptosis	222
	LF–OA	treatment of cancer cells (HepG2, HT29, MCF) with lactoferrin–oleic acid complexes (LF–OA) induced the activation of mitochondria apoptosis pathway, which corresponded with expression of caspase-3 and pro-apoptotic Bax protein; overexpression of p-JKN regulator confirmed the possibility of death receptor-mediated apoptosis pathway	223
antibacterial	apo-LF	limitation of the availability of iron to microorganisms resulting in direct binding of this element in the protein structure contributing to host defense against pathogens; antibacterial activity of LF against Gram-positive bacteria (<i>S. epidermidis</i> , <i>B. cereus</i>) was greater than against Gram-negative (<i>C. jejuni</i> , <i>Salmonella</i>), which suggests their higher sensitivity to iron deficiency; direct interactions of LF with components of microbial cells with lead to increase the permeability of their membranes resulting in destabilization of structure; prevalence of basic amino acid in LF sequence provides its high binding affinity to negatively charged lipopolysaccharides of bacteria membranes; interaction of LF with LPS caused the inhibition of growth of Gram-negative bacteria resulting in damage to cell; antibacterial activity of LF can be weakened in presence of some cations (Mg ²⁺ and Ca ²⁺) and anions (HEPES, phosphate, and citrate), which indicates the importance of electrostatic interactions during binding of LF to bacteria surface; citrate is known as a strong chelator for ferric ions which will compete with LF for metal binding and consequently will modify the protein antimicrobial properties; higher antibacterial activity LF peptides (lactoferricin) in comparison to native protein might be related to less branched structure which facilitates its interaction with bacteria cell	7, 224–228
	holo-LF	antibacterial effect of LF (0.1–2.0 mg/mL) against <i>P. aeruginosa</i> was based on the destructive of biofilm around its cell surface; addition of FeCl ₃ significantly decreased antibiofilm effect of LF, suggesting that free ferric ions may participate in the process of biofilm formation	229
	Ag–LF	Ag–LF complex showed germicidal activity against pathogenic bacteria; inhibition of Gram-positive and Gram-negative bacteria (<i>E. faecalis</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , and <i>S. aureus</i>) growth was caused by the antibiofilm activity of the complex	230, 231
antiviral	bLF	LF is able to interact with viral particles and also competes with them for binding to common receptors, avoiding in that way the adsorption and entering of viruses into the cells; neutralization of HCV resulting in rapid interactions of virus with bLF occurred much faster than entering of HCV into the cells; it was reported that sialic acid as part of LF glycan chain is not involved in these interactions, but it was reported that desialylated bLF exhibited a higher antiviral effect against rotavirus compared to native bLF; enhancing of antirotavirus activity after removing of sialic acid is related to facilitation of binding of LF with virus; incorporated ferric ion could contribute to the antiviral activity because holo-LF exerted more effective inhibition of HCV than apo-LF; different activity could be also caused by conformational alterations that occur in LF structure after metal binding; moreover, LF saturated with such metals as Fe ²⁺ , Mn ²⁺ , and Zn ²⁺ was characterized by higher activity against HIV compared to apo-LF	126, 151, 232, 233
	Zn–LF	antiviral activity against poliovirus of Zn–LF complex was directly correlated with degree of saturation of LF; inhibition of viral replication process was based on the binding of LF–metal complex to cell surface and transport of metal ion across cell membrane that led to interference of virus maturation	234, 235
	holo-LF	Fe–LF exhibits higher anti-HIV activity in T-cell line than other Mn–LF and Zn–LF complexes; as the authors remarked, preincubation of cells with protein–metal complex induced more effective inhibition of HIV infection; Fe–LF may be applied as potential antiviral agent in some diet supplements	233
prebiotic	bLF	prebiotic activity of bovine lactoferrin was compared <i>in vitro</i> as well as in fresh cheese samples; it was indicated that bLF promoted the growth of probiotic bacterial strains (<i>Lactobacillus casei</i>) only <i>in vitro</i> , while their population in fresh cheese samples was not changed probably because of the presence of psychotropic bacteria	236
	holo-LF	iron-saturated form of LF stimulated the growth of LAB strains (<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> , <i>Streptococcus thermophilus</i>) in the yogurt which might be directly related with chelated metal	237
	Mn–LF	prebiotic potential of Mn–LF complexes was examined monitoring the number of <i>Lactobacillus</i> strains (<i>L. plantarum</i> and <i>L. rhamnosus</i>); significant population growth was observed after 24 h of incubation of bacterial culture with Mn–LF; the authors related such effect to manganese uptake which contributes probiotic viability	24
anti-inflammatory	bLF	LF supplementation reduced the activation of the NF-κB signaling pathway which corresponded with suppressed expression of pro-inflammatory cytokines (TNF-α, IL-1β) in uterine tissue	238

LF denaturation. For example, it was proved that LF heating from 5 to 115 °C (at a heating rate of 2 °C/min) provided irreversible changes in the protein structure without any opportunity of conformation recovery upon cooling.⁷¹ Thus, a DSC study might deliver the essential information on the thermal behavior of a protein and its susceptibility to denaturation, which is especially required for the optimization of an isolation method to receive the biologically active protein.

8.7. Spectrometric Techniques. **8.7.1. Inductively Coupled Plasma Mass Spectrometry (ICP-MS).** ICP-MS is a powerful analytical tool widely applied for the comprehensive characterization of protein–ligand complex formation. Generally, it provides an accurate determination of the level of metal saturation in proteins.⁵⁷ Besides, the research group headed by Pomastowski has been studying the sorption mechanism between milk proteins and metals ions for a long time by using the ICP-MS technique. The basic principle of such an approach relies on the quantification of unbounded metal in the filtrate. The obtained results might be sufficiently implemented in modeling protein–metal sorption and kinetic isotherms and selecting the most optimal conditions of complex synthesis. The Freundlich and Langmuir models are known as the simplest adsorption isotherm models which are traditionally utilized in the analysis of binding processes. For example, Pryshchepa et al. have determined that LF saturation with ferric ions occurs mostly according to the Freundlich model ($K_F = 4.956 \text{ mg/g}$, $1/n = 0.454$, $R^2 = 0.9994$), which implies the heterogeneity of the protein surface and the multilayer character of metal sorption.¹⁸⁷ On the other hand, the formation of the Ag–LF complex showed more fitting to the Langmuir model ($K_L = 19.94 \text{ L/mg}$, $g_m = 1.55 \text{ mg/g}$, $R^2 = 0.971$), indicating binding of metal ions as a monolayer on the homogeneous adsorbent.¹³¹

8.7.2. Matrix-Assisted Laser Desorption/Ionization with Time-of-Flight Analyzer Mass Spectrometry (MALDI-TOF/MS). MALDI-TOF/MS represents an innovative instrumental approach for the accurate identification of native proteins as well as their peptides. The determined molecular weight of bLF ranges from 78 to 84 kDa and varies in dependence of the presence of metal and the level of protein glycosylation.^{131,214} Pryshchepa et al. has noticed the increase of molecular mass after bLF modification with silver ions.¹³¹ In addition, MALDI-TOF/MS is a useful tool widely applied for evaluation of the purity grade of bLF by detection of the presence of additional biomolecules.¹³⁰ Thus, it was indicated that preliminary ultrafiltration by membranes with a molecular weight cutoff of 50 kDa allowed for separation of bLF from smaller peptides.¹⁸⁰ MALDI-TOF/MS coupled with SDS-PAGE is an important approach in the determination of bioactive protein fragments resulting in proteolytic digestion. MALDI-TOF/MS analysis performed by Jia et al. sufficiently expressed glycosylation patterns of bLF during a particular lactation period.¹³ According to the obtained spectrometric profile, the authors noted that the transition milk contained the highest total amount of sialic acid in LF compared to colostrum and mature milk. Previous studies have been mentioned sialic acid as an important carbohydrate moiety which might contribute to protein antimicrobial and antiviral activity.^{149,151} These findings might be helpful in verifying the optimal conditions for LF isolation, which will allow obtaining the protein with relevant biological potential.²¹⁵

9. BIOLOGICAL POTENTIAL OF LF MOLECULE

LF is commonly known as a multifunctional biomolecule which has great potential as an antibacterial, antiviral, immunomodulatory, antioxidant, prebiotic, and anticancer agent. The considerable nutraceutical and therapeutic properties might be successfully realized in biomedical applications.^{30,216} In addition, the recent trend of LF functionalization with other molecules (polyphenols, fatty acids, metal ions) might contribute not only to the improvement of the protein primary features but also to the development of novel complexes with a broader functionality range (Table 3).

The development of advanced LF-based formulations accompanies a number of issues which are mainly related to the appropriate delivery of such biologically active preparations. In order to enhance the LF bioavailability to a human organism simultaneously allowing for the maintenance of the required structural and biological features, several key delivery carriers were proposed.²³⁹ For instance, LF conjugates with silver nanoparticles (AgNPs) have shown a great antiviral effect against herpes simplex virus type 2 (HSV-2).¹⁷⁸ The inhibition mechanism might be related to strong interactions between the LF–AgNPs complex and virus, which enabled prevention of the invasion of host cells. In contrast, Abdalla et al. reported that chitosan-stabilized LF–AgNPs composite exhibited a strong antibiofilm activity against pathogenic bacteria (*S. aureus*, *P. aeruginosa*) with no cytotoxic effect on the living cells.²³¹ Besides, Softisan based LF nanoemulsion (W/O/W) revealed a great antimicrobial effect against some Gram-positive bacteria strains (*S. aureus*, *Listeria innocua*) and yeast (*Candida albicans*) and potentially might be used as a natural oral antiseptic rinse.²⁴⁰ Recently, the activity of an alginate-enclosed calcium phosphate iron-saturated bLF formulation was evaluated.²⁴¹ The suggested nanocarriers showed promising results in cancer therapy by suppressing the proliferation of Caco-2 cells. The synthesized LF–AuNP nanoconjugate stabilized by polyethylene glycol (PEG) represented an efficient therapeutic agent against glioblastoma (GBM).²⁴² Additionally, the treatment was assisted with photothermal therapy (PTT) laser irradiation. The proliferation of cancer cells significantly reduced resulting in targeting of LF–PEG–AuNP through LF receptors of GBM tissue.

It is worth remembering that the selection of the appropriate delivery carrier is directly influenced by numerous factors, such as the target site of action and the route of drug administration.

LF has been widely known by strong bactericidal and bacteriostatic properties against numerous pathogenic microorganisms. It is worthwhile to underline that the mechanism of LF antibacterial activity is closely related to its iron saturation level. For instance, the populations of Gram-negative strains of *E. coli* and *Klebsiella pneumoniae* were reduced as the LF form was less saturated with iron.²⁰ Additionally, Dionysius et al. reported that iron-free LF and native LF efficiently inhibited the growth of *E. coli* strains, whereas the bacterial treatment with holo-LF showed no satisfactory result.²⁴³ A similar tendency was observed for *Pseudomonas syringae*, whose growth inhibition was dose dependent and required 0.9, 7.5, and 15 mg/mL apo-LF, native LF, and holo-LF, respectively.²⁴⁴ In contrast, none of the supplemented bLF forms (native LF, apo-LF, holo-LF) exhibited an evident antibacterial effect against Gram-negative *S. aureus* strains.²⁰ Remarkably, Lu et al. pointed out that both the apo and holo forms were able to inhibit the viability of *Streptococcus agalactiae* species,

indicating the possibility of LF to act through iron-dependent and iron-independent mechanisms.²⁴⁵

Iron is commonly known an essential element required in the formation of bacteria membranes and regulation of their growth. Generally, two mechanisms of iron acquisition by microorganisms are recognized. The first one relies on siderophore secretion by microorganisms which are highly susceptible to iron chelating and following the transport of such a complex across the outer membrane into the cell. For example, *E. coli* bacteria tend to produce aerobactin-type siderophores which are characterized by high binding affinity to iron.²⁴³ The other proposed mechanism concerns the interactions between microorganism receptors and iron-saturated proteins from the transferrin family, e.g. TF and LF.^{246,247} The iron-dependent (bacteriostatic) antimicrobial mechanism is mainly based on the high iron-chelation potential of LF and the limitation of the availability of that element; thus it provides inhibition of bacteria growth.²⁴⁸ The protein affinity to ferric ions tends to decrease as the saturation level of LF increases; thus the described mechanism is considered inefficient in the case of holo-LF. Besides, the bacteriostatic activity of LF might be interrupted after high-temperature treatment (over 75 °C), leading to protein destruction and the loss of the ability to bind iron.¹⁶

Another bactericidal mechanism is caused by direct interactions between the LF molecule and the microorganism, which in consequence leads to bacteria death.²⁴⁹ Ellison et al. revealed a high binding affinity of a positively charged LF molecule to the outer membrane components of bacteria, in particular, negatively charged lipopolysaccharides (LPS) found in Gram-negative bacteria.²⁵⁰ As usual, such interactions have a destabilization character, providing release of the LPS and following destruction of the cell membrane. Besides, Duarte et al. pointed that such binding might be disrupted in the presence of high concentrations of divalent cations (Fe^{2+} , Cu^{2+} , Zn^{2+} , Mn^{2+}) which will compete with LF for potential binding sites on the surface of bacteria cells.²⁵¹ Remarkably, LF nanoparticles produced by complexation with gellan gum showed a higher antimicrobial activity against *S. aureus* strains in comparison to native LF, probably due to stronger electrostatic interactions with LPS.²⁵¹

In recent time, the greatest attention has been paid to peptides derived from LF enzymatic hydrolysis, e.g. lactoferricin B (LFcin B) and lactoferrampin (LFampin). The derived peptides might be characterized by the same or even higher biological potential in comparison to native protein. Interestingly, such high antibacterial activities of LF peptides are not influenced by iron sequestering but rather by direct interactions with bacteria membranes (bactericidal) leading to the destabilization of their structures. LFcin B belongs to one of the most known peptides of LF which was identified between 17 and 41 residues in the N-terminal region.⁶⁶ Inhibition of bacteria growth in the presence of LFcin B was considered even more effective than with untreated LF. Moreover, Dionysius et al. reported that peptide 1 (pep1) located in the same region as LFcin B with one more additional amino acid (17–42) showed 3 times higher antimicrobial properties toward *E. coli* strains compared to native protein.²⁵² Another active fragment of lactoferrin, known as lactoferrampin (LFampin), might be obtained as a product of LF hydrolysis.²⁵³ LFampin belongs to the cationic amphipathic peptides (268–284) located in the N_1 -domain of bLF. The peptide structure is divided into two major regions:

the hydrophobic N-amphipathic helix and the positively charged C-terminal cluster. The hydrophilic fragment is a key element of the hydrolysate since it initiates an antimicrobial mechanism against Gram-positive and Gram-negative pathogens. The neutralization of LFampin's net charge tends to reduce its permeability through the bacteria membrane, which corresponds with the immediate loss of biological activity.²⁵⁴ Van Der Kraan et al. showed a great bactericidal effect of LFampin against series of bacteria strains, e.g. *E. coli*, *P. aeruginosa*, and *Bacillus subtilis*.²⁵⁵ Importantly, that analyzed hydrolysate was characterized by higher antibacterial activity in comparison with that of native protein. The authors also related a strong bactericidal effect of these peptides to the electrostatic character of binding with bacteria membrane. The described investigations prove a promising perspective of LF as an efficient antibacterial agent for biomedical applications.

Previous works indicated strong antiviral properties of LF against several dangerous microorganisms. The fundamental mechanism is based on the specific interactions of LF with host cell receptors as well as virus particles, preventing entry of the last one into the cytoplasm environment.²³² Superti et al. reported the highest effect of bLF on rotavirus inhibition during the initial step of infection (adhesion).¹⁵¹ On the other hand, Ikeda et al. did not observe any activity of bLF against the hepatitis C virus (HCV) after the absorption of the virus into the cell.¹²⁶ The type of chelated metal as well as saturation level may contribute to the protein antiviral potential. Indeed, Puddu et al. revealed the greater efficacy in the case of treatment with Me–LF complexes, which might be related to their stronger interactions with LF receptors located on host cells.²³³ The presence of Fe^{3+} ions led to much higher LF antiviral activity in comparison to Mn^{2+} and Zn^{2+} ions. Superti et al. also had noticed a pH-dependent character of binding between bLF and the influenza virus.²⁵⁶ The authors revealed that bLF treatment against a viral infection was the most effective at acidic conditions (pH 4–5). In the last years, searching for potential drug candidates against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was extremely topical. LF, widely known as a strong immunomodulator, was not beyond the testing. Conducted studies have showed the ambiguous effect of the protein in the treatment and prevention of COVID-19. The proposed antiviral mechanism of LF against SARS-CoV-2 was comparable to those against other viral infections. It was assumed that LF mediates in the binding with negatively charged molecules of heparan sulfate proteoglycans (HSPGs) found on the cell surfaces of host cells and thereby prevents the viral entry.²⁵⁷ Despite the inhibition of the viral replication, LF significantly reduced the level of pro-inflammatory cytokines, such as IL-1 β and IL-6.²⁵⁸ On the contrary, *in vivo* results were not satisfactory enough. According to a recent clinical trial, bLF supplementation (600 mg/day) did not ensure the total protection of the care personnel of a hospital against SARS-CoV-2 infection.²⁵⁹ SARS-CoV-2 symptoms were confirmed in 11 out of 104 participants (10.6%). Nor was any evident effect observed during a 30 day treatment of SARS-CoV-2 infected patients with a daily dose of 800 mg of bLF.²⁶⁰ Thus, the practical application of bLF against COVID-19 requires more clinical testing, taking into account the individual characteristics of each patient.

LF's antioxidant potential is questionable according to controversial results obtained from various research. It has

been reported that the free-radical scavenging properties of LF are highly affected by the Fe^{3+} saturation level. Belizi et al. pointed out that holo-LF exhibited 15.4% lower antioxidant activity in comparison to apo-LF.²⁶¹ It is widely known that ferrous ions (Fe^{2+}) in aqueous medium are capable of inducing the formation of ROS, as a result of the Fenton reaction (4).



Excessive production of ROS leads to a prooxidative/antioxidative imbalance in the organism followed by oxidative stress. Miller et al. have explained the importance of iron coordination by strong ligands, e.g. EDTA and DTPA.²⁶² In our case, apo-LF acts as a chelating agent forming a stable Fe–LF complex. Recently, it has been revealed that interactions of apo-LF with red blood cell receptors led to the reduced production of ROS inside the cell in comparison to holo-LF.²⁶³ The authors assumed the great influence of the free binding sites of apo-LF in prevention of the Fenton reaction, while the completely saturated bLF was not able to chelate free ferric ions. In addition, antioxidative properties of hLF are also determined by overexpression of antioxidative enzymes (SOD1, GPX, PRDX), which are known as the first line of host defense against oxidative stress in the initial stages.²¹⁹ bLF supplementation allows prevention of lipid peroxidation developed by chronic hepatitis C.²⁶⁴ The level of 8-isoprostane, which is considered a biomarker of oxidative stress, was reduced after bLF treatment, and its concentration in plasma was not correlated with iron metabolism. Condurache et al. pointed out that LF enzymatic digestion led to the release of bioactive peptides with greater antioxidant activities in comparison to native protein.²⁶⁵ The antioxidant potential of the obtained hydrolysates was positively correlated with the time of proteolysis. On the contrary, ROS generation inside macrophages promotes phagocytosis activation and inhibits the growth of parasites.²⁶³ In particular, holo-LF isolated from human neutrophils enhanced the production of highly reactive hydroxyl radicals. A similar mechanism was observed in the presence of iron-saturated transferrin. On the other hand, the amount of hydroxyl radical derived from another family of iron-saturated proteins, e.g. ferritin and desferrioxamine, was insignificant.²⁶⁶ Such an effect is probably induced by the high sequence similarity between TF and LF. Indeed, unsaturated forms of TF and LF are capable of specifically interacting with ferrous ions (Fe^{2+}) by generation of some ROS, including hydroxyl radicals ($\text{OH}^\bullet$) and hydrogen peroxide (H_2O_2).²⁶⁷ Importantly, bLF exhibited a damaging effect in leukemia cell lines by the enhanced production of ROS and activation of caspase mediators in the mitochondrial pathway of apoptosis.²⁶⁸

Metal-based complexes of LF have great potential as nutritional additives, especially in dairy products. A number of studies have confirmed a great antibacterial effect of LF against some pathogenic microorganisms (*E. coli*, *K. pneumoniae*, *P. syringae*).²⁰ Indeed, it has been reported that the addition of LF stimulates the growth of beneficial bacterial strains—probiotics—such as *Lactobacillus* and *Bifidobacterium* (*L. plantarum*, *L. paracasei*, *L. rhamnosus*, *B. longum*, *B. bifidum*, *B. infantis*, *B. breve*).^{20,269} Fermented dairy products (kefir, yogurt) represent a rich source of probiotics whose regular consumption improves gut health. Importantly, the prebiotic activity of bLF might be highly affected by the source of isolation. bLF isolated from bovine milk showed a high growth promotion activity which was positively correlated with the

protein concentration. On the contrary, the prebiotic properties of colostrum bLF were irrelevant.²⁷⁰ Glycosylation heterogeneity is one of the key factors which might entail the difference in prebiotic activity of bLF from milk and colostrum. Remarkably, N-glycans enzymatically released from whey proteins provided more significant growth of *B. infantis* in comparison to native glycosylated biomolecules.²⁷¹ Thus, free glycans represent a more available carbon source for bacteria growth and provide for their much higher probiotic activity than carbohydrates linked to macromolecules. Interestingly, iron accessibility does not cause any significant difference in the growth promotion of *Bifidobacterium* strains.²⁷⁰ The combination of probiotics and lactoferrin represents a novel strategy of food supplements which promotes a healthier gut environment and decreases the risk of the gastrointestinal infections. For example, Chen et al. confirmed a synergistic effect of bLF and its hydrolysates together with probiotic bacteria strains (*L. fermentum*, *B. longum*, *B. lactis*) against foodborne pathogens (*E. coli*, *Salmonella typhi*, *Salmonella typhimurium*).²⁷² The authors underlined that the appropriate combination of an LF form and a probiotic is required to avoid the growth inhibition of the beneficial bacteria. Similar studies proved that the supplementation of apo-LF with *L. fermentum* was the most optimal against methicillin-resistant *Staphylococcus aureus* (MSRA) infection.²⁷³ Besides, the recent studies reported the ability of an LF–probiotic–oligosaccharide mixture to modulate the expression of cytokines; thus such preparations might be efficient for the treatment of the abundant neonates' intestinal disease, such as necrotizing enterocolitis (NEC).²⁷⁴ Similarly, the clinical research confirmed that bLF supplementation in combination with probiotics showed a beneficial effect in the prevention of invasive fungal infections (IFI) in newborns.²⁷⁵ Thus, based on the promising results, the combination of LF and a probiotic might represent a highly perspective approach for the application in infant formula.

Another significantly important feature of bLF is enzymatic activity. It was noticed that LF can act in the same way as peroxidase, amylase, phosphatase, protease, and ATPase; however, its activity was relatively lower compared to those of standard enzymes and was highly influenced by the breed of cow.²⁷⁶ The authors related the difference in enzymatic activity to the glycosylation level of bLF. Glycan chains could be potential active centers which participate in catalysis. Diversity of glycosylation in the cow's milk may specifically modify the enzymatic activity of bLF.

bLF effectively acts as an anti-inflammatory agent, which is capable of interacting with immune cells and signaling molecules and controlling the expression of pro-inflammatory and anti-inflammatory genes. NF- κ B is one of the major inflammation biomarkers, which stimulates the secretion of pro-inflammatory cytokines.²⁷⁷ LPS are the driving molecules which participate in the activation of the NF- κ B pathway, immediately after bacterial infection. Accordingly to the bactericidal mechanism, LF tends to interact strongly with LPS of Gram-negative bacteria, in turn preventing the activation of the NF- κ B pathway.⁷⁹ The anti-inflammatory potential of bLF is mainly contributed by its capacity to bind with macrophage receptors providing changes in plasma cytokine expression. The level of pro-inflammatory cytokines (interleukin-6, IL-6; interleukin-1 β , IL-1 β ; tumor necrosis factor α , TNF- α) was surpassed after bLF treatment, while the expression of anti-inflammatory cytokine (IL-10) increased.²⁷⁸

The nonsaturated form of LF revealed a higher inhibitory effect against pro-inflammatory cytokines.²⁴³ LF interaction with epithelial cells modulates the expression of pro-inflammatory cytokines.²⁷⁹ The concentration of IL-8 was significantly reduced resulting in treatment of infected cells by *E. coli* with native-bLF and holo-bLF.

bLF plays a significant role in stimulation of the human immune system, particularly by interactions with immune system cells. LPS are known as characteristic outer membrane components of Gram-negative bacteria which are also capable of binding with LF and activating the innate immune response.²⁸⁰ The high level of LPS leads to overproduction of ROS in neutrophils and cell damage.²⁸¹ LF is able to compete with neutrophils for LPS binding. The complexation of LPS by LF allows inhibition of superoxide radical production in neutrophils and prevents oxidative stress.²⁸² LF is capable of modulating the activity of lymphocytes and regulating the adaptive immune response by the level of expressed antibodies. Moreover, LF participates in the activation of the TLR receptors signaling pathway.²⁶³ TLR receptors are localized in macrophages, fibroblasts, dendritic cells, and epithelial cells.²⁸³ bLF glycosylation has an impact on the nuclear factor κ B (NF- κ B) signaling pathway, which allows modulation of the therapeutic properties of protein. Heterogeneity of N-glycan chains in bLF induces the diverse effect on NF- κ B expression. Cell treatment with the desialated LF form provided a lower release of NF- κ B in TLR receptors compared to sialated bLF. In contrast, demannosylation of bLF corresponded to activation of NF- κ B production.²⁸⁴ It can be assumed that sialic acid acts as a mediator in signal recognition via TLR receptors providing release of NF- κ B, while the interactions between mannose residues and TLR are not favorable. Moreover, bLF supplementation promotes the expression of total (CD3⁺), helper (CD4⁺), and cytotoxic (CD8⁺) T-cell activations which regulate cytokine secretion.²⁸⁵

In recent years, LF has attracted wide attention in the scientific community as a potential anticancer agent. Many previous studies confirmed that LF is capable of inhibiting the growth and metastasis of tumor. In many cases, uncontrolled plasminogen activation becomes the reason for tumor cell invasion.²⁸⁶ The positively charged N-terminal fragment of LF is capable of inhibiting plasminogen binding to the host cell surface. This interaction precludes plasminogen transformation into plasmin by the urokinase-type plasminogen activator.²⁸⁷ bLF treatment led to prominently reduced proliferation of canine mammary tumor cells (CIPp, CHMp).²⁸⁸ The experimental finding confirmed that the antitumor effect of LF was highly affected by the concentration of added protein. Interestingly, LF oligomers also might be sufficient in cancer treatment. It has been reported that high molecular weight LF (HMW-LF) induced a cytotoxic effect against human breast carcinoma and human colorectal adenocarcinoma cell lines.¹⁵ Ebrahim et al. reported that HWM-LF at the concentration of 3200 μ g/mL showed the highest cytotoxic effect against human breast carcinoma (MDA-MB-231) and human colorectal adenocarcinoma (SW480) in comparison to native LF, apo-LF, and holo-LF with the same concentration.¹⁵ The greater antitumor potential of HMW-LF might be caused by enhanced activity of caspase-3 enzyme mediated in the mitochondrial pathway of tumor cell death (apoptosis). On the other hand, the fairly difficult question arises of how to maintain LF biological activity during gastric digestion. In recent times, the milk proteins have been widely studied in

cancer prophylaxis. Sakai et al. showed that enzymatically obtained bLF peptides also showed cytotoxic activity against human oral squamous carcinoma cell line (SAS).²⁸⁹ The efficiency of potential anticancer agents was estimated by monitoring the release of the LDH marker in tumor cells. Generally, LDH overexpression indicates that cancer invaded living cells.²⁹⁰ In addition, a synergetic cytotoxic effect of a whey biomolecule combination of LPO and LF in breast cancer cell lines (MCF-7, MDA) has been proven.²⁹¹ The relationship between the metal saturation level and LF anticancer potential has not been clearly determined so far. For, example, Zhang et al. suggested that Fe-LF addition causes ROS overproduction, facilitating the apoptosis (ferroptosis) of tumor cells.¹⁷³

The biological activity of LF could be influenced by the presence of LF receptors (LFR) in some host cells. LF is capable of interacting with various types of cells. Many studies confirmed that LFR are located on the surface of monocytes, macrophages, lymphocytes, erythrocytes, hepatocytes, enterocytes, dendritic cells, and epithelial cells. It was observed that the activity of LFR in respiratory epithelial cells was enhanced with the exposure of metals (Fe³⁺, VO²⁺), which suggests that LFR could be involved in a detoxication process and reduction of oxidative stress.²⁹² Some pathogens, including different species of the Neisseriaceae and Pasteurellaceae families, contain iron-glycoprotein receptors on their surfaces which directly interact with LF. In turn, iron is taken up by pathogens, stimulating their growth.^{293,294} LbpA and LbpB genes encode the LF receptor in bacterial membrane.²⁹⁵ It has been reported that LF-receptor interactions occur preferentially by binding between the LF N-lobe and the LbpB C-lobe, whether LbpA participates in the metal uptake process.²⁴⁷ On the other hand, the binding affinity of these receptors becomes limited in the medium of iron excess. Interactions of LF with monocyte cells provided a slight decrease of the protein isoelectric point, followed by reducing the affinity for immune receptors.²⁹⁶ Based on this, it was suggested that electrostatic type interactions through positively charged amino acids are favored between LF and monocytes. Interestingly other parameters of LF, like molecular weight and iron-binding affinity, were maintained. Thus, LF interaction with monocytes concerned exclusively the availability of basic amino acids on the protein surface not leading to other modifications in the LF structure.

10. FUTURE PERSPECTIVES

Lately, LF attracts more attention due its multifunctionality. This review described the major features of the LF molecular structure, such as the tendency to iron chelation and glycosylation variations. The development of an LF isolation method is currently focused on finding the universal highly efficient approach that will be compatible for different types of raw materials. The combination of chromatographic and membrane techniques has the potential to become the most optimal approach in LF production due to the high purity level of the final product and the maximum recovery. Importantly, the separation conditions (pH, temperature, ionic strength, pressure, drying method) have a considerable impact on the protein conformational stability; thus they should be strictly controlled to prevent the loss of LF biological activity. The appropriate analytical tools, including spectroscopic, spectrometric, chromatographic, and electromigration techniques, provide a comprehensive analysis of the LF physicochemical

properties (molecular weight, isoelectric point, iron saturation degree, glycosylation patterns). Since LF is naturally found as a mixture of apo-LF and holo-LF, a more advanced instrumental approach is required for the distinction and quantification of each individual form in native protein. Besides, the detailed insights into the MALDI-TOF/MS technique is essential for understanding the relationship between the LF glycosylation heterogeneity and the protein biological activity.

The remarkable health benefits of LF indicate its wide range of potential applications, including in dietary supplements, infant formula, the food industry, and medicine. LF and its related peptides (LFcin, LFampin) are commonly known for their strong antibacterial properties, and they might be used as natural food preservatives.²⁹⁷ Despite a high nutritional value, the products fortified in LF will be characterized by an extended shelf life. LF's binding capacity to numerous essential biomolecules, such as microelements, fatty acids, and polyphenols, indicates its promising delivery properties. Experimental findings showed that the supplementation of holo-LF facilitates the metal absorption by intestinal mucosa and might be efficient in the treatment of iron deficiency diseases.²⁹⁸ The greatest demand for such LF preparations is predicted for groups of people with the poorest iron bioavailability, such as neonates, pregnant women, vegetarians, and older adults. The ability of LF to boost the immune system and stimulate the growth of beneficial bacteria (probiotics), e.g. *Lactobacillus* and *Bifidobacterium*, could be especially topical for infant formula.²⁰ Remarkably, LF in combination with probiotics might act synergistically, protecting the gut microenvironment against pathogens more effectively. Besides, one of the key fields of LF consumption is medical treatment. The high efficiency of LF against a number of bacterial and viral infections has been proven. Additionally, recent *in vitro* studies have introduced the antiviral mechanism of LF against SARS-CoV-2. Remarkably, LF supplementation also has demonstrated promising results in cancer therapy. Nevertheless, the major problem of the delivery of LF to the target site of action is related to the sensitivity of the protein structure, especially in gastrointestinal conditions. In order to prevent LF degradation, various delivery strategies have been developed. The encapsulation of LF by using natural-based materials, e.g. polysaccharides (chitosan, alginate, pectin, gum Arabic, heparin), lipids (phospholipids, cholesterol, soy lecithin), and proteins (caseins, whey proteins, gelatin), has become one of the fast-growing trends used for improving protein bioavailability.^{299,300} Although LF is considered entirely safe for humans, more detailed clinical trials are required before its inclusion in medicinal products.

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ABBREVIATIONS USED

ACE, affinity capillary electrophoresis; AgNPs, silver nanoparticles; AMF, anhydrous milk fat; ATR, attenuated total reflection; AuNPs, gold nanoparticles; bLF, bovine lactoferrin; BOD, biological oxygen demand; bTF, bovine transferrin; CA, carrier ampholyte; CD, circular dichroism; CE, capillary electrophoresis; CGA, chlorogenic acid; cIEF, capillary isoelectric focusing; CM, carboxymethyl; CN, casein; COD, chemical oxygen demand; COVID-19, coronavirus disease 2019; DEAE, diethylaminoethyl; DHB, 2,5-dihydroxybenzoic acid; DLS, dynamic light scattering; DTPA, diethylenetriaminepentaacetic acid; DSC, differential scanning calorimetry; EDA, ethylenediamine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EOF, electroosmotic flow; ESI, electrospray ionization; Fe-NTA, ferric nitrilotriacetate; FTIR, Fourier-transform infrared; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GBM, glioblastoma; GPC, gel permeation chromatography; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; HA, hyaluronic acid; HCV, hepatitis C virus; hLF, human lactoferrin; HMW, high molecular weight; HPLC, high-performance liquid chromatography; HSV, herpes simplex virus; hTF, human transferrin; HTST, high temperature–short time; ICP-MS, inductively coupled plasma mass spectrometry; IEF, isoelectric focusing; IFI, invasive fungal infections; Ig, immunoglobulins; IL-1 β , interleukin 1 β ; ITC, isothermal titration calorimetry; LAB, lactic acid bacteria; LC, liquid chromatography; LDH, lactate dehydrogenase; LF, lactoferrin; LFampin, lactoferrampin; LFcin, lactoferricin; LFR, lactoferrin receptor; LMCT, ligand–metal charge transfer; LPO, lactoperoxidase; LPS, lipopolysaccharides; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; Man, mannose; MD, molecular docking; MES, 2-(N-morpholino)ethanesulfonic acid; MF, microfiltration; MRM, multiple reaction monitoring; NEC, necrotizing enterocolitis; Nd:YAG, neodymium-doped yttrium aluminum garnet; NeuAc, N-acetylneuraminic acid; NF, nanofiltration; NF- κ B, nuclear factor κ B; pI, isoelectric point; PMF, peptide mass fingerprint; OA, oleic acid; PDB, Protein Data Bank; PEG, polyethylene glycol; PRDX, peroxiredoxin; PTT, photothermal therapy; PVA, poly(vinyl alcohol); RO, reverse osmosis; ROS, reactive oxygen species; rTF, rabbit transferrin; SA, serum albumin; SARS-CoV-2, severe acute respiratory

syndrome coronavirus 2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; SERS, surface enhanced Raman spectroscopy; SOD1, superoxide dismutase 1; SP, sulfopropyl; TEM, transmission electron microscopy; TF, transferrin; TFA, trifluoroacetic acid; TFF, tangential flow filtration; TNF- α , tumor necrosis factor- α ; UF, ultrafiltration; UHT, ultrahigh temperature; UV-vis, ultraviolet-visible; WPs, whey proteins; XRD, X-ray diffraction; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

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**[P3] Impact of Ultrafiltration on the Physicochemical Properties
of Bovine Lactoferrin: Insights into Molecular Mass, Surface
Morphology, and Elemental Composition**

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Impact of ultrafiltration on the physicochemical properties of bovine lactoferrin: Insights into molecular mass, surface morphology, and elemental composition

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ABSTRACT

The large-scale isolation of bovine lactoferrin (bLF) typically involves using large amounts of concentrated eluents, which might introduce impurities to the final product. Sometimes, protein preconcentration is required for the greater accuracy of experimental results. In this research, the supplied bLF sample was subjected to additional UF to eliminate possible small impurities, such as salts and peptides of bLF. Beforehand, the basic characterization of native bLF, including surface charge properties and structural sensitivity to various pH conditions, was performed. The study aimed to evaluate the difference in molecular mass, primary structure, surface morphology, and elemental composition of the protein before and after UF. The research was provided by application of spectroscopic, spectrometric, electrophoretic, and microscopic techniques. Evident changes in the surface morphology of bLF were observed after UF, but the molecular masses of both proteins were comparable. According to MALDI-TOF/MS results, UF had a positive effect on bLF sample representation, improving the identification parameters, such as sequence coverage and intensity coverage.

Key words: bovine lactoferrin, ultrafiltration, SDS-PAGE, MALDI-TOF/MS

INTRODUCTION

The modern lifestyle requires finding healing agents that provide the maximum health benefits to the human organism. The growing demand for bovine lactoferrin (bLF) is directly associated with its wide spectrum of biological activity. Bovine lactoferrin (LF) is commonly known as iron-binding glycoprotein, which is characterizing by strong antibacterial, antiviral, anti-in-

flammatory, prebiotic, and antitumor properties. The application of LF-based formulations is becoming a more popular practice in the prevention of numerous diseases, including sepsis, hepatitis C, rotavirus, COVID-19, and colorectal and breast cancer (Pammi and Abrams, 2019; Khalafi et al., 2020; Ramírez-Rico et al., 2022; Sinopoli et al., 2022; Matino et al., 2023). In addition to the potential therapeutic uses, LF is recognized as a highly valuable nutrient. Bovine LF as an iron-binding biomolecule has been widely tested in clinical trials as a potential agent aimed to control iron homeostasis. The large number of LF receptors present in the intestinal epithelial cells might be potentially involved in the iron uptake mechanism (Conesa et al., 2023). Previous studies have shown that the oral administration of bLF showed promising results in treating anemia in pregnant women (Lepanto et al., 2018; Artym et al., 2021). Indeed, infant formula production was considered the key direction of bLF distribution according to the statistics of the LF global market from 2020 (Grand View Research, 2021). It was reported that LF supplementation promotes the growth of beneficial bacteria strains (*Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*) and normalize the gut microbiota health (Petschow et al., 1999). The ability of bLF to sequester the iron and interact with the bacterial cell membranes contribute to its antibacterial mechanisms, thus LF plays an essential role in the prevention of gut infections. Majka et al. (2020) showed a satisfactory effect of LF supplementation against the growth of pathogenic gram-negative bacteria strains, such as *Escherichia coli* and *Klebsiella pneumoniae*. Indeed, Chen et al. (2013) pointed out that LF in combination with particular probiotics (*Lactobacillus fermentum*, *Bifidobacterium longum*, *Bifidobacterium lactis*) acted synergistically against foodborne pathogens, for example, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. Bovine LF is approved as a safe dietary component that might be broadly applied in

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

the commercial products, including food, skin care, and oral care products. The fortification of food with bLF represents a recent trend in the functional food development (Jańczuk et al., 2023). Previously, LF was supplemented to various products, including milk beverages, ice cream, powdered milk, cakes, candies, and cheese products (Cutone et al., 2020). As it was reported, the yogurt enriched in bLF showed the improved nutritional value and sensory parameters and were characterized by an extended storage time (Franco et al., 2010; Jańczuk et al., 2023). As a cosmetic ingredient, LF has gained extreme popularity, especially in Asian countries, for example, Japan and South Korea (Wakabayashi et al., 2006). Because of its evident antioxidant activity and as a result its anti-aging effect the applicability of bLF has expanded into various beauty products, such as creams, lotions, and face-wash gels.

The multifunctionality of bLF is closely related to its specific molecular structure. It is a comparatively big protein (78–84 kDa), composed of 689 AA (Dyrda-Terniuk and Pomastowski, 2023). The 3-dimensional structure of bLF is represented by 2 globular lobes (N-lobe and C-lobe) linked by a 3-turn α -helix (Moore et al., 1997). Previously, the molecular studies indicated 2 constant iron-binding sites in the biomolecule, which are located within the interdomain clefts of both lobes. Based on the percentage of the bound iron, 3 forms of bLF might be distinguished: unsaturated (**apo-LF**), saturated (**holo-LF**), and native LF, representing a mixture of apo- and holo-LF (Bokkhim et al., 2013). Remarkably, the higher metal content provides a more stable protein conformation, which is especially evident under extreme environmental conditions, such as low pH, high salt concentration, and heating (Brisson et al., 2007). The bLF molecule contains a highly basic N-terminal region, which ensures a relatively high isoelectric point of the biomolecule (pI 8–9; Shimazaki et al., 1993). This region is also crucial for the antibacterial function of the protein and its interactions with various cell receptors (Vogel, 2012). Glycosylation is another key feature of LF structure. The N-type of glycosylation, including high-mannose, hybrid, and complex, is characteristic for the bLF molecule. The presence of the specific glycan moieties might play a fundamental role on the bLF molecule in various aspects, including variation in molecular masses and surface charge, proteolytic resistance, iron absorption efficiency, antibacterial, antiviral, and immunomodulatory activities (Moguilevsky et al., 1985; Superti et al., 2001; van Veen et al., 2004; Yu et al., 2011; O’Riordan et al., 2014). To the major factors affecting the N-glycan profile of bLF are the referred lactation period, season, cow’s age, diet, genetics, and physiological state (Barboza et al., 2012). Furthermore, the isolation processing and handling also might cause modifications

in the protein glycosylation, but this aspect has not been comprehensively studied until now.

The most common sources of bLF are colostrum and mature milk, which contain about 1 to 5 and 0.02 to 1 g of protein per liter, respectively (McGrath et al., 2016). For this reason, LF is considered as one of the most challenging proteins to isolate from milk. The purity and recovery of the final product is influenced by several factors, including the quality of the raw material, type of applied isolation methods, processing conditions, and storage conditions (Wakabayashi et al., 2018). Large-scale bLF production is a complicated multistage process performed by coupling of membrane and chromatographic techniques (Lu et al., 2007). Skim milk or cheese whey are mostly used as raw materials (Wakabayashi et al., 2018). The major group of impurities constitute other milk proteins, especially lactoperoxidase (**LPO**), which exhibits a high similarity to LF in molecular mass and isoelectric point parameters. For instance, the step of cation-exchange requires the application of huge amounts of highly concentrated salt buffer (sodium chloride, sodium phosphate). As a result, salts remain in the final product, reducing its purity grade. In this case, additional manipulations in purification are required. Generally, UF is considered the most relevant technique for the removal of smaller molecules, such as salts, peptides, sugars, and nonaqueous solvents. Centrifugal filters are predominantly used for protein concentration on a laboratory scale (Phillips and Signs, 2004). Regenerated cellulose, as the most common membrane material, is characterized by high hydrophobicity and neutral charge surface, thus a nonspecific type of binding between protein and membrane is favored (Valiño et al., 2014).

According to the manufacturers’ specifications, the purity grade of the commercially available bLF is in the range of from 85% to 98%. Currently, the product with the highest purity ($\geq 95\%$) is provided by a few companies: Fonterra (Auckland, New Zealand), Tatua (Morrinsville, New Zealand), Milei GmbH (Leutkirch, Germany), Royal FrieslandCampina (Amersfoort, the Netherlands), and WAKO (Osaka, Japan; Billakanti et al., 2019; Krolitzki et al., 2022; Morel et al., 2022). Several complementary techniques are commonly used for the verification of the protein purity, such as ELISA, HPLC, SDS-PAGE, capillary electrophoresis, and MS (Zhang et al., 2021; Yan et al., 2022). The comprehensive study of bLF purity is crucial for several reasons. First, the purity characterization of bLF determines its safety and the prospect of utilization as a bioactive ingredient in food, cosmetics, and pharmaceuticals (Jańczuk et al., 2023). Second, the detection of impurities provides more detailed information on the physicochemical properties of the biomolecule. For instance, the present contaminants might significantly modify the molecular conformation, iron-binding

capacity, solubility, thermal stability, molecular mass, particle size, and surface charge (Anema, 2021; Xiong et al., 2021). The high tendency of LF to interact with milk proteins (caseins, β -lactoglobulin, serum albumin) leads to formation of heterocomplexes (Lampreave et al., 1990). In turn, Xiong et al. (2021) reported the improved digestibility of bLF at gastric conditions, resulting in formation of disulfide-linked aggregates with β -LG in contrast to the bLF alone. The influence of salinity on LF extends to the stability of the protein itself, as well as to the degradation of glycosylated sugars. Glycosylation, the attachment of sugar molecules to proteins, is crucial for proper protein folding, stability, and function. However, in high-salinity environments, the degradation of these glycosylated sugars can be accelerated, leading to a reduction in protein functionality and stability. This is particularly relevant for LF, given its role in immune regulation, iron homeostasis, and antimicrobial activity (Dyrda-Terniuk and Pomastowski, 2023). Moreover, it is worth noting that although high levels of salinity can be detrimental, salts present in minor amounts might act as stabilizers for proteins in their final formulations (Sikand et al., 2013; Thakral et al., 2021). The underlying mechanism for salt-induced protein stabilization primarily revolves around the concept of preferential hydration of the biomolecule, accompanied by salt exclusion from its surface (Shimizu et al., 2006). Regarding the mentioned mechanism, the improved protein solubility might be explained in the presence of ionic salts, such as NaCl (Yousefi and Abbasi, 2022). Remarkably, Jiménez-Barrios et al. (2022) showed that the presence of salts, such as potassium phosphate, sodium phosphate, potassium citrate, and sodium citrate, induced the changes of pH and zeta potential of the protein solutions. Furthermore, the authors observed a decrease of α -helix content along with an increase of β -sheet content, indicating a relevant effect of added salts on the protein secondary structure. Indeed, the salt addition also might affect the thermal behavior of proteins. For instance, Hebishy et al. (2019) pointed out that the presence of calcium-binding salts (trisodium citrate, tripotassium citrate) enhanced the thermal stability of whey protein isolate dispersions.

Our study was focused on the application of the additional UF step at appropriate conditions for the more thorough desalting of the protein. The salt removal might positively affect the accuracy and reliability of results of bLF analysis. The understanding the impact of present salts on the physicochemical features of bLF will provide the insight into the necessity of conduction of more diligent purification procedures for the improvement of sample representation. Therefore, the changes in surface morphology, electrophoretic mobility, mass profile, and AA sequence of bLF before and after UF were compared. Additionally the overall

characterization of bLF, including surface charge properties (zeta potential, isoelectric point) at 3 different electrolyte concentrations and structural stability over the wide pH range, was performed. In this research, bLF was analyzed by series of microscopic (scanning electron microscopy [ScEM] and energy-dispersive x-ray spectroscopy [EDS]), electrophoretic (SDS-PAGE), spectroscopic (zeta potential measurements), and spectrometric (MALDI-TOF/MS) techniques.

MATERIALS AND METHODS

Materials

Bovine LF was provided by Ferilac Sp. z o. o. (Poznań, Poland). The estimated physicochemical parameters and compositional factors of LF M95 FNE product were the following: total protein in DM ($N \times 6.38$) = 99.6%; LF (% of total protein) = 95.44%; moisture = 4.2%; ash = 0.2%; pH 6.6; iron = 242 mg/kg (equal to 17.3% iron saturation); solubility (2%, 20°C) = pass; scorched particles = Disc A (7.5 mg of scorched particles in the dried milk product); nitrate = 30 mg/kg; and nitrite <0.5 mg/kg. In addition, the protein mineral profile was characterized by using inductively coupled plasma optical emission spectroscopy, thus the determined content of Na, Ca, and Mg in the bLF structure was (mean $\pm$ SD) 4.76 ± 0.33 mg/g, 460.4 ± 29.1 μ g/g, and 484.7 ± 1.7 μ g/g. The commercially available bLF (purity determined by SDS-PAGE $\geq 85\%$) was purchased from Sigma-Aldrich (St. Louis, MO). Trypsin Gold, MS grade, was obtained from Promega (Madison, WI). Reagents, including hydrochloric acid 36.5%–38% (wt/wt), ammonium bicarbonate, trifluoroacetic acid (TFA), acetonitrile (ACN), dithiothreitol, iodoacetamide, α -cyano-4-hydroxycinnamic acid, synaptic acid, were obtained from Sigma-Aldrich (St. Louis, MO). 2-(N-morpholino)ethanesulfonic acid SDS running buffer (20 $\times$), sample reducing buffer, and lithium dodecyl sulfate sample buffer were supplied by Thermo Fisher Scientific (Waltham, MA). Sodium chloride and sodium hydroxide were provided by StanLab (Lublin, Poland) and POCH S. A. (Bydgoszcz, Poland), respectively.

Primary Characterization of bLF Protein by Ferilac

Determination of Isoelectric Point. Particle charge characteristics of bLF protein (Ferilac) in dependence of ionic strength was examined by a batch-type analysis method. First, sodium chloride solutions were prepared at the following concentrations: 0.9%, 0.09%, and 0.009% wt/wt. Subsequently, a series of NaCl solutions at pH 2, 3, 4, 5, 6, 7, 8, 9, and 10 were prepared by gradual addition of 0.01 M NaOH and 0.01 M HCl. The

pH adjustment was controlled by FiveEasy Plus pH meter (Mettler Toledo, Columbus, OH). Bovine LF samples were dissolved under the appropriate pH conditions to gain the final protein concentration of 1 mg/mL. The obtained solutions were sonicated (20 s, 25°C) by using the ultrasonic cleaner (USC THD model with 45 kHz ultrasonic frequency, VWR International, Poland). Zeta potential measurements of bLF dispersions at various pH conditions were performed with a Zetasizer Nano-ZS (Malvern Instruments, UK). Each sample was analyzed in triplicate using DTS1070 cuvettes (Malvern Instruments, UK) at 25°C.

Study of the pH Effect on the Stability of bLF. Bovine LF samples remaining from the previous experimental part (in 0.09% [wt/wt] NaCl solution) were subjected to SDS-PAGE analysis to evaluate the effect of the applied pH conditions on the protein structural stability. To minimize the salt effect on the protein migration, the samples were diluted to 0.5 mg/mL by adding deionized water. Protein samples were loaded on 4%–12% Bis-Tris Plus polyacrylamide gel (Thermo Scientific, Waltham, MA) using Perfect Color Protein Ladder as a marker. The protein separation was carried out for 22 min at 25°C using 2-(N-morpholino)ethanesulfonic acid running buffer (Novex Life Technologies, Carlsbad, CA). The gel staining was carried out by incubation in Coomassie Blue for 1 h. Finally, the gel was destained in deionized water overnight.

Comparison of Salted and Desalted bLF

Protein Filtration. Briefly, the appropriate conditions of bLF UF were selected to avoid protein degradation. Solvent choice is one of the major factors that has a significant effect on the structural stability of biomolecule. The efficiency of bLF purification by UF was examined by testing of 2 types of solvent solutions: ultrapure water (from a Milli-Q water purification system; MilliPore, Burlington, MA) and 0.9% NaCl solution. Prepared bLF suspension (5 mg/mL) was loaded to centrifugal filters (Amicon Utra-4 Centrifugal filters, 50 kDa, Merck, Rahway, NJ). Ultrafiltration was conducted under the following conditions: rotation speed $6,300 \times g$, time 20 min, and temperature 4°C. After the first spin cycle, the retentate was washed with water to remove residual salts. In total, the washing-out process was performed in triplicate under the conditions described here. After each of spinning cycle, the presence of chlorides in filtrate was monitored by addition of silver nitrate (AgNO_3) solution. The obtained protein concentrate was collected and then subjected to freeze-drying to remove solvent.

SDS-PAGE Analysis of bLF. Three different forms of bLF were subjected to analysis by SDS-PAGE: (1) native bLF from Ferilac (before UF), (2) bLF received after UF,

and (3) LF from bovine milk supplied from Sigma as a control sample. The bLF samples were dissolved in water at 3 different concentrations: 0.2, 0.5, and 1.0 mg/mL. Filtrated bLF (dissolved in saline before UF) was analyzed at only 0.5 and 1.0 mg/mL. Protein separation was performed in both, reduced and nonreduced conditions, according to standard procedure described in the “Study of the pH effect on the stability of bLF” section.

Microscopy Analysis of bLF. The impact of UF on the structural changes in bLF was examined by microscopy analysis. Dried protein samples (before and after UF) were placed onto carbon adhesive disks (Leit Tabs) and fixed on ScEM specimen stubs. Morphology and elemental composition of bLF (before and after UF) was investigated by light microscopy (AXIO Zoom.V16, Carl Zeiss Microscopy GmbH, Jena, Germany) and ScEM (EVO 15, Carl Zeiss Microscopy GmbH, Oberkochen, Germany) coupled with an EDS detector (SmartEDX, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Scanning electron microscopy imaging was performed using a high-definition 4 segment backscattered electron detector. All images were taken from the same region to provide more effective comparative analysis.

Characterization of the Intact bLF by MALDI-TOF/MS. In this research the mass profile of bLF was examined by MALDI-TOF/TOF MS (Bruker Daltonik, Bremen, Germany) using a spectrometer equipped with a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser (2 kHz frequency and 355 nm wavelength). The reagents required for the MALDI-TOF/MS analysis were of the highest commercially available purity and were supplied from Sigma-Aldrich (St. Louis, MO). First, bLF samples were dispersed in 0.1% TFA to get a protein concentration of 2 mg/mL. A saturated solution of sinapinic acid dissolved in TA30 (mixture of 0.1% TFA and ACN at 70:30, vol/vol) was used as a matrix solution, and the Protein Calibration Standard II (Bruker Daltonics, Bremen) was selected for calibration. The matrix solution was mixed with the sample solution at 1:1 (vol/vol) in PCR tubes and the obtained mixture (1 μL) was applied on a ground steel target plate (Bruker Daltonics, Bremen) by the dried-droplet method. The MS spectra of intact proteins were recorded in the positive ion linear mode in the m/z range of 25,000 to 250,000 using an accelerating voltage of 25 kV. The raw data were processed by flexControl and flexAnalysis software (Bruker Daltonik).

In-Gel Tryptic Digestion and the Protein Identification. The in-gel tryptic digestion of bLF (before and after UF) was performed according to the protocol described previously (Shevchenko et al., 1996). The process consisted of several steps, including: (1) excision of protein bands from SDS-PAGE gel (obtained as described in the “SDS-PAGE Analysis of bLF” section), (2) washing of gel pieces and removal of the Coomassie Blue Stain with

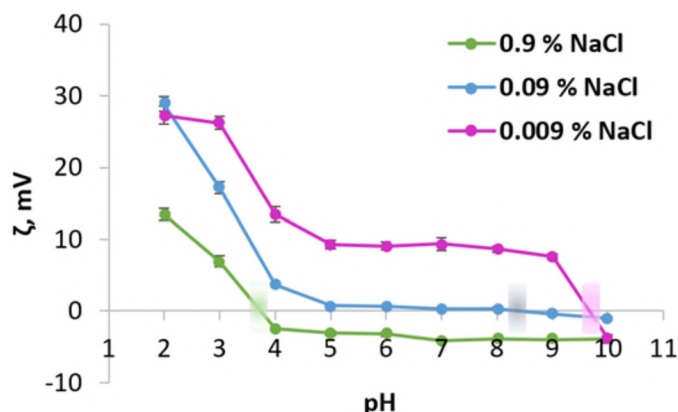


Figure 1. Zeta potential (ζ) of bovine lactoferrin dissolved in NaCl within a pH range of 2 to 10. The shaded areas on the graph indicate the isoelectric point values, whereas the error bars indicate SD.

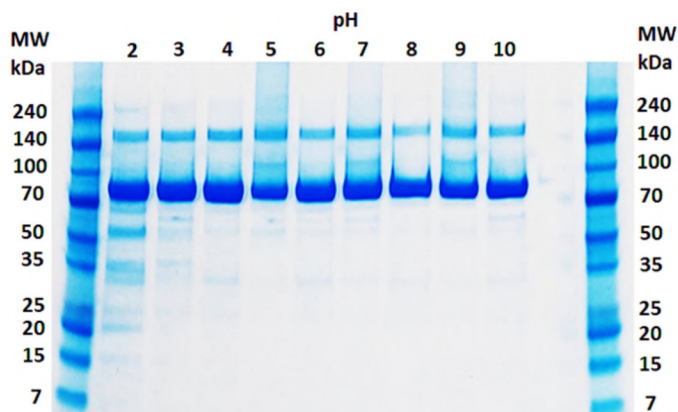


Figure 2. Native bovine LF (0.5 mg/mL) SDS-PAGE electropherogram performed in nonreduced conditions at a pH range of 2 to 10. The obtained electropherogram indicates the sensitivity of bLF structure over a wide pH range. MW = molecular weight.

a mixture of 10 mM ammonium bicarbonate (NH_4HCO_3) and 50% ACN, (3) the reduction of disulfide bridges by 10 mM dithiothreitol in 10 mM NH_4HCO_3 and alkylation of cysteine residues by 55 mM iodoacetamide in 10 mM NH_4HCO_3 ; (4) in-gel digestion with trypsin (40 ng/ μL in 10 mM NH_4HCO_3) and incubation at 37°C overnight (the ratio of substrate to enzyme was 1:50), and (5) extraction of peptides with 50% ACN and 1% TFA. Saturated α -cyano-4-hydroxycinnamic acid in TA30 was used as a matrix solution, and the Peptide Calibration Standard II (Bruker Daltonics, Bremen) was applied as a calibrant. The sample was mixed with matrix solution in a 1:1 (vol/vol) ratio in the PCR tube. The prepared mixture (1 μL) was applied on the ground steel target plate by dry the dried-droplet method. The peptide mass fingerprint (PMF) spectra were monitored in the reflectron positive ion mode within an m/z range of 500 to 3,500. Additionally, the MS/MS analysis was performed in laser ionization fragmentation technology mode to ensure the most accurate protein identification. Cysteine carbamidomethylation and methionine oxidation were selected as global and variable modifications, respectively. Mascot database, along with BioTools, was applied for results interpretation.

RESULTS AND DISCUSSION

Influence of Electrolyte Concentration on the bLF Isoelectric Point

A graph of zeta potential values (ζ) of LF at various pH values in the presence of 3 different NaCl (wt/wt) concentrations: 0.9%, 0.09%, and 0.009% is shown in Figure 1. The isoelectric point of bLF at the mentioned electrolyte concentrations was equal to 3.7, 8.2 and 9.8, respectively. Thus, the more cationic character of bLF

was observed as the salt concentration increased. As shown, the electrokinetic potential of bLF ranged from +13.46 to -3.88 mV in 0.9% NaCl solution. The zeta potential values of the bLF dispersed in 0.09% NaCl sharply decreased from +28.8 to +0.77 mV at pH 2 to 5 and then smoothly reached -1.06 mV at pH 10. The measured ζ values remained relatively constant in the pH range of 5 to 10. As is commonly known, the colloidal dispersions are considered stable because the measured ζ values were beyond the region beginning at -30 mV and ending at +30 mV. Dispersion destabilization typically accompanies the weakening of the repulsion forces between the particles and their increased tendency to aggregate (Mahbubul, 2019). The described effect might be referred to the subsequent results observed during SDS-PAGE analysis and also explains the presence of the protein multimers (Figures 2 and 3). Although based on the SDS-PAGE results, bLF was characterized by the highest structural stability at a pH range of 7 to 9 (Figure 2); however, the observed stability of the colloidal dispersion was especially low under such conditions. Meanwhile, the surface of bLF dissolved in 0.009% NaCl maintained a positive charge (from +27.3 to +7.55 mV) over the wide pH range, and the charge inversion occurs as the pH value of the solution exceeds 9.

The high abundance of basic AA, such as lysine (pKa 10.5), arginine (pKa 12.5), and histidine (pKa 6.1), is characteristic for the N-terminal region of bLF. For instance, the isoelectric points of N-terminal peptides, such as LF 1–11, LF 17–41, and lactoferrampin 268–284 were 11.70, 10.95, and 9.70 respectively (Sinha et al., 2013). The predominance of basic AA determines the cationic character of bLF and as a result its high isoelectric point at low-concentration electrolyte solutions. Oppositely charged ions (counter ions) might be adsorbed on the

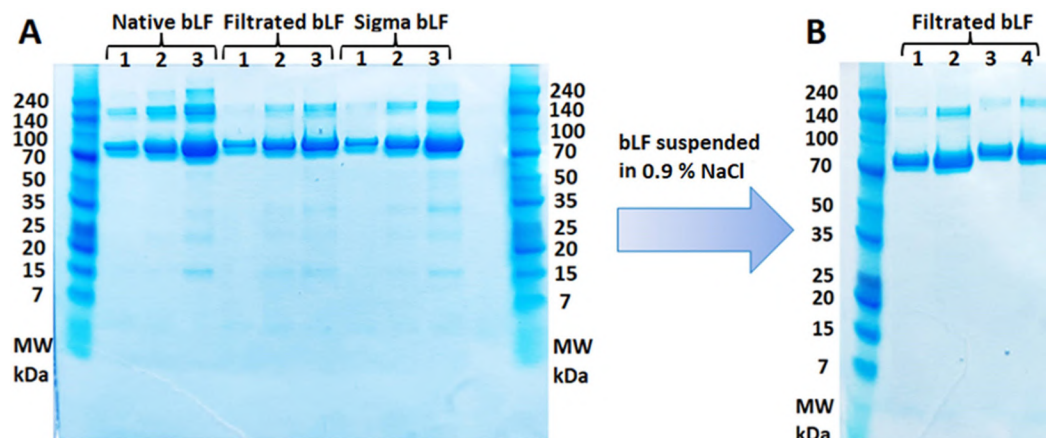


Figure 3. Comparison of SDS-PAGE mass profile of different bLF forms, where filtrated bLF was dissolved (A) in water under nonreduced conditions at concentrations of (1) 0.2 mg/mL, (2) 0.5 mg/mL, and (3) 1.0 mg/mL and (B) in 0.9% NaCl solution under nonreduced conditions at concentrations of (1) 0.5 mg/mL and (2) 1.0 mg/mL, as well as under reduced conditions at concentrations of (3) 0.5 mg/mL, (4) 1.0 mg/mL. The commercial Sigma bLF ($\geq 85\%$ [SDS-PAGE]) was applied as control sample in order to examine the purity grade of analyzed filtrated protein.

protein's surface, thereby forming a Stern layer. In turn, the rest of the unbound ions deriving from electrolyte solution represent the diffusive layer. Measured zeta potential is defined as electrokinetic potential within the electrical double layer (shear plane). The thickness of the electric double layer tends to decrease as the concentration of the electrolyte increases, and as a result a decrease of zeta potential is typically observed. Ionic strength is not the only factor that might influence the protein electrical charge properties. For example, Valiño et al. (2014) pointed out that the type of electrolyte used, as well as the protein concentration, are capable of modifying the isoelectric point of LF. Additionally, Bokkhim et al. (2013) observed a visible drop of pI value from 8.5 to 6.3 as iron desaturation occurs in the LF molecule. The conformational difference between apo- and holo-LF might alter the availability of ionizable AA and thereby the formation of the electric double layer. The difference in pI between both bLF forms plays a significant role, especially during isolation processing. As was previously mentioned, the native bLF represents a mixture of saturated and unsaturated LF. However, the quantification of the apo/holo ratio found in native bLF remains an unresolved task. For instance, Voswinkel et al. (2016) pointed out that apo-LF showed a much lower binding affinity to cation-exchange membrane in contrast to holo-LF under acidic conditions (pH 4.8). Nevertheless, the author related the difference in binding affinity to the protein structure specificity rather than to difference in pI values between apo- and holo-LF. The variations in the protein glycosylation also have a particular effect on the LF surface charge properties. It was proved that sialic acid (pK_a 2.6) tends to decrease the pI of glycoproteins

and the shift was directly affected by the number of the monosaccharide and its position in the biomolecule's structure (Barrabés et al., 2010). Obviously, the type of instrumental technique used has an effect on the pI results, but nevertheless this aspect requires more comprehensive research in the future.

Evaluation of the pH Effect on the Structural Stability of bLF

The bLF samples, previously prepared for pI analysis, were subjected to SDS-PAGE study to explore the susceptibility of the protein structure to degradation under various pH conditions. It is worthwhile to note that the sample dilution to 0.5 mg/mL is essential not only for the optimal bLF visualization but also to enable elimination of the unfavorable effects of high electrolyte concentration on protein mobility and accurate interpretation of results (See et al., 1985). The obtained electropherogram confirmed the presence of the monomeric bLF at approximately 81 to 84 kDa as well protein dimers at 170 to 185 kDa (Figure 2). In some cases, smaller peptides appeared, indicating the sensitivity of bLF to the particular pH conditions. As was expected, bLF undergoes the greatest damage at pH 2. Several fragments deriving from the native bLF were observed at 67, 55, 35, 32, 25, 20, and 15 kDa. In addition, protein degradation was visible at pH 3, 4, 5, 6, and 10. The bLF revealed the highest structural stability at neutral and slightly alkaline conditions of pH 7 to 9. As is widely known, LF structure is characterized by high sensitivity to physicochemical conditions, especially pH. For this reason, the appropriate pH con-

ditions during bLF production should be maintained to preserve its structure and biological role. Remarkably, the selection of raw material plays a crucial role because it determines the quality of the final product. Acid whey belongs to the popular raw materials widely used for LF extraction (Chen et al., 2007; Matijašić et al., 2020). Typically, acid whey is known as a by-product obtained during the manufacturing process of various dairy products, for example, cottage cheese, cream cheese, ricotta, and strained (Greek) yogurt (Chandrapala et al., 2015; Lievore et al., 2015). However, the acid coagulation of milk provides to relatively low pH of whey which is on average 4.2 to 4.6 (Rocha-Mendoza et al., 2021; Zou and Chang, 2022). Therefore, the use of this type of raw material might correspond with the increased level of denaturated protein and the reduction of bLF primary functionality. In contrast, sweet whey and skim milk, characterized by pH ranges of 6.0 to 6.5 and 6.6 to 6.8, respectively, are considered more relevant raw materials for the large-scale isolation of LF because the possible acidic hydrolysis of the bLF molecule is restricted (Mehra et al., 2021; Aydogdu et al., 2023). For instance, Sreedhara et al. (2010b) pointed out the importance of the pH conditions on protein conformational changes, including iron-binding capacity, surface hydrophobicity, denaturation degree, and tendency to form aggregates. Additionally, authors noticed a prominent decrease of the α -helical structure in bLF as the pH in the medium decreased from 5 to 2, which implies a partial disruption in the protein conformation (Sreedhara et al., 2010a). Moreover, our previous experiment clearly showed the pH effect on the macromolecule's charge properties, changing the ionization of the basic and acidic amino acids. The LF digestion in gastric acid (pH 1.5–3.5) is frequently used to produce biologically active peptides, such as lactoferricin and lactoferrampin (Bruni et al., 2016). The microbiological studies proved that the derived fragments exhibit much higher antibacterial activity in contrast to intact bLF. The changes that occur in LF structure under alkaline conditions have been poorly studied until now. However, the recent studies confirmed the tendency of bLF molecule to quickly degrade after treatment with 0.1 M NaOH solution (Oselt et al., 2021). Furthermore, Abe et al. (1991) pointed out that LF heating under neutral and alkaline conditions was accompanied by increased turbidity and the initiation of the gelation process. What is important is that the molecular dynamics simulation study performed by Nhan et al. (2019) showed a higher susceptibility of the apo-LF structure to disruption at extreme basic conditions (pH 14) compared with acidic conditions (pH 1). To summarize, dissolving bLF at a pH between 7 and 9 is considered the most optimal because minimal structural destabilization of the protein is possible at this range.

SDS-PAGE Analysis of bLF

The direct influence of UF conditions, in particular solvent composition, on the efficiency of protein purification was explored by the SDS-PAGE study. As shown in Figure 3A, the molecular mass of 3 analyzed bLF forms did not vary significantly and ranged from 80 to 85 kDa. The band position of the monomeric protein was in good agreement with previous results shown in Figure 2. The presence of large aggregates at approximately 190 to 210 kDa and smaller fragments at 50, 35, 25, and 15 kDa might be an issue of the protein isolation methodology as well as storage conditions. Lactoferrin conformation is known to be highly sensitive to the physicochemical conditions of the prepared solution, for example, pH, ionic strength, and protein concentration. Remarkably, the degradation effect in the protein structure increased after UF of bLF and is especially visible at the concentration of 0.5 mg/mL. In this case, mechanical denaturation should not be excluded. Although UF is considered a nondestructive separation method, it also might subject proteins to mechanical stresses. The key operating factors that might affect the protein stability during UF are temperature, pressure, and time (Miron et al., 2021). For instance, Belmejdoub et al. (2012) proved that LF structure was mechanically sensitive after the application of transmembrane pressure of 200 kPa; however, the damage did not exceed 15%. In addition, a 55%–60% loss of the antibacterial activity of lysozyme was observed after UF using a transmembrane pressure of 1.2 MPa (Miron et al., 2021). As shown in Figure 3B, prior dissolution of bLF in saline sufficiently provided the removal of peptides. It was established that the preliminary dissolution of bLF in the saline ensured the effective purification of bLF from smaller fragments, reducing the clogging of the membrane pores. This phenomenon might be explained by specific protein-salt interactions and the increase of electrostatic repulsions between negatively charged protein particles, which was previously confirmed by zeta potential measurements. As is commonly known, electrolyte concentration plays a crucial role in the stabilization of the protein solution. The present ions tend to interact directly with charged functional groups of the biomolecule, resulting in the evident conformational changes. The mentioned phenomenon is widely described as protein charge screening. Lactoferrin is a biomolecule that is characterized by a highly positive N-terminal region (Baker and Baker, 2012). Therefore, the present chloride ions are potentially capable of reducing the electrostatic repulsion in this area of the molecule, ensuring a more compact protein conformation (Beauchamp and Khajepour, 2012). For example, Millan et al. (2020) showed that the hydrodynamic radius of cytoplasmic protein (CRABP I) gradually decreased as

NaCl concentration rose from 0 to 0.15 M (Millan et al., 2020). The salt-induced protein stabilization also might occur indirectly as a result of electrolyte interaction with the biomolecule's hydration shell. The character of these interactions is mainly influenced by the type of the dissolved ions. Based on the Hofmeister series, electrolytes are basically classified as kosmotropes (promote protein stabilization by enhancing the intermolecular hydrogen bonding in the aqueous solutions) and chaotropes (induce protein denaturation by breaking the water structure; Zhang et al., 2012). Sodium chloride is characterized by weak kosmotropic properties, indicating its capability to mediate in the formation of a more structured and organized hydration shell and to restrict the preferential binding of salt to the protein surface (Moelbert et al., 2004). Thus, it could be assumed that the added sodium chloride contributed to a more compact conformation of bLF, facilitating the purification of the biomolecule. The molecular mass of the bLF filtrated in 0.9% (wt/wt) NaCl solution was at about 80 kDa. Interestingly, a visible increase of protein size to about 88 kDa was observed in the presence of reducing agent. The difference in the protein electrophoretic mobility might be explained by the substantial conformational changes after breaking 17 disulfide bridges, leading to extension of the bLF structure and thereby slowing its migration in the gel (Ray and Ghatak, 2014).

Microscopy Studies

The microscopy analysis revealed the evident changes in surface morphology and between filtrated and nonfiltrated proteins. As shown in light microscopy images, the native bLF was characterized by a crystalline structure; however, the filtrated protein rearranged into lighter amorphous assemblies (Figure 4A and 4B). Scanning electron microscopy results confirmed a smooth homogeneous structure of native bLF, but it became more porous after UF (Figures 4C and 4D).

The EDS spectra indicated the elemental composition of bLF samples in the analyzed area (Figure 5). The content of carbon, nitrogen, and oxygen as major components of protein molecules, exceeded 96% of total mass composition. Minor amounts of sulfur derived from sulfur-rich amino acids, for example, cysteine and methionine, and the iron presence might be explained by the high binding capacity of bLF to ferric ions. In turn, sodium and chlorine might remain in protein as a result of elution during the isolation process. What is interesting is that the amounts of these elements were reduced after UF, indicating the desalting effect. Ultrafiltration is one of relevant methods widely used for protein concentration and removal of low-molecular-weight compounds, especially salts. The difference in the transmembrane pressure is

considered the major driving force that enables sample fractionation by UF (Evans et al., 2009). In contrast to dialysis, UF is known to be a faster, more versatile, and economical method. The major reason for salt contamination of proteins is associated with the specificity of the isolation methodology. Indeed, the efficient separation of LF from LPO typically corresponds to the application of highly concentrated buffers with ionic strengths greater than 0.5 M (Yoshida and Ye-Xiuyun, 1991; Uchida et al., 1996). For example, Burling et al. (1989) carried out LF separation using 0.5 to 2.0 M NaCl solution as a mobile phase. Remarkably, manufacturers rarely provide the information about minerals content in proteins. Bokkhim et al. (2013) indicated that the sodium content in the commercial samples of native and apo-LF was 1,300 and 1,317 µg/g LF, respectively. Previously, the results of electron paramagnetic resonance spectrometric titration confirmed a high binding affinity of chloride ions to the transferrin (TF)-family proteins, such as ovo-TF ($n = 1.87 \pm 0.32$; $\log K = 1.49 \pm 0.03$; mean $\pm$ SE) and holo-LF ($n = 1.95 \pm 0.34$; $\log K = 1.41 \pm 0.06$; Chasteen et al., 1994). In turn, Thompson et al. (1986) reported that several salts (NaCl, NaClO₄) could induce conformational changes in TF structure, affecting its ability for iron binding. Trace amounts of magnesium and calcium might be recognized as soluble-phase components from cow's milk (de la Fuente et al., 1997). The distribution of minerals in milk between soluble (serum) and insoluble (colloidal) phases is variable and might depend on numerous environmental factors: lactation stage, breed, type of feeding, seasonality, and the animals' health status (e.g., mastitis; NRC, 1988; Zamberlin et al., 2011). However, the origin of the aluminum might be assigned to the spatula used in the sample preparation.

MALDI-TOF/MS Study

bLF Molecular Mass Profiling by MALDI-TOF/MS. The MALDI-TOF/MS study provided essential information on the protein molecular structure and stability, as well as sample purity. The position of both peaks assigned to monomeric bLF $[M+H]^+$ before and after UF were observed in the range of 82 to 83 kDa, and the peaks of the protein dimers $[2M+H]^+$ were reported at approximately 166 kDa (Figure 6). The obtained values were in good accordance with the SDS-PAGE results. It might be assumed that UF did not induce the evident changes in the molecular mass of bLF. A slight difference in mass might be caused by minor changes in the protein sequence as well as in glycosylation variations. The results previously obtained by Pryshchepa et al. (2022) showed a high similarity to ours; the peaks corresponding to singly charged protonated protein appeared at 83.207 and 83.227 kDa for nonfiltrated and

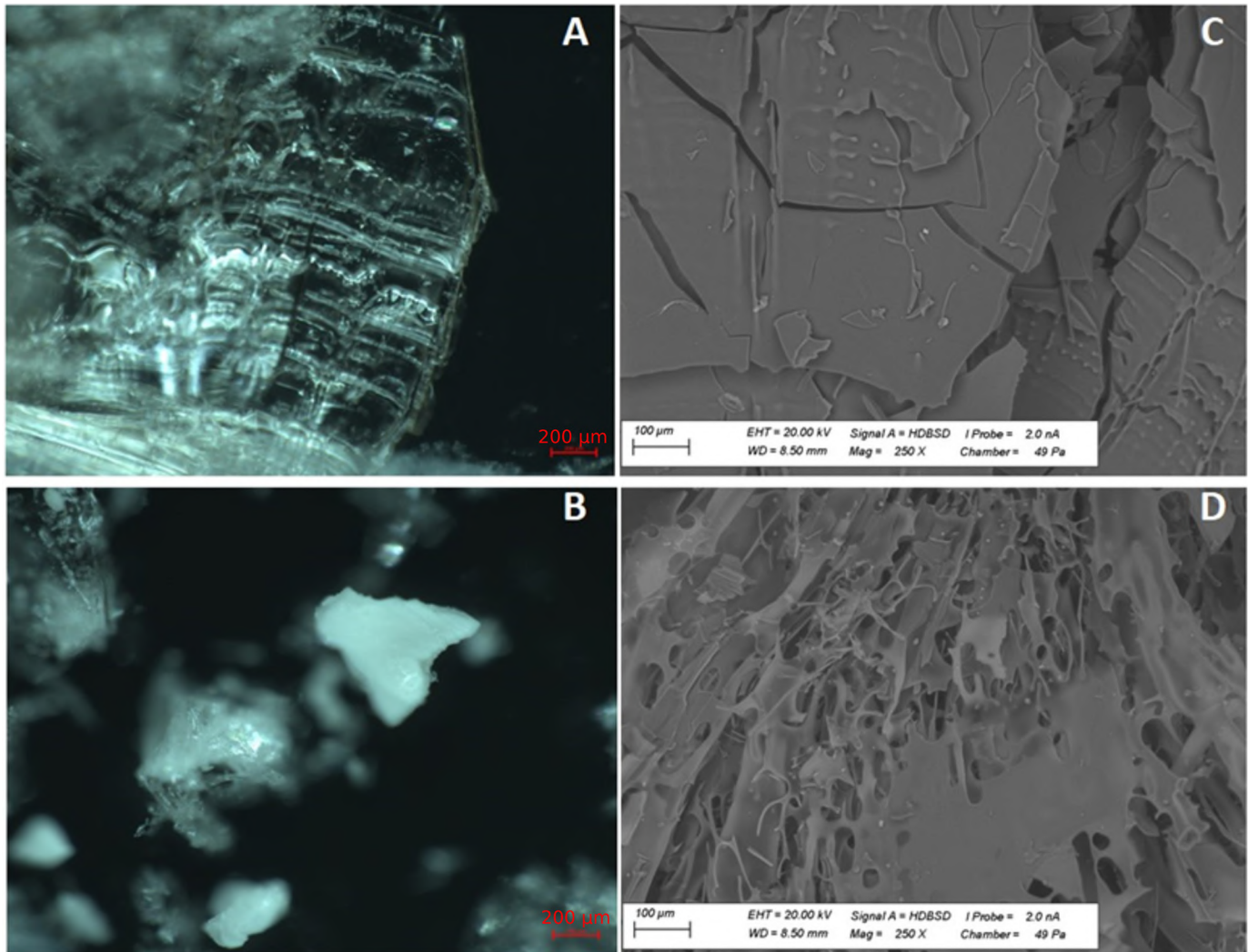


Figure 4. Light microscopy (A, B) and scanning electron microscopy images (C, D) of bovine LF before (A, C) and after UF (B, D). According to LM images, the structure of bLF before UF was more crystalline and organized, while it was broken into smaller irregular assemblies after UF. Scanning electron microscopy images indicated the difference in the surface morphology of both bLF. The protein surface before UF was smooth, whereas it became more porous after UF. EHT = electron high tension; WD = working distance.

filtrated protein, respectively. In contrast, Pomastowski et al. (2016) demonstrated that the average mass of bLF varied from 77 to 82 kDa. Indeed, the molecular mass of bLF might be affected by numerous factors, including iron-saturation level, lactation stage, seasonality, feeding type, cow's breed, isolation method, and the applied analytical technique. For instance, a slight difference was found between the molecular masses of the colostrum, transitional milk, and mature milk, which were reported at 82.733, 83.019, and 82.691 kDa, respectively (Jia et al., 2021). It is worthwhile to mention that the present salts were able to suppress protein ionization. As shown in Figure 5, the peak intensity of pseudomolecular ion increased from 2,500 to 5,200 after the desalting. Previously, this phenomenon was comprehensively described,

especially in the case of electrospray ionization (Chen et al., 2021). For instance, Chen et al. (2021) pointed out that charge competition between salts and a target protein is considered one of the major interfering factors that induce the ion suppression (Metwally et al., 2015). Indeed, the signal labeled as fragment (F) was unobservable at 25 kDa after UF. The same effect was also clearly shown on the SDS-PAGE gel (Figure 3).

Lactoferrin Identification by MALDI-TOF/MS Spectrometry. The PMF spectra reflect a series of peptides generated after the protein digestion with trypsin (Figure 7). All of the samples were identified as TRFL_BOVIN (lactotransferrin OS = *Bos taurus*). The obtained theoretical molecular mass of analyzed proteins was approximately 80 kDa, and their isoelectric point was

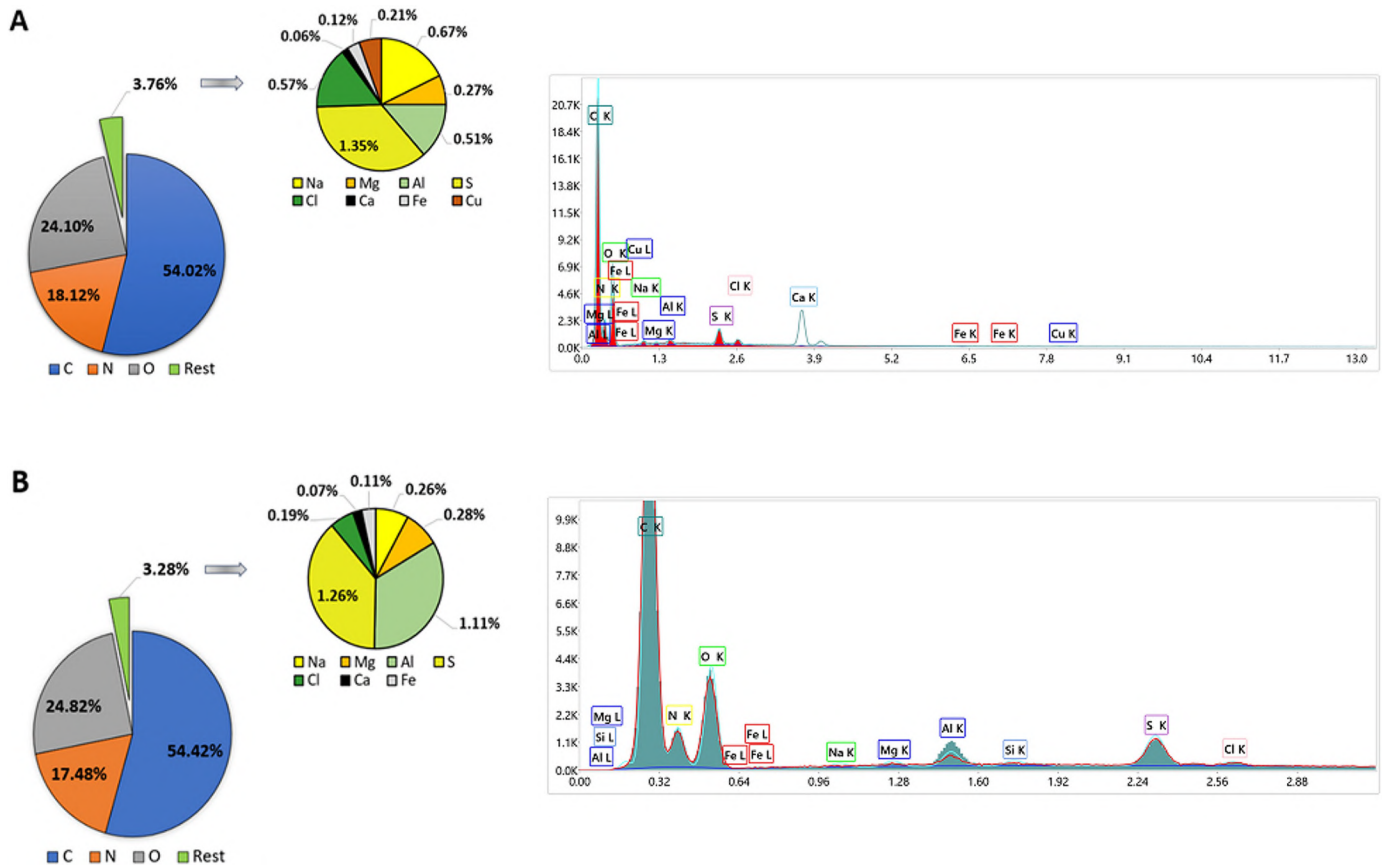


Figure 5. Energy-dispersive spectroscopy analysis of bovine LF before (A) and after UF (B) along with the corresponding pie charts describing the weight percentage each of elements present in the analyzed samples.

equal to 8.69. The digest score was found in the range of 220 to 270 (Figure 8), which implies a reliable and accurate match between the experimental data and the database sequences. Previously, Yan et al. (2022) indicated a mascot score of bLF at 119. Peptides matched in MS mode are presented in Supplemental Tables S1–S4, see Notes). The number of matching peptides for the nonfiltrated bLF (N), (R) and for the filtrated bLF (N), (R) was 45, 45 and 46, 38, respectively (Supplemental Table S5, see Notes). The difference might appear to be the result of nonspecific trypsin cleavages, miscleavages, and artificial modifications (Lin et al., 2020). The typical proteolytic degradation by trypsin occurs at the carboxyl side of lysine and arginine residues; however, trypsin cannot cleave peptide bonds if the next amino acid in the sequence is Pro (Rastogi et al., 2014). The nonspecific action of trypsin might be caused by several reasons, such as substrate:enzyme ratio, temperature, and time of incubation. For, instance, Lin et al. (2020) indicated a relatively high number of miscleavages at a substrate: enzyme ratio of 1:50. Furthermore, the preliminary UF of bLF also could affect the accessibility of specific cleav-

age sites. According to the obtained spectra, the difference in detected peptides between nonfiltrated bLF under reduced and nonreduced conditions was negligible. The most significant changes in the peptide profile occurred after the protein UF. Generally, the predominance of peptides derived from the N-lobe was observed. The following paragraph lists the peptides identified for series of bLF samples in MS mode; the range of numbers in parentheses indicates the amino acid positions of the analyzed peptide in the bLF molecule, and the string of letters reflects the identified peptide sequence. Remarkably, the fragments of bovine lactoferricin were detected at m/z 1,106 (40–46; RWQWRMK), m/z 1,073 (48–57; LGAPSITCVR), m/z 831 (40–44; RWQWR), and m/z 675 (41–44; WQWR) in all samples (Furlund et al., 2013). Additionally, one more fragment at m/z 1,201 (47–57; KLGAPSITCVR) was found after the UF of bLF. The high number of fragments deriving from bovine lactoferricin is related to a basic character of peptides and the presence of high numbers of arginine and lysine residues (FKCRRWQWRMKKLGAPSITCVRRAF), which are known as trypsin cleavage sites (Sinha et al., 2013).

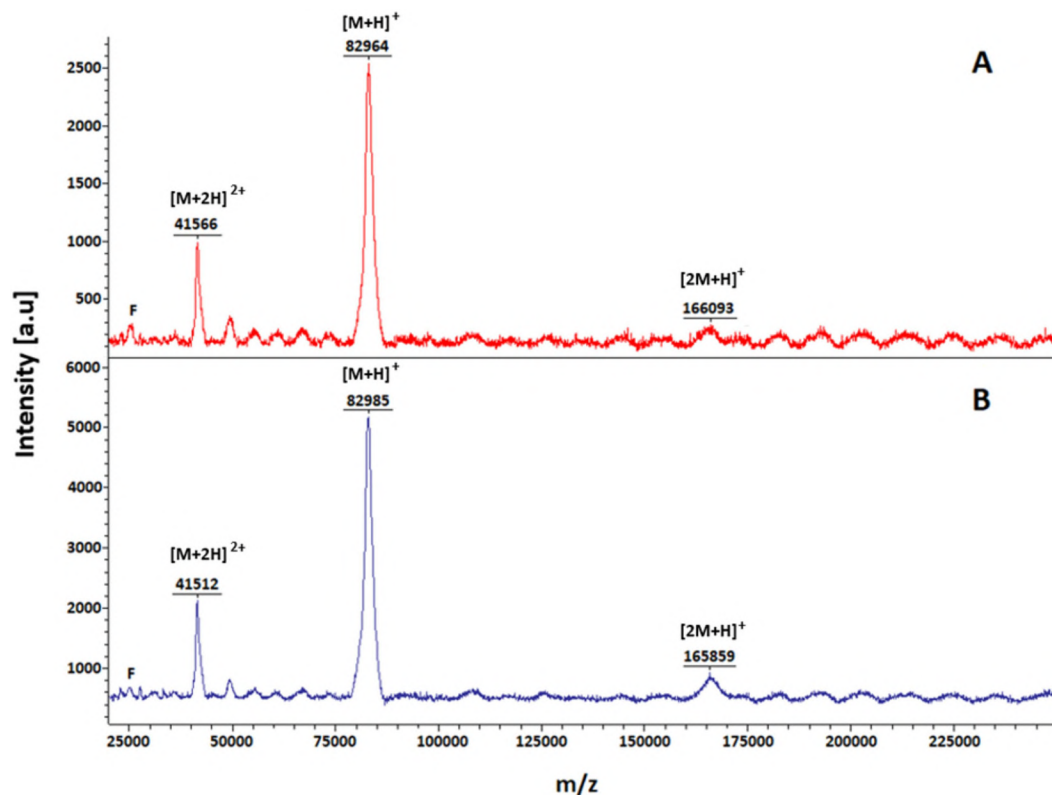


Figure 6. MALDI-TOF/MS spectra of intact bovine lactoferrin before (A) and after UF (B). F = fragment. The intensity changes of F signal provides the information on the purification effectiveness of bLF from the smaller fragments (peptides). a.u. = arbitrary units.

The signal of another biologically active peptide, bovine lactoferrampin (WKLLSKAQEKFGKNKSR) was not found; nevertheless, the fragment—in all samples, except for nonfiltrated bLF (N)—at m/z 1,730 (278–292; SVDG-KEDLIWKLLSK) contained a part of the peptide. In the case of the filtrated bLF (N), a few additional peptides were matched in the m/z range of 2,200 to 2,600 at m/z 2,558 (670–692; LGGRPTYEEYLGTEYVTAIANLK); m/z 2,314 (439–459; HSSLDCVLRPTEGYLAVAVVK), and m/z 2,240 (72–92; KADAVTLDGGMVFEAGRDPYK). The obtained identification data showed a high similarity to the results from a previous study in which the number of matched peptides obtained by Binita et al. (2014) was 17 (mascot score 1,630, sequence coverage 63%). Based on our studies, bLF (N) after UF showed the 11 matched peptides in common, and nonfiltrated samples had 10 matches.

Ultrafiltration could potentially modify the protein structure, leading to the destabilization of glycan chains and the release of sugar residues. In turn, the thiol groups of cysteine mediate as potential glycosylation sites. Indeed, the bLF macromolecule includes 17 disulfide bridges. Several authors revealed the possibility of nonenzymatic S-glycosylation of thiol groups induced by reductive sugars (Qin et al., 2018). The reduction

of the filtrated bLF by β -mercaptoethanol might cause considerable changes in the position of glycans and the appearance of new glycosites, and thus some of the signals became undetectable. Moreover, the number of cysteine-containing peptides increased from 20 to 21 after UF; nevertheless, it decreased to 17 under the reducing conditions. Therefore, the nonmatched signals (55, 55, 54, and 62) might arise from the potential glycopeptides. Based on the UniProt database (UniProtKB reviewed [Swiss-Prot]; www.uniprot.org), the bLF molecule (P24627 entry) contains 4.2% of Asn residues in its primary structure. Application of several variable modifications, such as HexNAc (N), Hex(1)HexNAc(2) (N), HexNAc(2)dHex(1) (N), Hex(1)HexNAc(1)dHex(1) (N), and Hex(1)HexNAc(1)NeuAc(1) (N), indicated matches (glycopeptides) representing the potential glycosylation sites at Asn300, Asn349, Asn537, and Asn640 in bLF (Supplemental Tables S6–S9, see Notes). Indeed, HexNAc (N) represents N-acetylhexosamine, which is typically attached to asparagine residue. HexNAc (N), as well as Hex(1)HexNAc(2) (N) (2 N-acetylhexosamines with 2 hexose [**Hex**]) are characteristic for core of N-glycan. These fragments are present in all N-glycan types (e.g. high mannose, complex, and hybrid). In turn, the fucose (**dHex**) presence in HexNAc(2)dHex(1) (N)

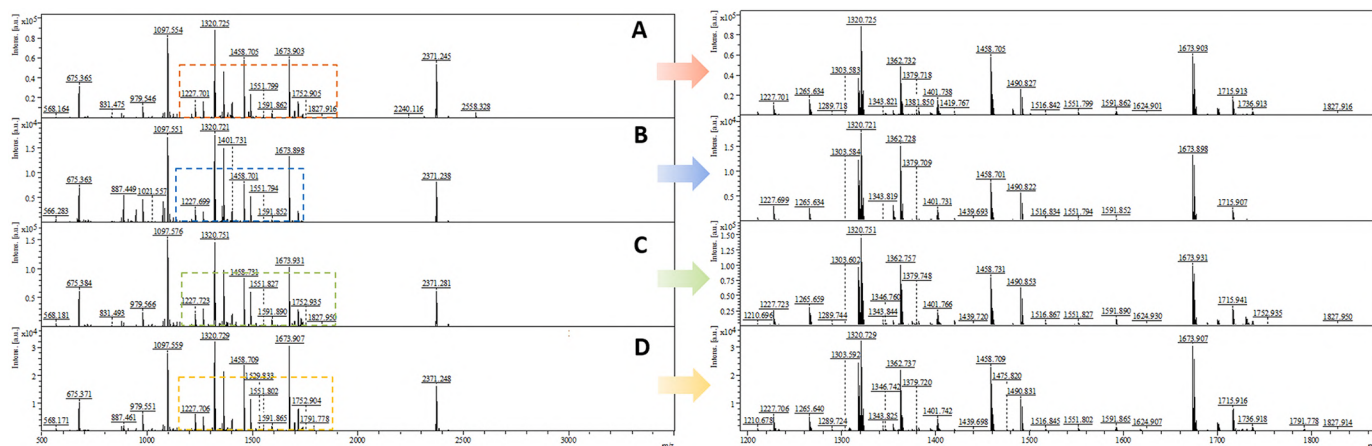


Figure 7. Peptide mass fingerprint spectra of trypsin-digested proteins: bovine lactoferrin after UF under nonreduced conditions (A); bovine lactoferrin after UF under reduced conditions (B); bovine lactoferrin before UF under nonreduced conditions (C); and bovine lactoferrin before UF under reduced conditions (D). a.u. = arbitrary units.

(N-glycan, with 2 N-acetylhexosamines and 1 fucose) and Hex(1)HexNAc(1)dHex(1) (N) (N-glycan with 1 N-acetylhexosamine, 1 hexose, and 1 fucose) is typical for complex glycans. Indeed, Hex(1)HexNAc(1)NeuAc(1) (N) contains sialic acid (NeuAc), which might be present in both, complex and hybrid N-glycans. Previously, Gnanesh Kumar and Mattad (2021) identified 5 glycosylation sites in bLF at Asn252, Asn300, Asn387, Asn495, and Asn584. The authors indicated a high-mannose type of glycosylation in 4 sites, the exception being Asn300. Sequence coverage of the target protein was found in the range of 49.0% to 61.6%, showing a high-accuracy of the identification results (Figure 8). In turn, the notable increase of the intensity coverage after UF of bLF implies a positive effect of the desalting procedure on sample representation. Regarding bLF analysis, the sample preparation plays a crucial role in ensuring the accuracy and representativeness of biological assays. The improved identification parameters (score, sequence coverage, and intensity coverage) obtained after the UF of bLF show a necessity of this procedure for the more accurate and comprehensive analysis of molecular structure. Indeed, the present salts induce the formation of a metastable colloidal system of bLF. Although UF is known as an efficient method for concentration and desalting of proteins, it has number of limitations. As was shown, UF of a water solution of bLF resulted in membrane fouling. In turn, the preliminary dissolution of protein in the 0.9% wt/wt NaCl solution ensured the efficient removal of impurities. Ding et al. (2019) noted that different factors, including pH, ionic strength, and temperature, might affect the UF membrane performance. Remarkably, the risk of membrane fouling increases as the membrane separation occurs in solutions with a pH close to the protein pI. Our studies showed that the addition of NaCl

contributed to the pI shift of bLF to 3.7 and, as a result, led to the enhancement of electrostatic repulsion between bLF molecules. Previously, it was reported that proteins are exposed to shear stress during UF, and thus their initial 3-dimensional structure undergoes modification (Van Audenhaege et al., 2013). Despite the UF operating conditions (pressure, temperature), the shear stress is closely related to the ratio of membrane pore size and the size of the loaded protein. For instance, Miron et al. (2021) showed that the application of membranes with low cut-off (1 kDa) promoted the increased shear stress for lysozyme (~14 kDa), which in consequence induced protein denaturation and loss of its antibacterial activity. In our study, the ratio of bLF size (~83 kDa) to the applied membrane cut-off (50 kDa) was relatively small, and thus the risk of protein exposure to the shear stress was minimized (Moino et al., 2024).

Currently, finding the appropriate validation methods to allow for the accurate physicochemical characterization of proteins is a problem (Brisson et al., 2007). Among the most abundant instrumental techniques used for bLF quantification, ELISA and HPLC should be mentioned. The combination of both techniques provides information about the overall bLF content, percentage of protein in a native state, and its degree of denaturation (Zhang et al., 2021). The iron-binding capacity is another physicochemical parameter that determines the protein biological activity. The accurate determination of the bound metal could be performed by application of inductively coupled plasma MS, inductively coupled plasma optical emission spectrometry, and UV-visible spectrophotometry (Majka et al., 2013; Bokkhim et al., 2014). Nevertheless, the last method restricts the occurrence of additional binding sites in biomolecule. As noted earlier, the iron content in the analyzed sample was 242 mg/kg

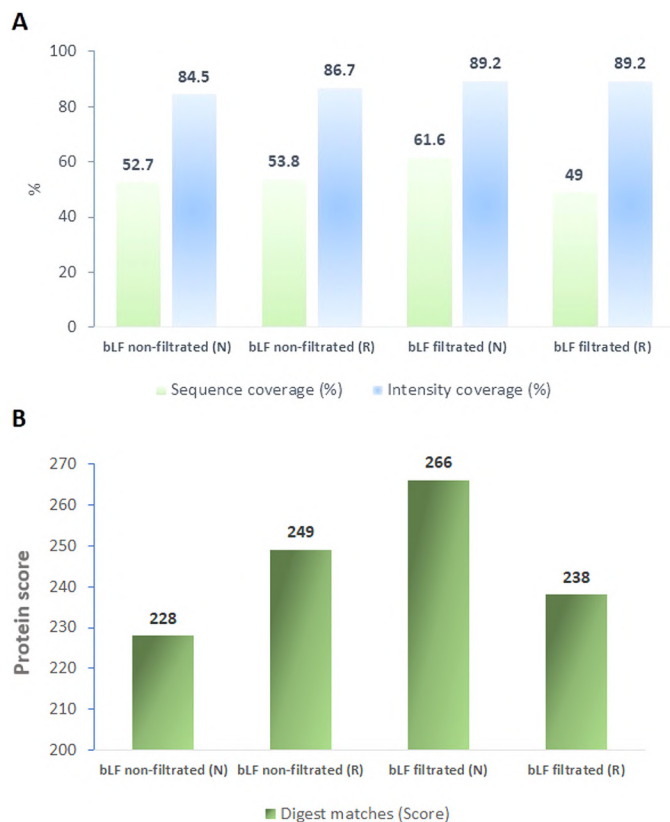


Figure 8. Identification parameters of bovine lactoferrin based on the PMF spectra. bLF nonfiltrated (N) = bLF before UF under nonreduced conditions; bLF nonfiltrated (R) = bLF before UF under reduced conditions; bLF filtrated (N) = bLF after UF under nonreduced conditions, and bLF filtrated (R) = bLF after UF under reduced conditions.

which is equal to 17.3% saturation. According to this result, bLF is classified as a native form, which implies the presence of both apo- and holo-LF in a certain ratio. Unfortunately, the current instrumental approach does not solve the problem of designating each individual form in the native bLF (Zhang et al., 2021).

Our studies showed that protein impurities might be efficiently monitored by application of various instrumental techniques, for example, EDS, SDS-PAGE, and MALDI-TOF/MS. The first one is relevant for the analysis of the mineral composition in a biomolecule, including salts. However, the latter 2 techniques provide the detection of protein fragments (peptides). The purity grade of the final product might be influenced by several factors, including the type of raw material, the presence of microbiological contaminants, processing conditions applied during separation, and storage conditions. The compositions of sweet and acid whey, as key raw materials, differ from each other by the content of minerals, such as Ca, K, Na, Mg, P, Cl (0.2%–0.5% and 0.4–0.7%, respectively), lactic acid (<0.1% and 0.3%–0.8%, respectively), and lactose (4.6%–5.2% and

3.5%–4.6%, respectively; Dufton et al., 2019; Talebi et al., 2020; Buchanan et al., 2023). These small molecules, potential impurities during bLF extraction, could be removed by application of membrane filtration techniques, such as UF or dialysis (Phillips and Signs, 2004).

Generally, UF is recognized as a widely integrated technological process in the dairy industry, and it has the biggest market share among the membrane techniques (Reig et al., 2021). On a large scale, the UF is mainly implemented in milk protein standardization for cheese production (e.g., cottage, Cheddar, feta, Havarti, mozzarella, and blue cheeses; El-Gazzar and Marth, 1991). Regarding the compositional aspect, cheese made by UF milk is characterized by a higher CN and colloidal salt content, along with a lower level of moisture and soluble-phase minerals in contrast to cheese made from the regular milk (Lauzin et al., 2020). In addition to cheesemaking, UF-obtained milk protein concentrate (MPC), containing approximately 40%–89% protein, might be potentially used in the production of high-protein yogurts, ice cream, dairy desserts, and nutritional beverages and bars (Khatkar et al., 2023). The major challenge restricting the widespread application of MPC is related to the presence of high amounts of solids, mainly calcium, owing to its poor solubility. To improve MPC solubility, the milk should be initially acidified to pH 4.6 to 6 and then subjected to the UF for efficient calcium removal (Meena et al., 2017). Additionally, membrane techniques are widely engaged in the management of dairy byproducts, such as whey. In particular, UF can be applied to the recovery of whey proteins and formulation of the commercial whey-based nutrition products, such as whey protein concentrate (protein content 30%–89%), and whey protein isolate (protein content >90%; Figueroa Pires et al., 2021). Remarkably, the obtained UF permeate represents a rich source of lactose, which is a valuable dairy ingredient. Lactose undergoes conversion to lactic acid in the presence of lactic acid bacteria strains, and thus its presence is essential during the production of fermented dairy products (Hettinga, 2019). More recently, lactose has been used as an additive to enhance the stability and shelf life of dried dairy products, such as skim milk powder, whey powder, and permeate powder (Huppertz and Gazi, 2016).

It is worthwhile to take into account the high sensitivity of bLF structure to physicochemical factors, including pH, ionic strength, and temperature. Previous work indicated that iron-saturation level is closely related to the conformational stability of bLF (Barros et al., 2021). Therefore, the iron-binding capacity of bLF is considered one of the key factors that determines its stability and biological potential. Protein exposure to highly acidic solutions is typically accompanied by iron release and structure loosening. Thus a significant difference

occurred in the percentage of bound metal remaining in holo-LF at pH 2 and pH 7, which was equal to 21% and 88%, respectively (Sreedhara et al., 2010a). Indeed, the evident modifications in the 3-dimensional structure related to bLF unfolding were initiated at pH < 4. Previously, it was mentioned that the use of acidic whey as a raw material increased the risk of acidic hydrolysis of a biomolecule. Importantly, the typical pH of the eluent used in cation-exchange separation of bLF is found in the range of 6.5 to 7.0, which is appropriate for maintaining the protein native structure (Sreedhara et al., 2010b). According to our studies, the greatest degradation of the bLF structure occurs in the pH range of 2 to 4, and the highest stability was observed at a pH of 7 to 9. Previously, it was reported that LF dissolving in highly concentrated salt solutions is accompanied by surface charge inversion and formation of negatively charged protein aggregates (Mela et al., 2010). For this reason, the pH as well as ionic strength of the buffer (eluent) applied during bLF isolation should be selected properly to minimize the damage to protein structure. The presence of peptides also might be associated with the specific drying method used as well as storage conditions. Temperature is known as one of critical parameters during protein drying. Although spray-drying is a popular drying method, the use of high temperatures (150–200°C) might negatively affect the quality of the final product. Indeed, Morel et al. (2022) noticed that bLF subjected to spray-drying was characterized by a much lower iron-binding capacity and higher denaturation degree than bLF after freeze-drying. It is worthwhile to highlight that the thermal stability of bLF substantially differs depending on iron-saturation state. Thus, it was reported that the unfolding of apo-LF starts at 63°C, and the thermal destabilization of holo-LF occurs at about 87°C (Xia et al., 2023). Furthermore, Goulding et al. (2021) pointed out that high-temperature, short-time processing of bLF in the temperature range of 72–95°C led to irreversible denaturation processes in the protein structure. Remarkably, the stability of the LF structure under high-temperature conditions might be influenced by the pH of the medium used. Thus, Saito et al. (1994) showed, that bLF heated to 100°C at pH 4 maintained a greater than 90% iron-binding capacity. Similarly, Williams et al. (2023) observed the highest amount of undenatured LF after high-temperature processing at pH 4, which was equal to 81%. In contrast, reduction of bLF thermal stability was observed at pH ≥ 6. The comprehensive study of bLF thermal properties provides insight into the biomolecule's conformational stability and functionality, as well as the potential application areas. Ultra-high-temperature processing is a frequently applied pretreatment procedure aimed at preserving dairy products against harmful microorganisms and extending its storage time. The selection of the

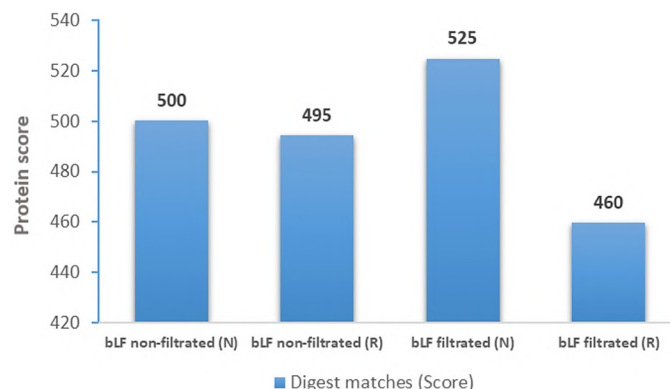


Figure 9. Identification data of bovine lactoferrin based on the MS/MS spectra. bLF nonfiltrated (N) = bLF before UF under nonreduced conditions; bLF nonfiltrated (R) = bLF before UF under reduced conditions; bLF filtrated (N) = bLF after UF under nonreduced conditions; and bLF filtrated (R) = bLF after UF under reduced conditions.

appropriate temperature range and the heating time will determine the further biological potential of individual milk components. Whey proteins are known to be a heat sensitive group of biomolecules that usually undergo unfolding and denaturation at high temperatures (Krishna et al., 2021). For instance, Williams et al. (2023) reported that approximately 10% of whey proteins maintained their native structure after UHT processing (142°C; 4s) of 8% (wt/wt) whey protein suspension (pH 7.5). It has been reported that UHT of bLF solution at 135°C for 4s provided complete degradation of the protein structure and as a result strongly reduced its antibacterial activity against *Escherichia coli* (Paulsson et al., 1993). Despite the tendency of proteins to denature at high temperatures, their stability also might be significantly affected at low temperatures. In this context, the term “cold denaturation” might be used. Proteins undergo unfolding and lose the their 3-dimensional structure, especially after long-term storage under cooling conditions. Remarkably, the level of LF in breast milk stored at –20°C did not differ significantly in the first 6 d (Kasiati et al., 2021). In contrast, Rollo et al. (2014) noted a 37% decrease in LF content in human milk as the time of low-temperature storage (–20°C) exceeded 3 mo. The evident changes in the LF content might be associated with degradation of protein structure as a result of low-temperature treatment.

More precise identification results were gained after peptide fragmentation using MS in the laser ionization fragmentation technology mode. The common criteria selected for the bLF identification was the peak intensity >1,000 arbitrary units. The only exception was nonfiltrated bLF (N), in which case the criterion was changed to a peak intensity >3,000 arbitrary units due to large number of signals. Peptides identified after MS/MS analysis are presented in Supplemental Tables S10–S13

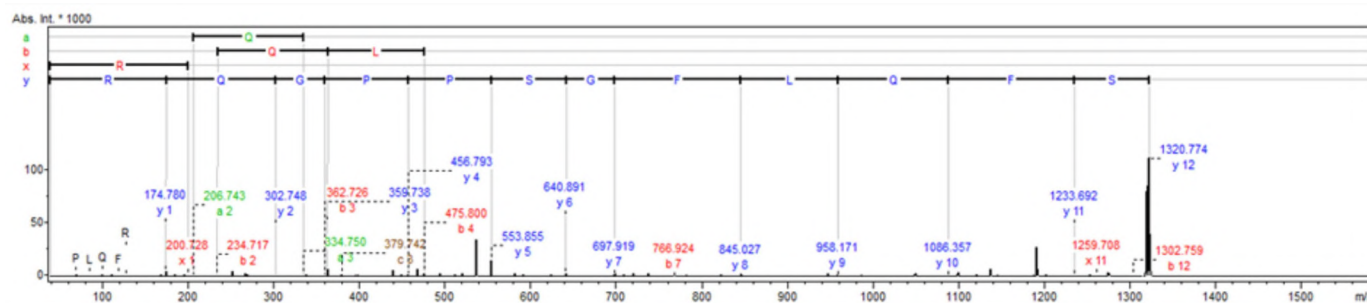


Figure 10. Peptide SFQLFGSPPGQR fragmentation (304–315). The ranges of S, F, Q, L, G, P, and R reflect the fragmentation pathway of the appropriate amino acid in the peptide. The b- and y-ions refer to fragments generated by breaking peptide bonds at N-terminal and C-terminal, respectively. In turn, a- and x-ions are produced by cleavage of the peptide backbone at N- and C- α bond, respectively. The numeral index indicates the number of amino acid the fragment contains.

(see Notes). The number of matching peptides decreased after MS/MS matching and was equal to 17, 16, 19, and 14 (Supplemental Table S5) for the nonfiltrated bLF (N), nonfiltrated bLF (R), filtrated bLF (N), and filtrated bLF (R), respectively, and the score values ranged from 460 to 525 (Figure 9). The highest mascot score was observed for filtrated bLF at nonreducing conditions, similar to during MS analysis. The fragmentation pathway of the selected peptide commonly matched in all samples was shown at the Figure 10. Importantly, the same peptide was identified by Xia et al. (2023); that study obtained score and number of matched peptides during bLF identification were 602 and 13, respectively. We can assume, that the better results of protein identification might be related to the specificity of the applied desalting method.

In the case of nonfiltrated bLF at nonreducing conditions, one more peptide assigned as m/z 1,289 (278–288; sequence of bLF peptides identified in the MS/MS mode: SVDGKEDLIWK) was matched in comparison to reducing conditions. Remarkably, this fragment also was not identified in the filtrated bLF (R) sample. A few additional matches appeared for bLF (N) after the UF at m/z 831 (40–44; RWQWR) and m/z 947 (590–597; LLCLDGTR). The fragment at m/z 945 (475–482; SHTAVDR) exceptionally matched in the filtrated bLF (R). It is worthwhile to emphasize that MS/MS spectra are more complicated and might provide more sequence-specific information in contrast to PMF. The other reason for the difference might induce a selected criteria that would restrict the number of analyzed peptides.

CONCLUSIONS

Ultrafiltration is known to be an efficient nondestructive technique that is relevant for protein concentration along with removal of low-molecular-weight compounds, such as salts. Precise protein desalting is crucial because it allows enhancement of the accuracy of experimental

results. In this study, the physicochemical and structural characteristics of bLF with and without UF were evaluated. The obtained results indicated the similarity in molecular masses between both bLF samples. The microscopy studies showed the evident transformation of the bLF surface structure after UF from smooth to more porous and rugged. Furthermore, the improvement of identification parameters, such as sequence coverage (from 52.7% to 61.6%) and intensity coverage (from 84.5% to 89.2%) confirms the significance of the prior UF step before further analysis of proteins to gain higher precision results.

NOTES

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Nonstandard abbreviations used: ACN = acetonitrile; apo-LF = unsaturated LF; a.u. = arbitrary units; bLF = bovine lactoferrin; CE = capillary electrophoresis; dHex = deoxyhexose; EDS = energy-dispersive spectroscopy; EHT = electron high tension; F = fragment; Hex

= hexose; HexNAc = N-acetyl-hexosamine; holo-LF = saturated LF; LPO = lactoperoxidase; MS/MS = tandem MS; MW = molecular weight; (N) = nonreducing conditions; Nd:YAG = neodymium-doped yttrium aluminum garnet; PDB = protein database; PMF = peptide mass fingerprint; (R) = reducing conditions; ScEM = scanning electron microscopy; TF = transferrin; TFA = trifluoroacetic acid; WD = working distance.

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Publikacja [P3]

Supplementary material

Materiały uzupełniające do tego artykułu są dostępne pod adresem:
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lub po zeskanowaniu następującego QR kodu:



[P4] The Impact of Lactiplantibacillus Plantarum on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition

Tetiana Dyrda-Terniuk, Viorica Railean, Aleksandra Florkiewicz, Justyna Walczak-Skierska, Mateusz Kolankowski, Joanna Rudnicka, Dorota Białczak, Paweł Pomastowski. The Impact of Lactiplantibacillus Plantarum on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition. *International Dairy Journal*, **2025**, 161, 106118. doi.org/10.1016/j.idairyj.2024.106118



Research paper

The impact of *Lactiplantibacillus plantarum* on the cream composition: Insight into changes of vitamin D3 content and fatty acid composition

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ABSTRACT

Food fortification is considered a promising strategy to enhance the bioavailability of vitamin D. This study aimed to analyze the impact of probiotic bacteria added to cream on vitamin D3 biosynthesis. Both the biomass and lyophilized form of *Lactiplantibacillus plantarum* were tested. In addition, changes in the fatty acid (FA) profile were evaluated. Supplementation with *L. plantarum* B/00401 lyophilisate resulted in more efficient vitamin D3 enrichment of the cream compared to the biomass, increasing its content from 1.71 to 15.96 $\mu\text{g g}^{-1}$. Moreover, a reduction in saturated fatty acids (SFA) accompanied by an increase in monounsaturated fatty acids (MUFA) was observed following the addition of *L. plantarum* B/00401 to the cream. The proposed fortification approach may help address poor vitamin D3 status. At the same time, the changes in the FA profile offer additional benefits to consumers by reducing the risk of heart disease development.

1. Introduction

Probiotics are live microorganisms that, when consumed in adequate amounts through the diet, provide health benefits beyond basic nutrition (Zayed & Roos, 2004). Key functions of probiotic bacteria include gut immunomodulation and the enhancement of internal defense mechanisms. Additionally, probiotics can produce hydrogen peroxide, organic acids, and bacteriocins, which contribute to the inhibition of pathogen growth in the gastrointestinal and urogenital tracts (Boris & Barbés, 2000; Reid & Burton, 2002; Velraeds et al., 1998). The recommended daily intake of probiotics is approximately 10^9 colony-forming units (CFU). (Latif et al., 2023). Dairy products, such as milk, yoghurt, cheese and butter, are defined as the most potential carriers of probiotic bacteria (Urrutia-Baca et al., 2024).

Probiotics can be incorporated into food in various forms, such as suspensions, powders, and capsules (Vera-Santander et al., 2023). Bacterial suspension or biomass is a commonly used form of probiotic

bacterial strains. It offers a wide spectrum of applications in functional dairy foods, animal feed, dietary supplements, and pharmaceuticals. (Ren et al., 2019). Although biomass production is relatively fast and cost-effective, the viability of the bacteria and their storage duration are significantly reduced compared to other forms. In addition to traditional bacterial biomass, probiotics can also be applied in a lyophilized (freeze-dried) form (Arellano-Ayala et al., 2021). Freeze-drying is a commonly used technique for producing dried probiotic powders (Tyagi et al., 2023). The higher viability of freeze-dried bacteria enhances their beneficial effects, making lyophilized probiotics increasingly popular in modern applications. Additionally, probiotics in their lyophilized form have an extended shelf life, allowing for longer storage without losing their fermentative activity (Shekh et al., 2020).

Lactic acid bacteria (LAB), particularly those from the *Lactobacillus* genus (with some now reclassified under the *Lactiplantibacillus* and *Lactiseibacillus* genera), are recognized as probiotics. Importantly, LAB exhibit diverse metabolic pathways, including carbohydrate

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metabolism, amino acid metabolism, and fatty acid (FA) metabolism (Hu et al., 2020). They produce numerous valuable compounds, such as organic acids, short-chain fatty acids (SCFA), exopolysaccharides, bacteriocins, amino acids, enzymes, and vitamins (Tang et al., 2023). As a result, numerous LAB species, including *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactocaseibacillus paracasei*, *Lactocaseibacillus rhamnosus*, and *Lactiplantibacillus plantarum* (*L. plantarum*) have found widespread application in functional food design (Abdollahzadeh et al., 2018; Li et al., 2020). In particular, *L. plantarum* is one of the most remarkable probiotic bacterial species which widely implemented in the production of the fermented products, such as pickles, juices, or certain types of cheese, and milk cream (Behera et al., 2018). Generally, *L. plantarum* promotes the transformation of milk sugars (mainly lactose) into lactic acid under anaerobic conditions, leading to a decrease in pH and the acidification of the product (Cui et al., 2021).

It is worth mentioning, that LAB are characterized as vitamin-producing microorganisms (Ribeiro et al., 2023). Vitamin deficiency is recognized as a rapidly growing issue in the modern world. Notably, approximately 1 billion people worldwide suffer from vitamin D3 deficiency (Saleem et al., 2024). Vitamin D3 is an essential steroid hormone, which can be delivered to human body through both endogenous and exogenous ways. The precursor of vitamin D3, 7-dehydrocholesterol (7-DHC), found in skin cells, is converted to D3 upon exposure to UVB radiation (282–310 nm) (Haines & Park, 2012). Alternatively, vitamin D3 can be obtained from animal-based foods such as meat, offal, fish, and eggs (Schmid & Walther, 2013). LAB strains are known to act as mediators in the production of several B-group vitamins (B2, B9, B12) and vitamin K (Ribeiro et al., 2023; Valle et al., 2011). However, information on vitamin D3, as a potential LAB metabolite remains limited. Nevertheless, Castagliuolo et al. (2021) pointed out that probiotic administration facilitates vitamin D3 absorption, leading to an increase in serum levels of 25-hydroxyvitamin D3 (25(OH)D3). Consequently, products supplemented with LAB could offer a natural way to improve vitamin D3 bioavailability, which is especially crucial during the winter season when sunlight exposure is reduced.

Therefore, the present work aimed to investigate the impact of the added *L. plantarum* B/00401 strain in two forms, as biomass and lyophilisate, on the production of vitamin D3 and changes in the FA profile of cream samples incubated under various light/dark and mixing/non-mixing conditions.

2. Materials and method

2.1. Reagents, solvents and materials

All reagents, solvents, and medium used in this study were of the highest available purity and were purchased from Sigma Aldrich (Steinheim, Germany). Small laboratory equipment and plastics were purchased from Alchem (Toruń, Poland). Deionized water (H₂O) was obtained using a system from Millipore (Schaffhausen, Switzerland). Cream samples were provided by Polmlek Sp. z o.o. (Grudziądz, Poland). The total fat percentage was 41 % (w/w), while the acidity (pH) was 6.72. The determined content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) was 548.7, 88.6, and 77.0 mg g⁻¹, respectively. More detailed information regarding the FA composition of the supplied cream is presented in Table S1, labeled as Control 2.

2.2. Preparation of bacterial biomass and lyophilisate

In the current study, *L. plantarum* isolated from cow milk 2 h after milking and deposited under no. B/00401 at the Polish Collection of Microorganisms (Hirsfeld Institute of Immunology and Experimental Therapy) was used. Initially, *L. plantarum* B/00401 was cultured on De Man, Rogosa and Sharpe Agar (MRSA) medium at 37 °C (24h) and subsequently subcultured in MRS broth (MRSB) under the same

conditions. Bacteria cells were collected by centrifugation (15 min; 12 000 rpm; 4 °C), and then the obtained biomass was washed three times with deionized water. A portion of the washed biomass was lyophilized using a freeze-dryer (LABCONCO FreeZone, Kansas City, MO, USA), while the remaining biomass was stored for further analysis. Both forms of *L. plantarum* B/00401, biomass and lyophilisate, were used in subsequent experimental designs.

2.3. The experimental design and designation of the experimental variants

The experimental design for this study is shown in Table 1. Both forms of *L. plantarum* B/00401 strain (biomass, lyophilisate) have been prepared in MRSB at 2.5×10^9 CFU. Finally, *L. plantarum* B/00401 inoculum was mixed with cream samples at 1:1 and 3:1 (v/v) ratios (cream/*L. plantarum* B/00401). These two different cream/bacteria ratios (1:1 and 3:1) were selected to highlight the significance of the added probiotics on the changes in vitamin D3 content and the overall FA profile. Cream samples without the addition of *L. plantarum* B/00401 strain were marked as controls. Additionally, experimental samples containing the of cream/*L. plantarum* B/00401 mixture without MRSB medium were included to assess the impact of the culture medium on changes in the FA profile.

The experiment was conducted under varying conditions including light, lack of light (darkness), shaking (mixing), non-shaking (non-mixing), and the used form of *L. plantarum* B/00401 strain (biomass, lyophilisate). Samples were incubated for 48 h at room temperature. Those stored under light conditions were exposed to fluorescent lamp radiation (T5 lamp; length 30 cm; power 4.5 W). Mixing conditions (150 rpm) were maintained using a Julabo SW22 water bath (without heating). Samples under light conditions without mixing were kept on a table, while those incubated in darkness without mixing were placed in an INCU-Line® Incubator at room temperature. The following abbreviations were used to mark the analyzed samples, such as: control cream samples incubated at light non-mixing (cL), mixing (cL/mix), dark non-mixing (cD) and mixing conditions (cD/mix); cream samples incubated with *L. plantarum* B/00401 biomass at 1:1 (v/v) ratio at light non-mixing (cBL (1:1)), mixing (cBL/mix (1:1)), dark non-mixing (cBD (1:1)) and mixing conditions (cBD/mix (1:1)); cream samples incubated with *L. plantarum* B/00401 lyophilisate at 1:1 (v/v) ratio at light non-mixing (cLL (1:1)), mixing (cLL/mix (1:1)), dark non-mixing (cLD (1:1)) and mixing conditions (cLD/mix (1:1)); cream samples incubated with *L. plantarum* B/00401 biomass at 3:1 (v/v) ratio at light non-mixing (cBL (3:1)), mixing (cBL/mix (3:1)), dark non-mixing (cBD (3:1)) and mixing conditions (cBD/mix (3:1)); cream samples incubated with *L. plantarum* B/00401 lyophilisate at 3:1 (v/v) ratio at light non-mixing (cLL (3:1)), mixing (cLL/mix (3:1)), dark non-mixing (cLD (3:1)) and mixing conditions (cLD/mix (3:1)). Samples of cream/*L. plantarum* B/00401 without MRSB medium were additionally labeled with (w/MRSB).

2.4. FA profile analysis

FA profile analysis was performed using an Agilent GC mass spectrometry (MS) system (6890 A series; Agilent Technologies, Waldbronn, Germany) coupled with a quadrupole mass spectrometer (GC-MS). Each sample (60 mg) was subjected to multi-step extraction followed by a derivatization procedure, as described by Zloch et al. (2022).

The gas chromatograph was equipped with a HP-5MS (30 m × 0.25 mm × 0.25 µm) capillary column (Agilent Technologies, California, USA), and helium was used as the carrier gas. A 5 µL injection volume was introduced into the system using split mode, with a split ratio of 50:1. The initial oven temperature (50 °C) was maintained for 2 min. In order to elute non-volatile compounds, the oven temperature program was set as follows: 50 °C for 2 min, ramped at 13 °C/min to 150 °C, then ramped at 5 °C/min to 230 °C (held for 8 min). The temperature of the MS transfer line was set to 250 °C. Chromatographic analyses were performed in scanning mode and MS spectra were recorded in the *m/z*

Table 1
The experimental design.

Samples	Experimental conditions			
	Light		Dark	
	Mixing	Non-mixing	Mixing	Non-mixing
- Mixture of cream and biomass/lyophilisate of <i>L. plantarum</i> B/00401 (2.5×10^9 CFU in MRSB) at 1:1 and 3:1 (v/v)	- Placed in Julabo SW 22 water bath (150 rpm; room temperature)	- Placed on the table at room temperature	- Placed in Julabo SW 22 water bath (150 rpm; room temperature)	- Placed in INCU-Line® Incubator (room temperature);
- Cream without added bacteria (control samples)	- Exposed to fluorescent lamp (4.5 W)	- Exposed to fluorescent lamp (4.5 W)	- Covered with aluminum foil;	- Incubation time: 48 h
	- Incubation time: 48 h	- Incubation time: 48 h	- Incubation time: 48 h	

50–600 range. The integrated peaks (previously deconvoluted and aligned) were identified using the NIST 17 library. The content of individual FAs was calculated as a percentage of the total peak areas of identified FAs for each sample, with the sum of FAs in a given sample equaling 100 %. The analysis was performed in triplicate for each sample.

2.5. Analysis of vitamin D3

Analyzed samples were subjected to saponification and extraction steps as it was previously described by Zloch et al. (2022). Briefly, a sample (0.15–0.25 g) was mixed with 2.5 mL of 96 % (w/w) ethanol and 0.5 mL of 60 % (v/v) potassium hydroxide. The prepared mixture was heated in a water bath at 80 °C (15 min) until the solution became clear. The saponified samples were then cooled to room temperature and subjected to liquid-liquid extraction with 5 mL of hexane. The aqueous layer was extracted with an additional 2 mL of hexane for two more times. Hexane extracts were filtered through anhydrous sodium sulfate and dried using a Multivap (Alchem, Toruń, Poland) at 36 °C. Subsequently, the obtained dry residue was dissolved in 0.3 mL of ethanol.

Vitamin D3 content was analyzed using a reverse-phase high-performance liquid chromatography (RP-HPLC) system (Prominence, Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD). Vitamin D3 was separated using an ACE 5 C8 column (4.6 × 100 mm) (Avantor, Gliwice, Poland) as the stationary phase with a mobile phase consisting of a mixture of 0.1 % (v/v) formic acid in methanol and 0.1 % (v/v) formic acid in water (90:10, v/v). Vitamin D3 quantification was

carried out at a wavelength of 265 nm. The calibration curve was prepared using a series of standard vitamin D3 solutions in ethanol at the following concentrations: 0.1; 0.5; 1; 2.5; 5; 10; and 25 µg mL⁻¹. Changes in vitamin D3 were monitored after 6, 24, and 48 h of cream incubation under specific conditions. All measurements were performed in triplicate. The experimental results, along with statistical data, are initially presented in Table S2.

2.6. Statistical analysis

The obtained data were processed using the Radar chart function and Excel’s Conditional Formatting with the Data Bar function. The mean values from the experiment for each sample were taken for data processing. The figures were then further refined using Origin 6.0 to generate the desired format.

3. Results

3.1. The influence of *L. plantarum* B/00401 on the production of vitamin D3 in cream

Fig. 1 presents the changes in D3 vitamin in cream samples incubated under various conditions. A total of 16 experimental variants, along with 4 controls, were prepared, taking into account factors such as light/dark exposure, mixing/non-mixing conditions, cream/*L. plantarum* B/00401 ratio (v/v), and the form of *L. plantarum* B/00401 strain used. Besides, vitamin D3 content was measured periodically, after 6, 24, and 48 h of

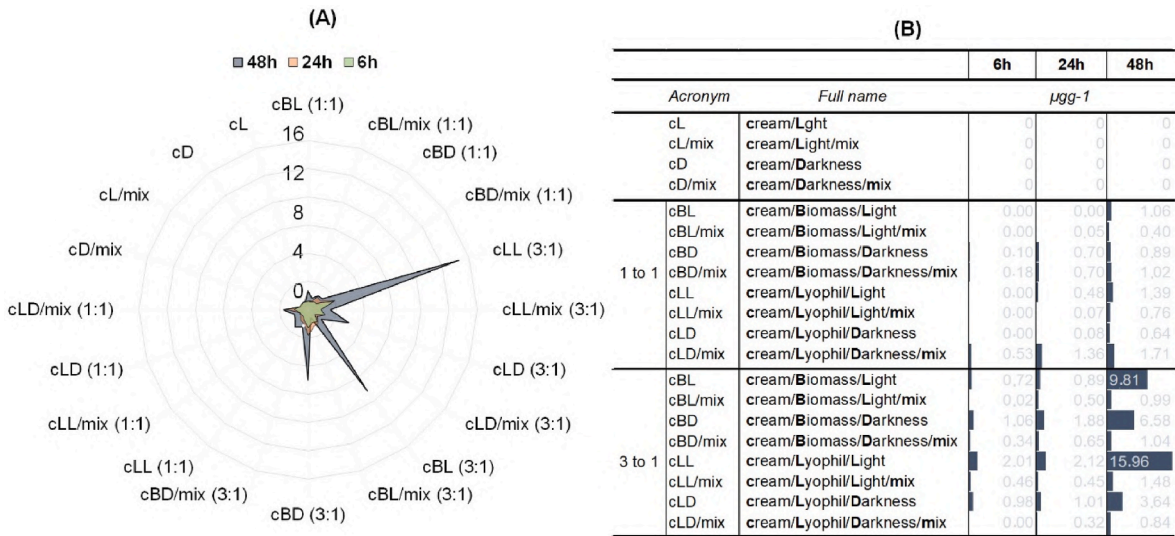


Fig. 1. Illustration of vitamin D3 produced in cream under different selected conditions over 48 h of incubation. (A) Radar chart showing the vitamin D3 concentrations measured at 6, 24, and 48 h. Each vertex represents a different treatment condition, with the concentric circles indicating the concentration of vitamin D3. The acronyms corresponds to those presented in Fig. 2B; (B) The bar chart proving a quantitative presentation of vitamin D3 concentrations for each treatment, illustrating a general increase in vitamin D3 content over time. Lyophil and Biomass refer to the lyophilisate and biomass forms of *L. plantarum* B/00401, while 1 to 1 and 3 to 1 represent the cream/*L. plantarum* B/00401 vol ratio (v/v).

incubation. As expected, vitamin D3 was not detected in the control samples; however, the addition of *L. plantarum* B/00401, either as biomass or lyophilisate, promoted vitamin D3 production. According to the radar chart, a dynamic increase in vitamin D3 content was observed over time, especially after 48 h (Fig. 1A). Moreover, samples with a 3:1 cream/bacteria ratio demonstrated more efficient vitamin D3 production compared to the 1:1 ratio (Fig. 1B). Vitamin D3 concentration in the 1:1 samples ranged between 0 and $1.71 \mu\text{g g}^{-1}$, while in the 3:1 samples, it was found between 0 and $15.96 \mu\text{g g}^{-1}$. The highest vitamin D3 content in the 1:1 cream/bacteria samples was observed in the cLL and cLD/mix variants. A remarkable increase in vitamin D3 was observed in the cBL (3:1) and cLL (3:1) variants, indicating these conditions as the most optimal for vitamin D3 biosynthesis. The vitamin D3 concentrations in the cBL (3:1) and cLL (3:1) variants were 9.81 and $15.96 \mu\text{g g}^{-1}$, respectively. Interestingly, vitamin D3 content was more influenced by mixing/non-mixing conditions than by light/dark exposure. For instance, vitamin D3 concentrations were 6.58 and $3.64 \mu\text{g g}^{-1}$ in the cBD (3:1) and cLD (3:1) variants, respectively, but decreased to 0.99 and $1.48 \mu\text{g g}^{-1}$ in the cBL/mix (3:1) and cLL/mix (3:1) variants, respectively.

3.2. The role of supplemented *L. plantarum* B/00401 on the modification of FA profile

Fig. 2 illustrates the changes in FA profiles among the analyzed samples. A detailed list of the identified FAs in the relevant experimental variants is provided in Table S1. SFA were found to be the predominant group of FAs in all cream samples, followed by MUFA and PUFA, with their content ranging at 488 – 907 , 93 – 512 , and 0 – 38 mg g^{-1} , respectively. The relationship between vitamin D3 content and the specific FA groups (SFA, MUFA, PUFA) is clearly presented for the 3:1 cream/*L. plantarum* B/00401 ratio after 48 h of incubation (Fig. 2A).

Among all variants, the cLL (3:1) was characterized by the highest concentration of both vitamin D3 and SFA (726 mg g^{-1}), but the lowest MUFA content (244 mg g^{-1}) (Fig. 2B). Additionally, it had a relatively high PUFA content of about 30 mg g^{-1} . In contrast, the opposite trend was observed for the cBL (3:1) variant. The highest MUFA content (280 mg g^{-1}) corresponded with the lowest PUFA level (10 mg g^{-1}). Remarkably, PUFA in cBL (3:1) increased to 35 mg g^{-1} under dark conditions.

For the 1:1 variants, the cBL (1:1) sample had the highest SFA content (769 mg g^{-1}) and the lowest MUFA content (204 mg g^{-1}). On the contrary, the lowest PUFA concentration (11 mg g^{-1}) was observed in the cBL/mix (1:1) sample, which had the highest MUFA content (329 mg g^{-1}). There was a noticeable negative correlation between the SFA and MUFA content, especially in the cream/*L. plantarum* B/00401 samples without MRSB medium. For example, the cBL/mix(w/MRSB) sample contained 907 mg g^{-1} of SFA and 93 mg g^{-1} of MUFA, while the cLL/mix(w/MRSB) sample had 488 mg g^{-1} of SFA and 512 mg g^{-1} of MUFA. PUFA was the least abundant group of FAs, with its content ranging from 0 to 38 mg g^{-1} . It is worth noting that PUFA was absent in experimental variants without the MRSB medium, except for cBL(w/MRSB) and cBD(w/MRSB) (Fig. 2B). In turn, the addition of MRSB medium (control) promoted PUFA production. Table 2 summarizes the overall results, highlighting the cream samples with the lowest and highest content of vitamin D3, SFA, MUFA, and PUFA.

4. Discussion

4.1. The impact of *L. plantarum* B/00401 supplementation on vitamin D3 biosynthesis

Naturally, dairy products are a poor source of vitamin D3, typically ranging from 0.001 to $0.006 \mu\text{g g}^{-1}$ depending on the type of product (Polzonetti et al., 2020). The only exception is fat-rich products, such as butter. Currently, the major strategies to increase vitamin D3 intake

include food fortification or the use of dietary supplements (Cashman, 2020). Our studies showed that vitamin D3 content in cream increased after the addition of *L. plantarum* B/00401, in both biomass and lyophilised forms. Previously, it was noticed that LAB strains promote the biosynthesis of B-group vitamins (B2, B9, B12) and vitamin K (Patel et al., 2013). Recent studies have also indicated the ability of probiotic bacteria to increase vitamin D3 content (Shang & Sun, 2016). For instance, Jones et al. (2013) reported, that the administration of *L. reuteri* strains to healthy hypercholesterolemic adults, aged of 20 and 75, led to an increase in $25(\text{OH})\text{D}_3$ levels, indicating a positive impact on poor vitamin D3 status in humans. Remarkably, the absorption of other fat-soluble vitamins (A and E) did not change significantly. This phenomenon may be explained by the ability of LAB strains to increase the secretion of lactate, acetate, and pyruvate metabolites, which are involved in 7-DHC biosynthesis (Gokhale & Bhaduri, 2019). In addition, Li et al. (2023) discovered, that *Carnobacterium maltaromaticum*, another LAB species, was directly involved in the production of vitamin D-related metabolites, similar to *L. plantarum* in this study.

The most efficient growth of vitamin D3 was observed in samples with a higher proportion of cream after 48 h of incubation under light conditions without mixing. Indeed, Zloch et al. (2022) reported a similar trend after the addition of *L. paracasei* biomass to cream samples, where the maximum vitamin D3 produced reached $15.7 \mu\text{g g}^{-1}$. Light accessibility plays a critical role in the photoactivation of 7-DHC and its subsequent conversion to vitamin D3. Previous studies have indicated that 7-DHC might be one of the metabolites produced by probiotic bacteria (Li et al., 2023). In addition, other cream components, particularly lipids containing double bonds in their carbon skeletons, exhibit a high photosensitivity (Nam, 2011). The light emitted by the fluorescent lamp could promote free radical generation and initiate the lipid peroxidation (LPO) pathway (Schaich, 2005). LPO is an exothermic process that is accompanied by the release of energy. The produced energy might contribute to the transformation of 7-DHC into vitamin D3.

The results of the current study showed that static conditions were more favorable for vitamin D3 biosynthesis. Although mixing typically stimulates the growth of probiotic strains, the colloidal nature of fat globules played a crucial role in this case (Leser & Michel, 2008). Mixing can affect the size and distribution of milk fat globules in cream, affecting its homogeneity. Remarkably, lipids are well known for their ability to encapsulate various compound, including fat-soluble vitamins (Maurya & Aggarwal, 2019). Under static conditions, fat globules and the vitamin D3 precursor tend to accumulate at the cream's surface where they are most exposed to light. As a result, vitamin D3 enrichment was more efficient in non-mixing cream samples compared to those under mixing conditions.

Recently, the application of freeze-dried LAB cultures has become increasingly popular in the food industry. Dairy products, particularly cream, represent an ideal matrix for the delivery of probiotics due to their nutrient-rich composition and tendency for fermentation (Ningtyas et al., 2019). While LAB can tolerate mildly acidic conditions in fermented foods such as cream (pH 3.8–4.9), it is important to note that their survivability significantly decreases at pH below 2 (Bahlol, 2015; Shepard et al., 2013). Indeed, LAB strains are known for their diverse metabolic pathways and their ability to synthesize various valuable compounds, which expands the functional properties of products. Our studies confirmed that vitamin D3 is one such metabolite. Thus, cream supplemented with *L. plantarum* B/00401 strain provides a source of naturally produced vitamin D3, offering a better alternative to chemically synthesized supplements. Moreover, the biological methods are more cost-efficient and environmentally friendly compared to chemical synthesis (Revuelta et al., 2016).

As demonstrated, the freeze-dried *L. plantarum* B/00401 strain promoted enhanced vitamin D3 biosynthesis. The higher viability and extended shelf life of lyophilized probiotic formulations improve their functionality and health-promoting properties compared to the biomass form of the bacteria. Despite it, freeze-dried bacterial formulations are

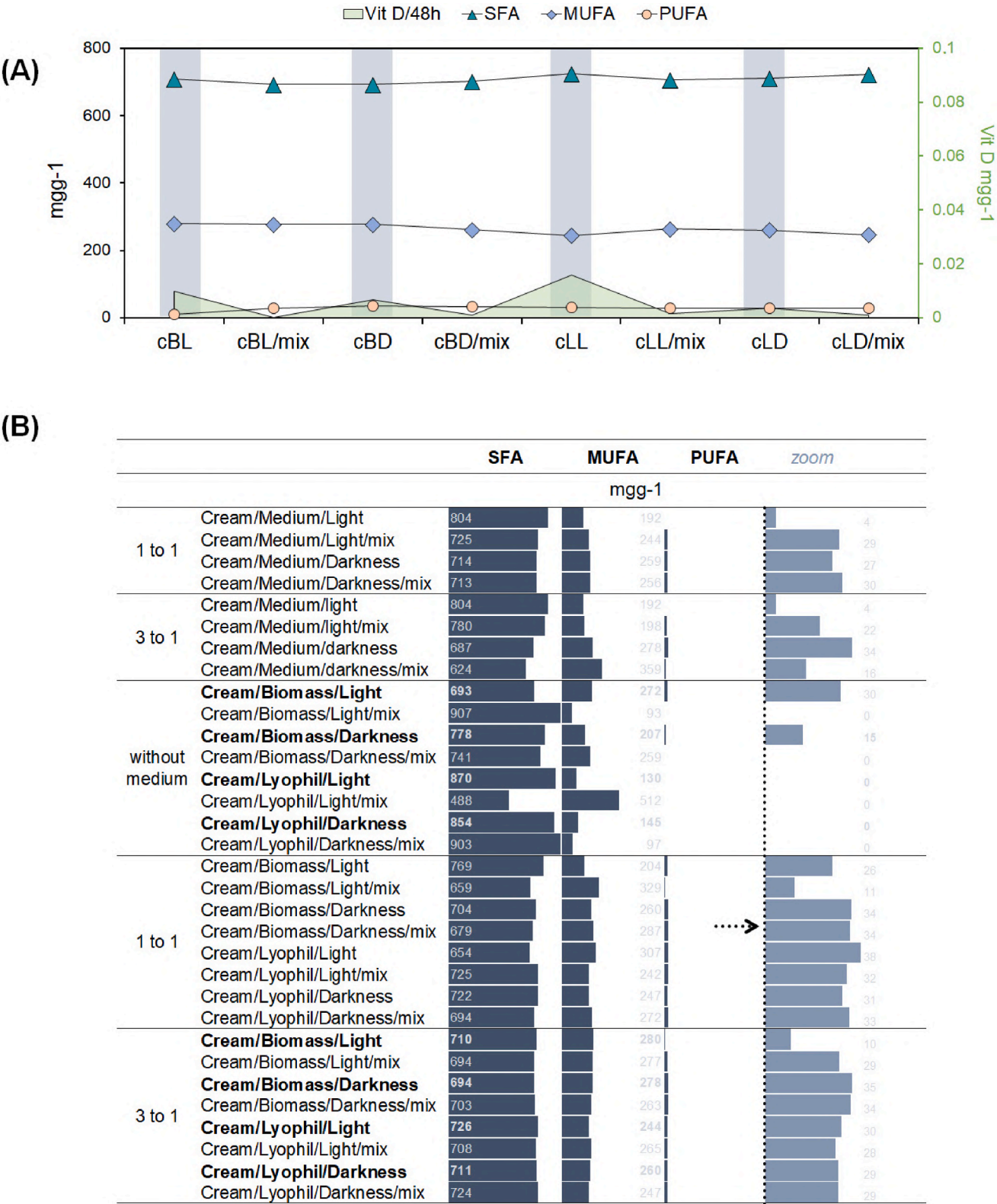


Fig. 2. (A) Relationship between FAs and vitamin D3 in cream/*L. plantarum* B/00401 at a 3:1 (v/v) ratio after 48 h, where the listed abbreviations are explained as follows: cBL is a cream incubated with *L. plantarum* biomass at light non-mixing conditions, cBL/mix is a cream incubated with *L. plantarum* biomass at light mixing conditions; cBD is a cream incubated with *L. plantarum* biomass at dark non-mixing conditions; cBD/mix is a cream incubated with *L. plantarum* biomass at dark mixing conditions; cLL is a cream incubated with *L. plantarum* lyophilisate at light non-mixing conditions; cLL/mix is a cream incubated with *L. plantarum* lyophilisate at light mixing conditions; cLD is a cream incubated with *L. plantarum* lyophilisate at dark non-mixing conditions; cLD/mix is a cream incubated with *L. plantarum* lyophilisate at dark mixing conditions. (B) Normalized bar graphs showing the content of SFA, MUFA and, PUFA in the designed experimental variants after 48 h of incubation. The zoom-in highlights PUFA content for better understanding.

Table 2
A table summarizing the overall results of the study.

Cream component	Highest content		Lowest content	
	Sample name	Content [mg g ⁻¹]	Sample name	Content [mg g ⁻¹]
Vitamin D3	cLL (3:1)	(15.96 ± 0.31 ^a) × 10 ⁻³	cBL/mix (1:1)	(4.0 ± 0.3) × 10 ⁻⁴
SFA	cBL (1:1)	769 ± 2.3 ^a	cLL (1:1)	654 ± 2.2 ^a
MUFA	cBL/mix (1:1)	329 ± 2.7 ^a	cBL (1:1)	204 ± 1.8 ^a
PUFA	cLL (1:1)	38 ± 2.5 ^a	cBL (3:1)	10 ± 0.9 ^a

^a SD – standard deviation.

more convenient for dosing and practical use, allowing for easier product standardization. On the other hand, the biofortification of cream with vitamin D3 using lyophilized probiotic bacteria may face several technological challenges, such as increased processing time and additional energy inputs, including the use of a freeze-drier and fluorescent lamp.

It is also worth noting that the stability of vitamin D3 is highly influenced by various factors, e.g. temperature, pH, medium, light exposure, and oxygen availability (Rakuša et al., 2021; Zareie et al., 2021). Indeed, the added LAB contribute to lactic acid production, which acidifies the cream (Bintsis, 2018). To prevent the degradation of vitamin D3, pH adjustment (pH > 5) is crucial to preserve its structure (Rakuša et al., 2021). In addition, Zareie et al. (2021) highlighted, that exposure to artificial light is a key factor contributing to the decrease of vitamin D3 content. In our study, light is essential for the production of vitamin D3; however, excessive light exposure can lead to vitamin D3 degradation, reducing the efficiency of its biosynthesis. Therefore, the duration of light exposure must be properly controlled. Moreover, practical aspects of enriching cream with vitamin D3, such as processing, storage, and packaging conditions, should be carefully considered in future developments.

4.2. The modification FA profile in the presence of *L. plantarum* B/00401

The analyzed cream represents a rich source of variable FAs, including SFA, MUFA, and PUFA. The composition of these FAs plays a valuable role in the product's nutritional value, stability, shelf life, texture, flavor, and health benefits (Temme et al., 2011). The proposed biotechnological approach resulted in significant modifications to the FA profile. The addition of *L. plantarum* B/00401 contributed to an increase in MUFA alongside a decrease in SFA content. Additionally, visible changes were observed in PUFA content. The highest content of PUFA was noticed in samples incubated with *L. plantarum* B/00401 biomass under dark conditions. Among FAs, PUFA are known to be the most susceptible to LPO, a process that can be triggered by light exposure (Liu et al., 2015).

It should be highlighted that the MRSB medium plays a key role in the metabolic activity of *L. plantarum*, modulating the content of produced FAs. As it was shown, PUFA were absent in all samples, except for cBL(w/MRSB) and cBD(w/MRSB). The content of SFA increased in cream samples without the added medium, except for cBL(w/MRSB) and cLL/mix(w/MRSB), while the opposite trend was observed for MUFA. For instance, cLL/mix(w/MRSB) had the lowest SFA content (488 mg g⁻¹) and the highest amount of MUFA (512 mg g⁻¹) among the samples. Numerous studies have previously confirmed the significance of the used culture medium on the bacterial metabolic activity and biodiversity of products, in particular FAs (Kim & Kim, 2017; Średnicka et al., 2023). Indeed, Markowiak-Kope and Slizewska (2020) reported a valuable impact of LAB fermentation on the increased production of SCFA metabolites, including acetic, propionic, lactic, butyric acid. Previous studies have also indicated that *L. plantarum* is able to convert PUFA, particularly linoleic acid, into FA derivatives (Aziz et al., 2021).

The observed changes in FA composition of cream after *L. plantarum* supplementation may offer substantial health benefits for consumers. Enrichment of food with MUFA helps normalize cholesterol levels, blood pressure, and blood glucose levels (Kim et al., 2023). Additionally, MUFA consumption has been associated with cancer prevention and improved immune function. In turn, reducing SFA intake positively contributes to lowering the risk of cardiovascular disease. Besides, numerous studies have highlighted the negative impact of SFA on gut microbiota diversity (Schoeler et al., 2023). Although the total elimination of SFA intake is impossible, most nutritionists recommend limiting solid fats in the daily diet (Vieira et al., 2015). In the previous studies, successful substitution of SFA with MUFA/PUFA has been achieved in dairy products (Givens, 2017). On the other hand, replacing SFA in the food industry may pose several challenges. Firstly, the presence of SFA enhances product stability and extends shelf life due to its greater resistance to LPO compared to MUFA and PUFA (Vieira et al., 2015). Secondly, reducing SFA can significantly impact the texture and consistency of dairy products, necessitating careful control of such modifications.

Understanding the relationship between vitamin D3 and FA content in cream is essential for developing high-quality products with appropriate nutritional value. It worth noting that FAs can modify the intestinal absorption of vitamin D3. For example, Gonçalves et al. (2013) highlighted the positive impact of MUFA on cholecalciferol absorption, while PUFA had the opposite effect. Similarly, Niramitmahapanya et al. (2011) observed increased serum levels of vitamin D3 in the presence of MUFA. On the other hand, Dawson-Hughes et al. (2015) indicated that both MUFA and PUFA played a negligible role in vitamin D3 absorption.

Remarkably, various studies have found out a relationship between vitamin D3 content and cholesterol levels. For instance, Surdu et al. (2021) noticed that an increase in vitamin D3 corresponded to a decrease in total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C), and triglycerides (TG) in blood serum. Thus, the level of cholesterol in cream, as a derivative of 7-DHC, might be indirectly correlated with the amount of produced vitamin D3.

5. Conclusions

In the current study, *L. plantarum* B/00401 strain showed great potential for fortifying cream with vitamin D3. The amount of produced vitamin D3 was highly dependent on various conditions, including light/dark exposure, mixing/non-mixing, the cream/bacteria ratio, and incubation time. Besides, the form of *L. plantarum* B/00401, whether biomass or lyophilisate, played a significant role in the enrichment of cream with vitamin D. The differences in metabolic activity between the two forms of *L. plantarum* B/00401 may be associated with their varying levels of survivability. Moreover, non-mixing conditions and light exposure were identified as the most valid criteria for enhancing vitamin D3 production, as 7-DHC is efficiently photoactivated and converted to vitamin D3 under these conditions. It is also worth noting that the addition of *L. plantarum* B/00401 led to modifications in the FA profile of the cream. The decrease in SFA was accompanied by an increase in MUFA content upon cream supplementation with the strain. SFA and MUFA contents varied slightly depending on the experimental conditions, while PUFA content was highly influenced by the presence of the MRSB culture medium and light exposure (mostly in the case of biomass). The changes observed in the FA composition of cream after *L. plantarum* B/00401 supplementation could potentially improve vitamin D3 bioavailability. Moreover, this remodeling of FA content in cream offers health benefits by reducing the risk of heart disease, although it may pose a risk to the product's shelf life. Future research could explore the use of compatible cryoprotectant to enhance the viability of the strain without interfering with the existing system. A well-designed probiotic-cryoprotectant system could deliver greater health benefits by boosting the metabolic activity of probiotic strains. Additionally, the potential application of encapsulated *L. plantarum*

should also be considered.

CRediT authorship contribution statement

Tetiana Dyrda-Teruiuk: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Viorica Railean:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Aleksandra Bogumiła Florkiewicz:** Writing – original draft, Formal analysis. **Justyna Walczak-Skierska:** Validation, Software, Investigation, Formal analysis. **Mateusz Kolankowski:** Investigation, Formal analysis. **Joanna Rudnicka:** Validation, Software, Investigation, Formal analysis. **Dorota Białczak:** Investigation. **Paweł Pomastowski:** Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Publikacja [P4]

Supplementary material

Table S1. Detailed information on the fatty acid composition of cream samples incubated under various experimental conditions.

Group of fatty acids	No.	Name of fatty acid	Structure	t _R [min]	mg g ⁻¹	RSD [%]
Control 1: Medium (MRSB)						
SFA	1	tetradecanoic acid	C14:0	26,67	23,4	0,1
	2	hexadecanoic acid	C16:0	31,06	550,9	0,2
	3	octadecanoic acid	C18:0	34,83	380,3	0,1
	total				954,6	0,1
MUFA	1	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	44,4	0,1
	total				44,4	0,1
PUFA	-	-	-	-	-	-
Control 2: Cream						
SFA	1	hexanoic acid	C6:0	6,48	56,3	0,5
	2	octanoic acid	C8:0	8,74	143,8	1,3
	3	pentadecanoic acid	C15:0	23,39	45,0	0,4
	4	decanoic acid	C10:0	11,32	34,1	0,8
	5	undecanoic acid	C11:0	12,93	9,0	0,1
	6	lauric acid	C12:0	14,90	21,1	0,9
	7	tridecanoic acid	C13:0	17,28	15,3	0,2
	8	octadecanoic acid	C18:0	34,82	10,1	2,5
	9	tetradecanoic acid	C14:0	20,15	15,1	3,5
	10	heptadecanoic acid	C17:0	30,82	73,0	2,0
	11	hexadecanoic acid	C16:0	27,05	32,2	2,1
	12	eicosanoic acid	C20:0	39,92	34,2	0,9
	13	eicosadienoic acid	C20:2	43,52	14,6	3,3
	14	docosanoic acid	C22:0	47,32	17,5	0,5
	15	tricosanoic acid	C23:0	50,66	3,1	0,1
	16	tetracosanoic acid	C24:0	51,50	24,2	3,0
	total				548,7	1,4
MUFA	1	<i>cis</i> -9-tetradecenoic acid	C14:1	21,29	24,5	0,8
	2	pentadecylic acid	C15:1	24,69	2,2	0,1
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	35,60	3,5	0,7
	4	<i>trans</i> -10-heptadecenoic acid	C17:1	31,81	40,0	1,1
	5	7-hexadecenoic acid	C16:1	27,95	8,1	2,1
	6	<i>cis</i> -11-eicosenoic acid	C20:1 n9	42,80	7,5	0,3
	7	<i>cis</i> -13-docosenoic acid	C22:1 n9	47,93	2,8	0,2
	total				88,6	0,7
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	37,36	45,9	3,4
	2	α -linolenic acid	C18:3 n3	38,57	21,8	1,0
	3	<i>cis</i> -13,16-docosadienoic acid	C22:2	49,92	9,3	0,2
	total				77,0	1,5
Cream/medium/light (1 to 1)						

SFA	1	decanoic acid	C10:0	17,30	25,2	0,5
	2	dodecanoic acid	C12:0	22,40	62,9	0,4
	3	tetradecanoic acid	C14:0	26,70	175,7	0,3
	4	pentadecanoic acid	C15:0	28,50	18,5	0,5
	5	octadecanoic acid	C18:0	34,80	93,0	0,2
	6	heptadecanoic acid	C17:0	32,50	152,8	0,5
	7	hexadecanoic acid	C16:0	31,10	275,9	0,6
	total				804,0	0,4
MUFA	1	<i>trans</i> -10-heptadecenoic acid	C17:1	32,60	3,2	0,8
	2	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,50	159,1	0,7
	3	<i>cis</i> -13-octadecenoic acid	C18:1 n9c	34,60	12,1	0,9
	4	7-hexadecenoic acid	C16:1	30,60	17,6	0,8
	total				192,0	0,8
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,30	3,2	0,0
	2	9,11, 13-octadecadienoic acid	C18:3	35,20	1,1	0,0
	total				4,3	0,0
Cream/medium/light/shaking (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	13,5	1,0
	2	decanoic acid	C10:0	17,31	51,1	0,9
	3	dodecanoic acid	C12:0	22,36	93,2	0,8
	4	tetradecanoic acid	C14:0	26,67	162,5	0,7
	5	pentadecanoic acid	C15:0	28,45	26,9	1,1
	6	hexadecanoic acid	C16:0	31,06	286,6	1,6
	7	heptadecanoic acid	C17:0	32,47	14,1	1,5
	8	octadecanoic acid	C18:0	34,83	77,1	1,9
	total				725,0	1,2
MUFA	1	4-decenoic acid	C10:1	17,12	5,2	0,6
	2	7-hexadecenoic acid	C16:1	30,64	20,1	0,8
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	175,6	1,0
	4	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,5	1,2
	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	35,9	0,9
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	3,7	0,7
	total				244,0	0,9
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	23,5	0,5
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,7	0,9
	total				29,2	0,7
Cream/medium/darkness (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	28,3	1,0
	2	decanoic acid	C10:0	17,31	53,3	1,5
	3	dodecanoic acid	C12:0	22,36	78,7	0,8
	4	12-methyl-tridecanoic acid	i-C14:0	26,15	3,4	0,3
	5	tetradecanoic acid	C14:0	26,67	121,2	0,9
	6	pentadecanoic acid	C15:0	28,45	33,0	1,6
	7	hexadecanoic acid	C16:0	31,06	276,1	1,8
	8	heptadecanoic acid	C17:0	32,47	20,5	1,7

	9	octadecanoic acid	C18:0	34,83	99,5	2,0
		total			714,0	1,6
MUFA	1	4-decenoic acid	C10:1	17,12	6,6	2,5
	2	7-hexadecenoic acid	C16:1	30,64	2,7	1,5
	3	9-hexadecanoic acid	C16:1	30,66	36,1	2,8
	4	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	4,3	3,1
	5	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	209,3	3,2
		total			259,0	2,6
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	26,6	2,0
		total			26,6	2,0
Cream/medium/darkness/mix (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	5,1	0,6
	2	decanoic acid	C10:0	17,31	44,0	0,9
	3	dodecanoic acid	C12:0	22,36	80,1	0,6
	4	tetradecanoic acid	C14:0	26,67	148,0	1,5
	5	pentadecanoic acid	C15:0	28,45	21,2	1,8
	6	hexadecanoic acid	C16:0	31,06	302,5	1,7
	7	heptadecanoic acid	C17:0	32,47	13,9	1,0
	8	octadecanoic acid	C18:0	34,83	98,2	0,9
		total			713,0	1,1
MUFA	1	4-decenoic acid	C10:1	17,12	3,0	0,9
	2	7-hexadecenoic acid	C16:1	30,64	20,4	1,8
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,5	1,7
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	196,0	2,0
	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	20,9	3,0
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	12,2	2,0
		total			256,0	1,9
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	25,2	0,7
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,2	0,9
		total			30,4	0,7
Cream/medium/light (3 to 1)						
SFA	1	hexanoic acid	C6:0	6,33	11,2	0,8
	2	octanoic acid	C8:0	11,76	21,6	0,9
	3	decanoic acid	C10:0	17,31	39,9	1,5
	4	dodecanoic acid	C12:0	22,36	68,4	1,4
	5	tetradecanoic acid	C14:0	26,67	121,3	2,5
	6	pentadecanoic acid	C15:0	28,45	20,2	2,9
	7	hexadecanoic acid	C16:0	31,06	224,7	1,8
	8	heptadecanoic acid	C17:0	32,47	231,8	0,9
	9	octadecanoic acid	C18:0	34,83	64,9	0,7
		total			804,0	1,5
MUFA	1	4-decenoic acid	C10:1	17,12	3,9	1,8
	2	7-hexadecenoic acid	C16:1	30,64	16,1	1,7
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	2,7	2,6
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	139,1	2,7

	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	9,0	2,4
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	21,2	3,3
				total	192,0	2,4
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	4,3	0,4
				total	4,3	0,4
Cream/medium/light/mix (3 to 1)						
SFA	1	hexanoic acid	C6:0	6,33	11,1	1,8
	2	octanoic acid	C8:0	11,76	10,3	1,2
	3	decanoic acid	C10:0	17,31	71,2	0,9
	4	dodecanoic acid	C12:0	22,36	219,4	2,7
	5	tridecanoic acid	C13:0	24,72	7,0	1,4
	6	12-methyl-tridecanoic acid	i-C14:0	26,15	9,0	0,7
	7	tetradecanoic acid	C14:0	26,67	95,5	1,4
	8	pentadecanoic acid	C15:0	28,45	91,2	2,5
	9	hexadecanoic acid	C16:0	31,06	15,9	3,0
	10	heptadecanoic acid	C17:0	32,47	20,3	2,4
	11	octadecanoic acid	C18:0	34,83	229,1	0,9
				total	780,0	1,7
MUFA	1	4-decenoic acid	C10:1	17,12	6,8	2,5
	2	7-hexadecenoic acid	C16:1	27,95	9,7	0,7
	3	9-hexadecanoic acid	C16:1	30,66	80,5	2,1
	4	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	11,7	1,4
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	89,5	0,9
				total	198,2	1,6
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,37	21,5	0,9
				total	21,5	0,9
Cream/medium/darkness (3 to 1)						
SFA	1	decanoic acid	C10:0	17,31	25,6	0,8
	2	dodecanoic acid	C12:0	22,36	65,0	0,6
	3	tetradecanoic acid	C14:0	26,67	137,7	0,7
	4	pentadecanoic acid	C15:0	28,45	21,4	1,6
	5	hexadecanoic acid	C16:0	31,06	303,4	1,2
	6	heptadecanoic acid	C17:0	32,47	24,8	0,7
	7	octadecanoic acid	C18:0	34,83	109,4	0,9
				total	687,3	0,9
MUFA	1	7-hexadecenoic acid	C16:1	30,64	19,7	0,8
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,9	1,6
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	210,1	1,2
	4	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	39,5	2,8
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	4,9	2,7
				total	278,1	1,8
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	27,7	0,8
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	6,7	1,3
				total	34,4	1,1
Cream/medium/darkness/mix (3 to 1)						

SFA	1	hexanoic acid	C6:0	6,33	8,4	1,5
	2	octanoic acid	C8:0	11,76	6,6	2,4
	3	tridecanoic acid	C13:0	24,72	3,0	3,6
	4	heptadecanoic acid	C17:0	32,47	4,0	3,5
	5	decanoic acid	C10:0	17,31	29,8	2,8
	6	pentadecanolic acid	C15:0	28,45	52,0	2,6
	7	dodecanoic acid	C12:0	22,36	85,6	1,0
	8	octadecanoic acid	C18:0	34,83	110,6	0,7
	9	hexadecanoic acid	C16:0	31,06	141,7	0,9
	10	tetradecanoic acid	C14:0	26,67	175,7	0,5
	11	12-methyl-tridecanoic acid	i-C14:0	26,15	3,8	0,7
	12	eicosanoic acid	C20:0	38,35	2,6	0,6
	total				623,8	1,8
MUFA	1	cis-9-tetradecenoic acid	C14:1	21,29	64,5	2,3
	2	4-decenoic acid	C10:1	17,12	10,1	0,4
	3	9-hexadecanoic acid	C16:1	30,66	87,2	1,2
	4	trans-10-heptadecenoic acid	C17:1	31,79	55,1	0,9
	5	cis-10-heptadecenoic acid	C17:1 n10c	32,55	20,0	1,6
	6	cis-13-octadecenoic acid	C18:1 n13c	34,62	86,1	0,5
		cis-11-eicosenoic acid	C20:1 n9	43,20	36,0	1,5
	total				359,0	1,2
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	34,29	12,5	3,5
	2	9,11, 13-octadecadienoic acid	C18:3	35,19	1,7	2,9
	3	5,8,11,14-eikosotetraenoic acid	C20:4	37,19	2,1	3,0
	total				16,3	3,1
Cream/biomass/light (without MRSB medium)						
SFA	1	decanoic acid	C10:0	17,31	27,8	0,9
	2	dodecanoic acid	C12:0	22,36	87,0	0,7
	3	tetradecanoic acid	C14:0	26,67	151,8	1,3
	4	pentadecanolic acid	C15:0	28,45	31,0	2,5
	5	hexadecanoic acid	C16:0	31,06	286,1	1,4
	6	heptadecanoic acid	C17:0	32,47	12,2	3,0
	7	octadecanoic acid	C18:0	34,83	97,1	1,2
	total				693,0	1,6
MUFA	1	4-decenoic acid	C10:1	17,12	2,8	0,9
	2	cis-10-heptadecenoic acid	C17:1 n10c	32,55	4,3	2,4
	3	9-hexadecanoic acid	C16:1	30,66	24,8	1,6
	4	cis-13-octadecenoic acid	C18:1 n13c	34,62	29,7	3,0
	5	trans-9-octadecenoic acid	C18:1 n9t	34,45	210,1	2,5
	total				271,7	2,1
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	34,27	22,5	2,0
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	7,4	1,5
	total				29,9	2,0
Cream/biomass/light/mix (without MRSB medium)						
SFA	1	dodecanoic acid	C12:0	22,36	2,3	0,4

	2	tetradecanoic acid	C14:0	26,67	15,4	0,7
	3	hexadecanoic acid	C16:0	31,06	202,6	1,8
	4	heptadecanoic acid	C17:0	32,47	601,9	2,1
	5	octadecanoic acid	C18:0	34,83	84,8	3,0
	total				907,0	1,6
MUFA	1	9-hexadecanoic acid	C16:1	30,66	8,7	1,2
	2	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	83,8	1,2
	total				92,5	1,2
PUFA	-	-	-	-	-	-
Cream/biomass/darkness (without MRSB medium)						
SFA	1	decanoic acid	C10:0	17,30	3,2	2,4
	2	dodecanoic acid	C12:0	22,40	29,8	3,0
	3	tetradecanoic acid	C14:0	26,70	443,6	1,3
	4	pentadecanolic acid	C15:0	28,50	3,4	2,4
	5	hexadecanoic acid	C16:0	31,10	276,3	3,0
	6	heptadecanoic acid	C17:0	32,50	15,0	1,4
	7	octadecanoic acid	C18:0	34,80	7,1	2,0
	total				778,4	2,2
MUFA	1	9-hexadecanoic acid	C16:1	30,70	47,9	0,9
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,60	123,5	3,0
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,50	35,1	1,4
	total				206,5	1,8
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,30	14,9	2,0
	total				14,9	2,0
Cream/biomass/darkness/mix (without MRSB medium)						
SFA	1	decanoic acid	C10:0	17,31	265,6	3,0
	2	dodecanoic acid	C12:0	22,36	110,9	2,9
	3	tetradecanoic acid	C14:0	26,67	225,9	1,5
	4	pentadecanolic acid	C15:0	28,45	99,4	3,0
	5	hexadecanoic acid	C16:0	31,06	21,5	2,4
	6	octadecanoic acid	C18:0	34,83	18,1	1,5
	total				741,4	2,4
MUFA	1	7-hexadecenoic acid	C16:1	30,64	23,4	2,4
	2	9-hexadecanoic acid	C16:1	30,66	17,5	3,0
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	217,7	2,9
	total				258,6	2,8
PUFA	-	-	-	-	-	-
Cream/Lyophil/light (without MRSB medium)						
SFA	1	dodecanoic acid	C12:0	22,36	15,0	0,5
	2	tetradecanoic acid	C14:0	26,67	35,2	3,0
	3	hexadecanoic acid	C16:0	31,06	573,6	2,4
	4	octadecanoic acid	C18:0	34,83	246,2	2,8
	total				870,0	2,2
MUFA	1	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	99,6	3,0
	2	9-dodecanoic acid		36,36	30,4	2,8
	total				130,0	2,9

PUFA	-	-	-	-	-	-
Cream/Lyophil/light/mix (without MRSB medium)						
SFA	1	dodecanoic acid	C12:0	36,36	4,6	2,7
	2	tridecanoic acid	C13:0	22,36	5,4	2,9
	3	12-methyl-tridecanoic acid	i-C14:0	26,67	33,5	3,5
	4	tetradecanoic acid	C14:0	34,45	52,0	1,4
	5	pentadecanoic acid	C15:0	34,83	195,4	3,8
	6	hexadecanoic acid	C16:0	38,60	197,1	2,7
	total				488,0	2,8
MUFA	1	trans-10-heptadecenoic acid	C17:1	30,63	287,1	2,8
	2	9-hexadecanoic acid	C16:1	31,06	224,9	2,4
	total				512,0	2,6
PUFA	-	-	-	-	-	-
Cream/Lyophil/darkness (without MRSB medium)						
SFA	1	dodecanoic acid	C12:0	22,36	10,4	1,4
	2	tetradecanoic acid	C14:0	26,67	31,0	2,6
	3	hexadecanoic acid	C16:0	31,06	537,5	1,7
	4	octadecanoic acid	C18:0	34,83	275,1	0,7
	total				854,0	1,6
MUFA	1	trans-10-heptadecenoic acid	C17:1	30,73	38,0	0,9
	2	trans-9-octadecenoic acid	C18:1 n9t	34,45	42,9	0,7
	3	cis-11-eicosenoic acid	C20:1 n9	43,00	64,1	0,5
	total				145,0	0,7
PUFA	-	-	-	-	-	-
Cream/Lyophil/darkness/mix (without MRSB medium)						
SFA	1	dodecanoic acid	C12:0	22,36	6,5	0,7
	2	tetradecanoic acid	C14:0	26,67	17,4	3,4
	3	hexadecanoic acid	C16:0	31,06	546,7	2,5
	4	octadecanoic acid	C18:0	34,83	332,4	3,7
	total				903,0	2,6
MUFA	1	9-hexadecanoic acid	C16:1	30,66	6,9	3,7
	2	trans-9-octadecenoic acid	C18:1 n9t	34,45	26,0	2,4
	3	cis-13-octadecenoic acid	C18:1 n13c	34,62	34,9	2,1
	4	cis-11-eicosenoic acid	C20:1 n9	42,92	28,9	3,7
	total				96,7	3,0
PUFA	-	-	-	-	-	-
Cream/biomass/light (1 to 1)						
SFA	1	decanoic acid	C10:0	17,31	9,2	0,7
	2	dodecanoic acid	C12:0	22,36	59,5	0,8
	3	tetradecanoic acid	C14:0	26,67	117,2	3,1
	4	pentadecanoic acid	C15:0	28,45	21,6	2,5
	5	hexadecanoic acid	C16:0	31,06	457,9	3,4
	6	heptadecanoic acid	C17:0	32,47	27,2	2,9
	7	octadecanoic acid	C18:0	34,83	76,4	3,0
	total				769,0	2,3
MUFA	1	cis-10-heptadecenoic acid	C17:1 n10c	32,55	3,0	0,7

	2	9-heksadecenoic acid	C16:1	30,66	16,9	0,8
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	147,8	3,8
	4	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	29,2	2,9
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	7,1	0,9
	total				204,0	1,8
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	35,16	5,3	1,6
	2	9,11, 13-octadecadienoic acid	C18:3	34,27	21,1	2,0
	total				26,4	1,8
Cream/biomass/light/mix (1 to 1)						
SFA	1	decanoic acid	C10:0	17,31	39,3	3,9
	2	dodecanoic acid	C12:0	22,36	92,4	2,6
	3	12-methyl-tridecanoic acid	i-C14:0	26,15	2,6	3,7
	4	tridecanoic acid	C13:0	24,72	3,5	3,1
	5	tetradecanoic acid	C14:0	26,67	156,7	2,8
	6	pentadecanoic acid	C15:0	28,45	49,5	2,1
	7	hexadecanoic acid	C16:0	31,06	215,5	1,6
	8	octadecanoic acid	C18:0	34,83	99,5	3,7
	total				659,0	2,9
MUFA	1	4-decenoic acid	C10:1	17,12	4,5	3,1
	2	9-hexadecenoic acid	C16:1	30,66	55,9	3,5
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	34,5	2,5
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	139,3	3,7
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	36,2	2,1
	6	<i>cis</i> -11-eicosenoic acid	C20:1 n9	42,92	48,2	2,4
	7	<i>cis</i> -13-docosenoic acid	C22:1 n9	47,93	10,8	1,5
	total				329,4	2,7
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	11,4	1,5
	total				11,4	1,5
Cream/biomass/darkness (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	4,5	1,7
	2	decanoic acid	C10:0	17,31	38,3	2,4
	3	dodecanoic acid	C12:0	22,36	85,8	3,5
	4	tetradecanoic acid	C14:0	26,67	152,8	2,1
	5	pentadecanoic acid	C15:0	28,45	27,0	1,4
	6	hexadecanoic acid	C16:0	31,06	296,5	3,0
	7	heptadecanoic acid	C17:0	32,47	0,2	2,8
	8	octadecanoic acid	C18:0	34,83	98,9	1,0
	total				704,0	2,2
MUFA	1	4-decenoic acid	C10:1	17,12	3,6	1,1
	2	9-heksadecenoic acid	C16:1	30,66	21,6	2,0
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,9	3,0
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	202,1	1,7
	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	24,6	2,0
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	4,6	0,9
	total				260,4	1,8

PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	27,5	0,7
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	6,8	0,4
	total				34,3	0,7
Cream/biomass/darkness/mix (1 to 1)						
SFA	1	decanoic acid	C10:0	17,31	28,9	0,7
	2	dodecanoic acid	C12:0	22,36	57,5	3,2
	3	tetradecanoic acid	C14:0	26,67	137,4	1,4
	4	pentadecanolic acid	C15:0	28,45	25,7	2,6
	5	hexadecanoic acid	C16:0	31,06	303,2	2,4
	6	octadecanoic acid	C18:0	34,83	126,2	1,2
	total				678,9	1,9
MUFA	1	7-hexadecanoic acid		30,64	20,7	0,9
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	4,1	0,7
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	209,2	0,5
	4	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,60	43,6	1,2
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	9,8	1,9
	total				287,4	1,0
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	25,8	2,0
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	7,7	2,5
	total				33,5	2,0
Cream/Lyophil/light (1 to 1)						
SFA	1	heptadecanoic acid	C17:0	32,47	14,5	1,3
	2	pentadecanolic acid	C15:0	28,45	16,5	3,7
	3	octanoic acid	C8:0	11,76	19,0	2,4
	4	decanoic acid	C10:0	17,31	34,3	1,0
	5	octadecanoic acid	C18:0	34,83	54,0	0,9
	6	dodecanoic acid	C12:0	22,36	56,0	2,5
	7	tetradecanoic acid	C14:0	26,67	95,5	2,5
	8	hexadecanoic acid	C16:0	31,06	364,2	3,0
	total				654,0	2,2
MUFA	1	4-decenoic acid	C10:1	17,12	3,2	0,9
	2	7-hexadecenoic acid	C16:1	30,64	23,7	0,7
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	1,6	0,6
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	232,1	2,0
	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,6	28,3	2,4
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	18,1	2,1
	total				307,0	1,5
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	30,5	2,5
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	7,4	3,0
	total				37,9	2,5
Cream/Lyophil/light/mix (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	4,1	1,6
	2	decanoic acid	C10:0	17,31	44,6	1,4
	3	dodecanoic acid	C12:0	22,36	106,9	1,9
	4	tetradecanoic acid	C14:0	26,67	157,8	2,0

	5	pentadecanoic acid	C15:0	28,45	26,4	3,8
	6	hexadecanoic acid	C16:0	31,06	284,7	3,4
	7	heptadecanoic acid	C17:0	32,47	14,7	0,9
	8	octadecanoic acid	C18:0	34,83	85,8	0,7
	total				725,0	2,0
MUFA	1	4-decenoic acid	C10:1	17,12	4,2	0,7
	2	7-hexadecenoic acid	C16:1	30,64	21,1	2,5
	3	cis-10-heptadecenoic acid	C17:1 n10c	32,55	3,6	3,0
	4	trans-9-octadecenoic acid	C18:1 n9t	34,45	174,5	2,4
	5	cis-11-octadecenoic acid	C18:1 n11c	34,6	33,8	2,7
	6	cis-13-octadecenoic acid	C18:1 n13c	34,62	4,8	1,5
	total				242,0	2,1
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	34,27	26,5	2,5
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,7	3,7
	total				32,2	2,5
Cream/Lyophil/darkness (1 to 1)						
SFA	1	octanoic acid	C8:0	11,76	29,0	2,4
	2	decanoic acid	C10:0	17,31	51,1	1,7
	3	dodecanoic acid	C12:0	22,36	87,2	3,0
	4	tetradecanoic acid	C14:0	26,67	147,4	2,9
	5	pentadecanoic acid	C15:0	28,45	26,1	2,7
	6	hexadecanoic acid	C16:0	31,06	280,5	3,0
	7	heptadecanoic acid	C17:0	32,47	14,6	1,7
	8	octadecanoic acid	C18:0	34,83	86,1	2,0
	total				722,0	2,4
MUFA	1	4-decenoic acid	C10:1	17,12	5,0	0,9
	2	7-hexadecenoic acid	C16:1	30,64	19,4	0,7
	3	cis-10-heptadecenoic acid	C17:1 n10c	32,55	3,5	3,0
	4	trans-9-octadecenoic acid	C18:1 n9t	34,45	175,4	2,5
	5	cis-11-octadecenoic acid	C18:1 n11c	34,6	36,9	3,4
	7	cis-13-octadecenoic acid	C18:1 n13c	34,62	6,8	1,0
	total				247,0	1,9
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	34,27	24,6	2,0
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,9	2,5
	total				30,5	2,3
Cream/Lyophil/darkness/mix (1 to 1)						
SFA	1	hexanoic acid	C6:0	6,33	3,4	1,5
	2	octanoic acid	C8:0	11,76	5,7	2,4
	3	decanoic acid	C10:0	17,31	19,9	3,0
	4	dodecanoic acid	C12:0	22,36	72,3	3,2
	5	tetradecanoic acid	C14:0	26,67	141,3	1,4
	6	pentadecanoic acid	C15:0	28,45	20,4	2,0
	7	hexadecanoic acid	C16:0	31,06	315,6	3,4
	8	heptadecanoic acid	C17:0	32,47	15,0	0,9
	9	octadecanoic acid	C18:0	34,83	100,4	0,9

				total	694,0	2,1
MUFA	1	7-hexadecenoic acid	C16:1	30,64	20,0	0,7
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,5	2,3
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	213,8	1,2
	4	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,6	34,7	2,3
				total	272,0	1,6
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	27,4	1,6
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,7	1,7
				total	33,1	1,7
Cream/biomass/light (3 to 1)						
SFA	1	decanoic acid	C10:0	17,31	267,8	0,8
	2	dodecanoic acid	C12:0	22,36	80,0	0,7
	3	tridecanoic acid	C13:0	24,72	2,8	1,5
	4	12-methyl-tridecanoic acid	i-C14:0	26,15	4,3	2,4
	5	12-methyl-tetradecanoic acid	i-C14:0	28,5	85,9	2,1
	6	tetradecanoic acid	C14:0	26,67	36,9	2,0
	7	pentadecanoic acid	C15:0	28,45	115,7	1,0
	8	hexadecanoic acid	C16:0	31,06	89,9	0,9
	9	heptadecanoic acid	C17:0	32,47	20,7	0,2
	10	octadecanoic acid	C18:0	34,83	5,8	0,6
				total	709,8	1,2
MUFA	1	4-decenoic acid	C10:1	17,12	3,1	3,0
	2	9-heksadecenoic acid	C16:1	30,66	88,6	2,0
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	94,2	1,2
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	94,4	0,8
				total	280,3	1,8
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	9,9	0,9
				total	9,9	0,9
Cream/biomass/light/mix (3 to 1)						
SFA	1	octanoic acid	C8:0	11,76	8,4	1,2
	2	decanoic acid	C10:0	17,31	35,7	3,0
	3	dodecanoic acid	C12:0	22,36	69,7	2,5
	4	tetradecanoic acid	C14:0	26,67	148,4	1,4
	5	pentadecanoic acid	C15:0	28,45	27,3	2,7
	6	hexadecanoic acid	C16:0	31,06	254,2	2,1
	7	heptadecanoic acid	C17:0	32,47	51,2	3,0
	8	octadecanoic acid	C18:0	34,83	98,6	1,2
				total	693,5	2,1
MUFA	1	4-decenoic acid	C10:1	17,12	3,8	2,1
	2	9-heksadecenoic acid	C16:1	30,66	21,9	1,4
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	4,3	1,9
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	210,1	3,0
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	37,1	1,4
				total	277,2	2,0
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	20,8	2,0

	2	9,11, 13-octadecadienoic acid	C18:3	35,16	8,5	3,0
				total	29,3	3,0
Cream/biomass/darkness (3 to 1)						
SFA	1	decanoic acid	C10:0	17,31	19,7	0,7
	2	dodecanoic acid	C12:0	22,36	70,4	2,0
	3	tetradecanoic acid	C14:0	26,67	144,3	2,0
	4	pentadecanoic acid	C15:0	28,45	27,0	1,6
	5	hexadecanoic acid	C16:0	31,06	298,6	1,6
	6	heptadecanoic acid	C17:0	32,47	17,1	0,9
	7	octadecanoic acid	C18:0	34,83	116,4	0,7
				total	693,5	1,4
MUFA	1	9-heksadecenoic acid	C16:1	30,66	21,4	0,9
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	3,8	0,4
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	204,4	2,5
	4	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,6	39,7	2,1
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	9,1	1,9
				total	278,4	1,6
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	27,3	0,2
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	7,3	0,2
				total	34,6	0,2
Cream/biomass/darkness/mix (3 to 1)						
SFA	1	decanoic acid	C10:0	17,3	30,3	0,2
	2	dodecanoic acid	C12:0	22,4	80,4	0,2
	3	tetradecanoic acid	C14:0	26,7	145,5	0,4
	4	pentadecanoic acid	C15:0	28,5	26,9	0,6
	5	hexadecanoic acid	C16:0	31,1	288,3	1,8
	6	heptadecanoic acid	C17:0	32,5	11,7	1,1
	7	octadecanoic acid	C18:0	34,8	119,9	0,2
				total	703,0	0,6
MUFA	1	9-heksadecenoic acid	C16:1	30,7	21,3	3,4
	2	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,6	4,0	2,6
	3	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,5	202,8	3,5
	4	7-hexadecenoic acid	C16:1	27,6	26,2	2,8
	5	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,6	8,8	0,3
				total	263,1	2,5
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,3	26,6	0,6
	2	9,11, 13-octadecadienoic acid	C18:3	35,2	7,2	0,1
				total	33,8	0,4
Cream/Lyophil/light (3 to 1)						
SFA	1	octanoic acid	C8:0	11,76	27,4	0,4
	2	decanoic acid	C10:0	17,31	50,6	2,6
	3	dodecanoic acid	C12:0	22,36	86,7	3,0
	4	tetradecanoic acid	C14:0	26,67	154,1	2,1
	5	pentadecanoic acid	C15:0	28,45	25,6	2,4
	6	hexadecanoic acid	C16:0	31,06	285,5	3,0

	7	heptadecanoic acid	C17:0	32,47	13,7	1,5
	8	octadecanoic acid	C18:0	34,83	82,4	3,1
				total	726,0	2,3
MUFA	1	4-decenoic acid	C10:1	17,12	5,0	0,8
	2	7-hexadecenoic acid	C16:1	30,64	20,5	0,7
	3	cis-10-heptadecenoic acid	C17:1 n10c	32,55	3,5	3,0
	4	trans-9-octadecenoic acid	C18:1 n9t	34,45	176,7	2,4
	5	cis-11-octadecenoic acid	C18:1 n11c	34,6	11,4	3,0
	6	cis-13-octadecenoic acid	C18:1 n13c	34,62	26,9	1,0
				total	244,0	1,8
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	34,27	24,6	0,6
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,4	0,6
				total	30,0	0,6
Cream/Lyophil/light/mix (3 to 1)						
SFA	1	hexanoic acid	C6:0	6,33	3,4	2,5
	3	octanoic acid	C8:0	11,76	5,2	1,3
	4	decanoic acid	C10:0	17,31	28,6	1,4
	5	dodecanoic acid	C12:0	22,36	76,8	3,0
	6	tetradecanoic acid	C14:0	26,67	142,0	3,7
	7	pentadecanolic acid	C15:0	28,45	22,3	2,6
	8	hexadecanoic acid	C16:0	31,06	326,2	1,4
	9	heptadecanoic acid	C17:0	32,47	14,0	3,6
	10	octadecanoic acid	C18:0	34,83	89,5	1,5
				total	708,0	2,3
MUFA	1	cis-9-tetradecenoic acid	C14:1	21,3	17,4	1,4
	2	trans-9-octadecenoic acid	C18:1 n9t	34,45	236,4	0,9
	3	cis-11-eicosenoic acid	C20:1 n9	41	11,2	1,4
				total	265,0	1,2
PUFA	1	cis,cis-9,12-octadecadienoic acid	C18:2	35,16	5,0	0,9
	2	9,11, 13-octadecadienoic acid	C18:3	34,27	22,7	0,4
				total	27,7	0,7
Cream/Lyophil/darkness (3 to 1)						
SFA	1	octanoic acid	C8:0	11,76	9,4	0,7
	2	decanoic acid	C10:0	17,31	39,2	3,2
	3	dodecanoic acid	C12:0	22,36	80,3	1,5
	4	tetradecanoic acid	C14:0	26,67	147,2	2,7
	5	pentadecanolic acid	C15:0	28,45	20,4	1,5
	6	hexadecanoic acid	C16:0	31,06	312,5	0,9
	7	heptadecanoic acid	C17:0	32,47	10,6	0,4
	8	octadecanoic acid	C18:0	34,83	91,4	3,1
				total	711,0	1,8
MUFA	1	4-decenoic acid	C10:1	17,12	3,1	0,7
	2	7-hexadecenoic acid	C16:1	30,64	19,2	2,5
	3	cis-10-heptadecenoic acid	C17:1 n10c	32,55	3,5	3,0
	4	trans-9-octadecenoic acid	C18:1 n9t	34,45	200,6	3,7

	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,6	21,2	2,1
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	12,4	2,0
				total	260,0	2,3
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	35,16	5,7	0,9
	2	9,11, 13-octadecadienoic acid	C18:3	34,27	23,4	0,9
				total	29,1	0,9
Cream/Lyophil/darkness/mix (3 to 1)						
SFA	1	octanoic acid	C8:0	11,76	7,2	0,7
	2	decanoic acid	C10:0	17,31	26,9	0,6
	3	dodecanoic acid	C12:0	22,36	121,2	0,6
	4	tetradecanoic acid	C14:0	26,67	201,9	0,7
	5	pentadecanoic acid	C15:0	28,45	26,2	1,5
	6	hexadecanoic acid	C16:0	31,06	302,9	0,8
	7	heptadecanoic acid	C17:0	32,47	7,4	0,9
	8	octadecanoic acid	C18:0	34,83	30,3	1,0
				total	724,0	0,9
MUFA	1	4-decenoic acid	C10:1	17,12	16,2	3,8
	2	7-hexadecenoic acid	C16:1	30,64	28,3	3,4
	3	<i>cis</i> -10-heptadecenoic acid	C17:1 n10c	32,55	4,0	0,9
	4	<i>trans</i> -9-octadecenoic acid	C18:1 n9t	34,45	145,5	2,1
	5	<i>cis</i> -11-octadecenoic acid	C18:1 n11c	34,6	18,3	2,6
	6	<i>cis</i> -13-octadecenoic acid	C18:1 n13c	34,62	34,7	0,6
				total	247,0	2,2
PUFA	1	<i>cis,cis</i> -9,12-octadecadienoic acid	C18:2	34,27	23,0	0,6
	2	9,11, 13-octadecadienoic acid	C18:3	35,16	5,9	0,6
				total	28,9	0,6

Where: SFA is saturated fatty acid, MUFA is monounsaturated fatty acid, PUFA is polyunsaturated fatty acid; t_R is retention time; RSD is relative standard deviation.

Table S2. Changes in vitamin D3 content in cream samples incubated under different conditions over 48 h.

Cream to bacteria ratio (v/v)	Abbreviation	Full name	$\mu\text{g g}^{-1}$		
			6h	24h	48h
Control samples	cL	cream/Light	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$
	cL/mix	cream/Light/mix	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$
	cD	cream/Darkness	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$
	cD/mix	cream/Darkness/mix	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$
1 to 1	cBL	cream/Biomass/Light	$0,00 \pm 0,00^*$	$0,00 \pm 0,00^*$	$1,06 \pm 0,01^*$
	cBL/mix	cream/Biomass/Light/mix	$0,00 \pm 0,00^*$	$0,05 \pm 0,01^*$	$0,40 \pm 0,03^*$
	cBD	cream/Biomass/Darkness	$0,10 \pm 0,01^*$	$0,70 \pm 0,00^*$	$0,89 \pm 0,02^*$
	cBD/mix	cream/Biomass/Darkness/mix	$0,18 \pm 0,01^*$	$0,70 \pm 0,01^*$	$1,02 \pm 0,02^*$
	cLL	cream/Lyophil/Light	$0,00 \pm 0,00^*$	$0,48 \pm 0,03^*$	$1,39 \pm 0,31^*$
	cLL/mix	cream/Lyophil/Light/mix	$0,00 \pm 0,00^*$	$0,07 \pm 0,02^*$	$0,76 \pm 0,01^*$
	cLD	cream/Lyophil/Darkness	$0,00 \pm 0,00^*$	$0,08 \pm 0,02^*$	$0,64 \pm 0,01^*$
	cLD/mix	cream/Lyophil/Darkness/mix	$0,53 \pm 0,02^*$	$1,36 \pm 0,22^*$	$1,71 \pm 0,00^*$
3 to 1	cBL	cream/Biomass/Light	$0,72 \pm 0,01^*$	$0,89 \pm 0,01^*$	$9,81 \pm 0,01^*$
	cBL/mix	cream/Biomass/Light/mix	$0,02 \pm 0,01^*$	$0,50 \pm 0,01^*$	$0,99 \pm 0,02^*$
	cBD	cream/Biomass/Darkness	$1,06 \pm 0,03$	$1,88 \pm 0,02^*$	$6,58 \pm 0,01^*$
	cBD/mix	cream/Biomass/Darkness/mix	$0,34 \pm 0,02^*$	$0,65 \pm 0,02^*$	$1,04 \pm 0,00^*$
	cLL	cream/Lyophil/Light	$2,01 \pm 0,02^*$	$2,12 \pm 0,31^*$	$15,96 \pm 0,31^*$
	cLL/mix	cream/Lyophil/Light/mix	$0,46 \pm 0,31^*$	$0,45 \pm 0,02^*$	$1,48 \pm 0,02^*$
	cLD	cream/Lyophil/Darkness	$0,98 \pm 0,01^*$	$1,01 \pm 0,01^*$	$3,64 \pm 0,01^*$
	cLD/mix	cream/Lyophil/Darkness/mix	$0,00 \pm 0,00^*$	$0,32 \pm 0,00^*$	$0,84 \pm 0,00^*$

Where: control cream samples incubated at light non-mixing (cL), mixing (cL/mix), dark non-mixing (cD) and mixing conditions (cD/mix); cream samples incubated with *L. plantarum* biomass at 1:1 (v/v) ratio at light non-mixing (cBL (1:1)), mixing (cBL/mix (1:1)), dark non-mixing (cBD (1:1)) and mixing conditions (cBD/mix (1:1)); cream samples incubated with *L. plantarum* lyophilisate at 1:1 (v/v) ratio at light non-mixing (cLL (1:1)), mixing (cLL/mix (1:1)), dark non-mixing (cLD (1:1)) and mixing conditions (cLD/mix (1:1)); cream samples incubated with *L. plantarum* biomass at 3:1 (v/v) ratio at light non-mixing (cBL (3:1)), mixing (cBL/mix (3:1)), dark non-mixing (cBD (3:1)) and mixing conditions (cBD/mix (3:1)); cream samples incubated with *L. plantarum* lyophilisate at 3:1 (v/v) ratio at light non-mixing (cLL (3:1)), mixing (cLL/mix (3:1)), dark non-mixing (cLD (3:1)) and mixing conditions (cLD/mix (3:1)); * is standard deviation (SD).

[P5] Lactoferrin as an Active Coordination Scaffold for Ruthenium(III)

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Lactoferrin as an active coordination scaffold for ruthenium(III)

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This study sought to elucidate the molecular principles by which bovine lactoferrin (bLF) governs ruthenium(III) coordination and to assess how protein-guided binding translates into structural and biological effects. The protein was modified using potassium aquapentachlororuthenate(III) ($K_2[RuCl_5(H_2O)]$) as a metal precursor. The obtained LF–Ru systems were comprehensively examined using spectroscopic (spectrofluorimetry, DLS, ATR-FTIR, SRCD), spectrometric (ICP-OES), microscopic (LM, SEM-EDS, TEM-EDS), scattering (SAXS), and electrophoretic (SDS-PAGE) techniques. Antibacterial activity was evaluated for LF and LF–100Ru against Gram-positive (*Enterococcus faecalis*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*) bacteria. The cellular response to LF–Ru complexes was investigated using murine fibroblast (L929), human hepatocellular carcinoma (HepG2), and human colorectal adenocarcinoma (Caco-2) cell lines. Molecular docking indicated favorable Ru(III) binding in histidine-containing regions related to native metal-binding motifs. Molecular dynamics simulations supported conformational stability upon metal binding, whereas quantum-mechanical calculations on simplified residue models were used only to assess qualitative donor preferences. The adsorption isotherm was better described by the Langmuir model, indicating saturation-like binding of Ru(III) to accessible LF regions at low and moderate loading. At the highest precursor concentrations, BET-style analysis together with TEM observations suggested additional secondary Ru accumulation within the protein matrix. Fluorescence quenching demonstrated strong interactions in the LF–Ru system ($K_a = 1.789 \times 10^4 \text{ M}^{-1}$). Desorption studies indicated only minor metal release from LF–100Ru after incubation in simulated gastric and intestinal fluids. Importantly, LF–100Ru reduced the viability of HepG2 and Caco-2 cells while showing comparatively low toxicity toward normal L929 fibroblasts under the tested conditions, indicating a differential cellular response.

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1. Introduction

Protein–metal interactions play a significant role in biological systems by controlling catalysis, oxygen transport and storage, electron transfer, structural organization of biomolecules, and cellular signaling and regulatory processes.¹ Understanding the mechanisms of molecular interactions in protein–metal systems is of great value for the design and development of promising therapeutic strategies.²

Ruthenium (Ru) complexes are recognized by their wide therapeutic spectrum, including antitumor, antibacterial, and antioxidant activities.^{3,4} The multiplicity of Ru oxidation states (from –II to +VIII) enables the synthesis of diverse complexes with a broad range of biological interactions and new mechanisms of action, including DNA damage, ROS-mediated apoptosis, and antimetastatic activity.⁵ Among them, Ru(II) and Ru(III) coordination compounds are considered the most appli-



cable due to them having the highest chemical stability. Lastly, Ru(III) complexes are considered a better alternative to commonly implemented platinum drugs for cancer treatment, which is mainly attributed to their lower toxicity and better drug resistance.^{6,7} However, the most promising Ru(III) drug candidates, namely NAMI-A and KP1019, did not achieve the expected efficiency in clinical chemotherapy studies due to severe side effects and limited bioavailability.⁸ A series of limitations necessitated the search for safer and at the same time efficient strategies for cancer treatment.

Targeted therapy is a relatively new and advanced approach in cancer therapy that is not as widely implemented as chemotherapy. The key principle of targeted therapy is based on drugs interacting with specific molecular targets (proteins, *e.g.* receptors, enzymes, factors) responsible for the survival and proliferation of cancer cells.⁹ Several advantages offered by targeted therapy, such as improved selectivity and better tolerability by the patient, enabled the development of diverse drug-delivery systems. In this case, a series of emerging challenges related to targeting efficiency, stability of delivery systems, and their biocompatibility must be overcome.

In recent decades, bovine lactoferrin (bLF), a member of the transferrin family of proteins, has been recognized as a potential carrier for biologically active substances, *e.g.* metal ions, nanoparticles, fatty acids, nucleic acids, lipopolysaccharides, vitamins, and other proteins.^{10,11} Additionally, bLF is known to be a biodegradable, non-toxic, and non-immunogenic protein that shows antibacterial, antiviral, antioxidant, anti-inflammatory and anticancer activities.¹² This diverse spectrum of biological properties makes bLF an attractive candidate for biomedical applications. It is well defined as a large glycoprotein ($M_w \sim 77\text{--}91$ kDa) characterized by a high binding affinity for iron ($K_A \sim 10^{20} \text{ M}^{-1}$).^{10,13} Therefore, LF naturally acts as an iron carrier, enabling its efficient intestinal absorption through a receptor-mediated endocytosis mechanism.¹⁴

LF receptors (LFRs) are expressed on the surface of various types of cells, *e.g.* intestinal epithelial cells, hepatocytes, fibroblasts, lymphocytes, neurons, neutrophils, and macrophages.¹⁵ What is important is that the overexpression of LFRs in cancer cells has been reported, suggesting high potential of LF as a drug carrier for targeted anticancer therapy.¹⁶ It is worthwhile noting the ability of ruthenium to mimic iron for binding to transferrin family proteins; thus it might be assumed that Ru uptake occurs in a similar way to that of Fe – by receptor-mediated endocytosis. Previous studies have primarily focused on investigating interactions between Ru(III) complexes and transferrin (TF); however, information regarding the LF–Ru binding mechanism remains limited.¹⁷ It was shown that KP1019 coordinated to the specific iron-binding sites of human apo-transferrin (apo-hTF) in a 2:1 molar ratio, whereas at higher molar ratios, it bound non-specifically.¹⁸ Analogously, Levina *et al.* noticed that KP1019 competed for the iron-binding site in holo-TF, which led to disruption interactions between TF and the TF receptor (TFR), resulting in poor metal uptake.¹⁹ In contrast, Wang *et al.* demonstrated that TF-mediated uptake of Ru(III) by human Caucasian pro-

myelocytic leukemia (HL-60) cells was not affected by coordinated Fe(III), as the two metals occupied distinct binding sites.²⁰ Although LF and TF belong to the same protein family and share about 60% sequence homology, their structural differences are substantial.²¹ For instance, iron release from LF occurs approximately 100 times more slowly than from TF; thus its structure shows a higher resistance to proteolytic digestion.²² Besides, the isoelectric point of LF is considerably higher (approximately 8–9) compared to TF, which is in the range of around 5–6.²³ The protein isoelectric point significantly affects surface charge properties and, consequently, the tendency to engage in electrostatic interactions with other molecules.²⁴ Thus, the design of LF–Ru complexes will allow for significant improvement of the delivery of Ru(III) complexes through receptor-mediated pathways.²⁵ Importantly, the design of LF–Ru complexes may provide new insights into both the structural characteristics and biological activity of the functionalized biomolecule.

This study aims to uncover the molecular principles governing Ru(III) coordination within LF and to evaluate how such protein-guided binding translates into structural and biological outcomes. For this purpose, potassium aquapentachlororuthenate(III) ($\text{K}_2[\text{RuCl}_5(\text{H}_2\text{O})]$) was selected as a precursor. In contrast to commonly used Ru(III) precursors such as ruthenium(III) chloride hydrate ($\text{RuCl}_3 \cdot x\text{H}_2\text{O}$), $\text{K}_2[\text{RuCl}_5(\text{H}_2\text{O})]$ is a well-defined coordination complex with enhanced hydrolytic stability in aqueous solution and reduced tendency toward uncontrolled polymerization.^{26–28} Consequently, its use for LF modification provides greater control and reproducibility in binding studies and the preparation of LF–Ru complexes. The given Ru(III) complex was not previously recognized by any specific biological properties; however, it is characterized by high solubility in water and compactness, and therefore we assume it may be easily bound within the LF biomolecule. The nature of interactions in the synthesized LF–Ru complexes was analyzed by a number of spectroscopic (fluorescence spectroscopy, ATR-FTIR, DLS), spectrometric (ICP-OES), scattering (SAXS), microscopic (LM, SEM-EDS, TEM-EDS), and electromigration (SDS-PAGE) techniques. The application of a number of computational methods, including molecular docking, molecular dynamics (MD), and density functional theory (DFT), enabled filling the gap with experimental data. The biological activity of the LF–Ru complexes was assessed by testing their antibacterial properties against Gram-negative (*E. coli*, *K. pneumoniae*) and Gram-positive (*E. faecalis*) bacteria. The impact of the LF–Ru complexes on cell viability along with their cytotoxicity was examined on several cell lines, including murine fibroblast (L929), human hepatocellular carcinoma (HepG2), and human colorectal adenocarcinoma (Caco-2).

2. Experimental

2.1. Materials

The bovine lactoferrin (bLF) used for analysis was the same as in the previous study.²⁹ Potassium aquapentachlororuthenate



(III) ($\text{K}_2[\text{RuCl}_5(\text{H}_2\text{O})]$), glacial acetic acid (CH_3COOH), and sodium acetate (CH_3COONa) were purchased from Sigma Aldrich (St Louis, Missouri, USA).

2.2. Batch isotherm study of Ru(III) binding to LF

The mechanism of LF–Ru binding was analyzed by using a batch sorption approach. In this study, potassium aquapentachlororuthenate(III) was applied as the ruthenium precursor. Appropriate amounts of the Ru(III) complex were dissolved in 0.1 M acetic buffer (pH 4) at the following concentrations: 1.25, 2.5, 5, 10, 25, 50, 75, 100, 150, 250, and 500 mg L^{-1} . Similarly, LF was suspended in acetic buffer (pH 4) at a concentration of 1000 mg L^{-1} and then mixed with the Ru(III) complex solution at a ratio of 1:1 (v/v). The Ru-to-protein molar ratios in the respective mixtures were 0.27/1, 0.53/1, 1.07/1, 2.14/1, 5.34/1, 10.68/1, 16.02/1, 21.36/1, 32.04/1, 53.40/1, and 106.79/1 (mol Ru per mol LF). The reaction mixture was incubated for 24 h at 37 °C and 400 rpm using an Eppendorf ThermoMixer (Hamburg, Germany). Afterwards, it was transferred to an Amicon-0.5 centrifugal filter with a 3 kDa molecular weight cut-off (MWCO) and centrifuged at 14 000 rpm for 5 min to separate the formed complex and the unbound metal ions. The collected filtrate was dissolved in 2% nitric acid and then the Ru(III) concentration was determined by ICP-OES.

2.3. Kinetic study of Ru(III) binding to LF

The mechanism of LF–Ru complex formation was further investigated by performing a kinetic binding study. Initially, the LF solution (1000 mg L^{-1}) was mixed with the Ru(III) complex solution at two different concentrations (10 and 100 mg L^{-1}) at a ratio of 1:1 (v/v). The mixture was incubated (37 °C; 400 rpm) for different time periods, namely 2, 5, 10, 20, 30, 60, 120, 300, 450, 600, and 1440 min. Unbound Ru(III) was quantified using the same procedure as that described in the previous step.

2.4. Preparation of LF–Ru complexes

Based on the isothermal and kinetic studies, three concentrations of potassium aquapentachlororuthenate(III) (1.25, 100, and 500 mg L^{-1}) were selected for the large-scale synthesis of LF–Ru complexes, designated as LF–1.25Ru, LF–100Ru, and LF–500Ru, respectively. According to the isothermal study, the molar ratios of bound Ru to bLF in the complexes were determined to be 0.10/1, 8.43/1, and 28.85/1 (mol Ru per mol LF), respectively. The synthesis conditions were identical to those described in section 2.2. After 24 h of incubation, the reaction mixture was transferred to Amicon-15 centrifugal filters with a 3 kDa MWCO and centrifuged (6000 rpm, 4 °C, 40 min). The concentrate containing the LF–Ru complex was washed twice with distilled water and subsequently freeze-dried (−54 °C, 0.080 mbar) overnight using a lyophilizer (Labconco, Kansas City, MO, USA) until a dry powder was obtained.

2.5. Study of the fluorescence properties of the LF–Ru complex

2.5.1. 3D spectra measurements. Initially, LF and LF–100Ru were dissolved in deionized water at a concentration of 100 mg L^{-1} . The 3D fluorescence spectra were recorded using a Jasco FP-8300 spectrofluorometer (JASCO, Easton, MD, USA) at specific excitation (200–730 nm) and emission (210–750 nm) ranges. The measurements were performed at an interval of 5 nm and a scan speed of 5000 nm min^{-1} .

2.5.2. Analysis of the quenching mechanism in the LF–Ru system. A series of solutions of the Ru(III) complex with concentrations of 1.25, 2.5, 5, 10, 25, 50, 75, 100, 150, 250, and 500 mg L^{-1} were prepared for the Stern–Volmer experiment. The protein solution (1000 mg L^{-1}) was mixed with the metal complex in a 1:1 (v/v) ratio. The specific maximum emission wavelength was used according to the previously recorded 3D spectra of LF (Fig. S1). The changes in fluorescence intensity were analyzed with a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 280/330 \text{ nm}$.

2.6. Particle size and zeta potential measurements

The changes in hydrodynamic diameter and zeta potential of the LF–100Ru complex and LF dispersed in different media (distilled water and 0.1 acetic buffer at pH 4) at a concentration of 1000 mg L^{-1} were analyzed using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). DTS0012 and DTS1070 cuvettes were used for the particle size and zeta potential measurements, respectively. The analyses were performed at 25 °C with an equilibration time of 120 s by using a manual mode of runs.

2.7. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

The lyophilized samples of bLF, LF–1.25Ru, LF–100Ru, and LF–500Ru were analyzed in the MIR range (3500–900 cm^{-1}) with a resolution of 4 cm^{-1} using an Alpha FTIR spectrometer (Bruker, Billerica, MA, USA) equipped with a diamond crystal. The collected spectra were subjected to baseline correction using the second derivative method (zeroes) followed by normalization using the OriginPro 2024 software (OriginLab Corporation, Northampton, MA, USA). The amide I band (1720–1580 cm^{-1}) was deconvoluted using the second derivative method (to identify hidden peaks) combined with the Savitzky–Golay smoothing derivative method with a polynomial order of 2 and a window of 20 points. The areas of the fitted peaks were calculated using the Gaussian function. The achieved coefficient of determination (R^2) was 0.999 and a chi-square tolerance value of 1×10^{-6} was reached for each sample. The percentage of each secondary structure type was determined as the ratio of the area of the individual fitted peak (assigned to a given secondary structure) to the sum of the areas of all fitted peaks.



2.8. Synchrotron radiation circular dichroism (SRCD) study

Circular dichroism (CD) spectra of protein samples were recorded on the AU-CD beamline of the ASTRID2 synchrotron radiation source at the Department of Physics and Astronomy, Aarhus University, Denmark. The spectra were collected at 25 °C using a Hellma quartz suprasil cuvette type 121.000 with a nominal 0.1 mm path length under a nitrogen atmosphere. The CD spectra were collected every 1 nm with an average of 2 per measurement. The final CD spectra were calculated as the smoothed average of five independently measured and baseline corrected spectra recorded between 178 and 280 nm. The spectra were smoothed using a seven point Savitzky–Golay filter. The protein concentration was determined using the biuret method. The secondary structure content of analyzed proteins was calculated using BestSel.³⁰

2.9. Small angle X-ray scattering (SAXS) analysis

SAXS curves were collected at beamline P12 of the Petra III storage ring at DESY (Deutsches Elektronen-Synchrotron) in Hamburg.³¹ Protein samples (20 µL) were analyzed either in deionized water or in phosphate buffer, and the corresponding solvent/buffer was used for background subtraction. Before measurement, the samples were centrifuged to remove aggregates. All data were collected at 15 °C with a scattering vector (s) range of 0.0088–5 nm^{−1}, and overlays of the merged data sets were used to detect concentration-dependent scattering in the lowest s region. All SAXS data were processed using the ATSAS package.³² Integration, scaling and buffer subtraction were performed using the PRIMUS program. The resulting curves were used for all further calculations and reconstructions. Models were calculated using DAMMIF.³³ Structural parameters and theoretical intensities were calculated using CRY SOL.³⁴ The radius of gyration (R_g), maximum diameter (D_{max}) and pair-distance distribution function $p(r)$ were determined using GNOM.³⁵

2.10. Microscopy imaging

2.10.1. Light microscopy (LM) and scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS). Small amounts of the dried bLF, LF-1.25Ru, LF-100Ru, and LF-500Ru samples were mounted on carbon adhesive disks (Leit Tabs) and fixed onto SEM specimen stubs. The morphology and elemental composition were examined using an Axio Zoom.V16 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and an EVO 15 scanning electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) equipped with a backscattered electron detector (BSE) and a SmartEDS energy dispersive X-ray spectrometer (EDS).

2.10.2. Transmission electron microscopy with energy-dispersive X-ray spectroscopy (TEM-EDS). TEM samples were prepared by dispersing a few milligrams of the material in 99.8% anhydrous ethanol, followed by sonication for 5 s in an ultrasonic bath. Subsequently, a 5 µL aliquot of the resulting suspension was deposited onto a carbon-coated copper grid with

holes (Lacey type, Cu 400 mesh, Plano). The solvent was allowed to evaporate under ambient conditions, and the dried material adhering to the grid was subjected to TEM analysis. The prepared samples were analyzed with a Tecnai X-Twin transmission electron microscope (FEI Company, Hillsboro, OR, USA) equipped with a dark field detector (BF), a high-angle annular dark field detector (HAADF), and an Edax RTEM energy dispersive X-ray spectrometer (EDS).

2.11. Desorption study

Primarily, the LF-100Ru complex was dissolved in water to prepare a solution at a concentration of 10 000 mg L^{−1}. Samples were mixed with gastric (pH 1.4) and intestinal fluids (pH 7.25), prepared according to European Pharmacopeia guidelines,³⁶ at a 1:4 ratio (v/v) and then incubated at 37 °C for 1, 2, and 3 h. Afterwards, the reaction mixture was transferred to Amicon centrifugal filters (cut-off 3 kDa) and centrifuged for 5 min at 14 000 rpm. The remaining filtrate was appropriately dissolved in 2% HNO₃ and the amount of released Ru(III) ions was analyzed using ICP-OES. In parallel, the LF-100Ru solution (10 000 mg L^{−1}) was mineralized in aqua regia at 80 °C for 3 h to determine the initial concentration of Ru(III) in the complex.

2.12. SDS-PAGE analysis

The electrophoretic separation of LF and the LF-100Ru complex was performed in 4–12% polyacrylamide gel (Thermo Scientific, Waltham, MA, USA) under non-reducing conditions. Non-reducing conditions were achieved by adding 5 µL of 4× Bolt lithium dodecyl sulfate (LDS) Sample Buffer (Thermo Scientific, Waltham, MA, USA) to 15 µL of the sample. Electrophoresis was run at 200 V for 22 min using the 1× MES running buffer. Band visualization was performed by gel incubation for 1 h in Coomassie Blue R-350, followed by overnight destaining in deionized water.

2.13. Peptic digestion kinetics

Peptic digestion kinetics were investigated in a similar way to the procedure previously described by Pryshchepa *et al.*³⁷ Initially, a pepsin stock solution (2000 U mL^{−1}) was prepared in deionized water and subsequently diluted to 5 U mL^{−1} with simulated gastric fluid. The substrates, LF and the 100LF–Ru complex, were individually dissolved in water to obtain working solutions at a concentration of 5000 mg L^{−1}. The enzyme/substrate mixture was prepared at a 0.1 U per 100 µg ratio, in which the final protein concentration was 1000 mg L^{−1}. A series of samples were incubated in an Eppendorf ThermoMixer (Hamburg, Germany) at 37 °C for 1, 2, 5, 15, and 30 min. The reaction was terminated by adding 0.7 M Na₂CO₃ (35% of the total sample volume) to the reaction mixture. Finally, all samples were analyzed by SDS-PAGE in reducing mode. Reducing conditions were achieved by adding 2 µL of 10× Bolt Sample Reducing Agent (Thermo Scientific, Waltham, MA, USA) containing 500 mM dithiothreitol (DTT) per 20 µL of the total reaction mixture.



2.14. Molecular docking in LF–Ru systems

Holo- and apo X-ray crystallography bLF structures were used for docking. In the PDB database, there is only one holo- (iron-saturated) X-ray crystallography bLF with two high affinity metal coordination sites – histidine 253 and 595. This structure (PDB ID: 1BLF) with a resolution of 2.80 Å comprises residues 5–689.³⁸ Also, there is only one apo- (iron-free) X-ray crystallography structure of bLF (PDB ID: 4OQO), with a resolution of 2.42 Å, which comprises residues 342–689.³⁹ In this structure, the histidine 253 residue is missing; thus, only the histidine 595 site can be studied. Protein structures were prepared in the Protein Preparation Module of the Schrödinger Suite.⁴⁰ Hydrogen atoms were added to the structure, and bond orders and disulfide bonds were assigned. Protein protonation states were assigned using PROPKA at pH 4.0.⁴¹ The protein energy minimization was performed in the OPLS4 force field.⁴² The $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex was constructed using Maestro and subjected to DFT geometry optimization and single point energy calculations using Jaguar, an application of the Schrödinger Suite.⁴³ For geometry optimization, B3LYP-D3 was used with the LANL2DZ basis set for the Ru atom and 6-31G** for the remaining atoms. The calculations were conducted in water solvent using the polarizable continuum model. After geometry optimization, electrostatic potential charges were determined using single point energy calculations, with the total complex charge set to -2 . In MetalDock, the ruthenium geometry optimization and single point energy calculations were part of the automated workflow, and were executed using ORCA 5.0.4.⁴⁴ DFT calculations utilized the B3LYP theory and the def2-TZVP basis set for all atoms. Molecular docking was performed in the Schrödinger Suite using the Glide application⁴⁵ and in the MetalDock⁴⁴ workflow, which uses the AutoDock4 docking engine. Two Glide-specific docking methods were used: standard precision and induced fit docking.⁴⁶ The docking grid box was centered on the protein histidine residues 253 and 595. MetalDock is an open-source workflow specifically developed for molecular docking of metal complexes to biomolecules. The vacancy in the first coordination sphere of the Ru atom was indicated, and a dummy atom was added to the metal complex for direct coordination of the metal atom to a protein residue. Molecular docking was also performed using AutoDock4 as a docking tool.

2.15. Molecular dynamics (MD) and density functional theory (DFT) studies

We carried out theoretical calculations in two stages: (1) DFT and (2) classical MD. In the DFT calculations, we optimized the structure of individual amino acids, the isolated $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex, and the $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex interacting with amino acids to obtain their minimum-energy ground-state configurations utilizing the Gaussian09 code.⁴⁷ The B3LYP hybrid functional was employed, with the GENECP keyword used to define the LANL2DZ basis set for Ru(III), while the 6-31G basis set was applied for Cl, H, O, C, and N atoms.^{48,49} The optimized structures and corresponding electron density distributions were visualized using GaussView 5.0.

Guided by the molecular docking results, only selected amino acids were considered for quantum mechanical calculations to reduce the computational cost. For each system, all possible binding sites were examined, and the most favorable configuration corresponding to the lowest ground-state energy was chosen for further interaction analysis.

For classical MD simulations, the initial structure of bovine lactoferrin (bLF) was obtained from the RCSB Protein Data Bank: 1BLF for the holo-form and 4OQO for the apo-form. After molecular docking of the three modeled configurations, the highest-scoring pose in each case was used as the starting structure for MD simulations. GROMACS was used for simulations with the CHARMM force field for the protein residues, and EasyPARAM-derived parameters were used for $[\text{RuCl}_5]^{2-}$, respectively.^{50–52} Before the MD run, the protein with the $[\text{RuCl}_5]^{2-}$ complex was solvated with SPC/E water molecules in a cubic box of 10 nm in edge length.⁵³ The total energy of the system was minimized to relax bonds and remove possible structural clashes. Particle Mesh Ewald (PME) was used to calculate long-range electrostatic interactions, and the cut-off for non-bonded interactions was set to 1.2 nm. After minimization, the system was heated to 300 K under the NVT ensemble using a Langevin thermostat. Subsequently, the system was equilibrated under the NPT ensemble at 1 bar and 310 K. Each equilibration step lasted 100 ps. Production runs were continued for 30 ns, after which the trajectories were analyzed and the root mean square deviation (RMSD) was calculated.

2.16. Resazurin-based assessment of the antibacterial activity of LF and LF–100Ru

The antibacterial response of LF and LF–100Ru was evaluated using three bacterial strains: *Escherichia coli* ATCC 33876, *Enterococcus faecalis* ATCC 51299, and *Klebsiella pneumoniae* B34. Initially, bacteria were inoculated in Mueller–Hinton broth (MHB) and incubated for 24 h under aerobic conditions. Afterwards, the prepared 0.5 McFarland bacterial suspension was diluted 100-fold. A series of solutions of LF and LF–100Ru were prepared in MHB at concentrations of 10 000, 5000, 2500, 1250, 625, and 312.5 mg L^{−1}. Then, 100 μL of bacterial suspension was mixed with 100 μL of the analyzed samples in a 96-well plate and incubated overnight at 37 °C. The bacterial suspension and culture medium alone were used as the positive and negative controls, respectively. Subsequently, 10 μL of resazurin solution (100 mg L^{−1}) was added to each well and the plate was additionally incubated at 37 °C for 1 h. Bacterial viability was determined from the changes in fluorescence intensity at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 560/590$ nm using a Varioskan™ LUX Multimode Microplate Reader. All analyses were performed in triplicate for both samples and controls. The value of the negative control was subtracted from each analyzed sample and from the positive control. The fluorescence value of the positive control was taken as 100% bacterial viability.

2.17. In vitro cell line assessment

The cytotoxic potential of the LF–Ru complexes was assessed on the L929 murine fibroblast cell line and human epithelial



colorectal adenocarcinoma Caco-2 cells, both obtained from the American Type Culture Collection (ATCC), as well as on hepatocellular carcinoma HepG2 cells acquired from the European Collection of Authenticated Cell Cultures (Sigma Aldrich). All cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Biowest) supplemented with 10% fetal bovine serum (Biological Industries) and antibiotics, namely penicillin (100 U mL^{-1}) and streptomycin ($100 \mu\text{g mL}^{-1}$) (Capricorn Scientific GmbH). Cultures were incubated at 37°C in a humidified incubator under a 5% CO_2 atmosphere. Subculturing was performed routinely, 2–3 times a week, using a 0.05% trypsin–EDTA solution (Capricorn Scientific GmbH) once the cells reached approximately 70% confluence.

2.17.1. Cell viability by the MTT assay. The proliferative response of cells to LF–Ru complexes was assessed *via* the MTT assay (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; Thermo Fisher Scientific). Initially, L929, HepG2, and Caco-2 cells were seeded into 96-well culture plates (Jet Biofil) at a concentration of 5×10^4 cells per mL within $100 \mu\text{L}$ of supplemented DMEM per well and pre-incubated at 37°C for 24 h. Following this incubation, cells were exposed to the LF–Ru complexes for 48 h. Stock solutions of the complexes were prepared and then subjected to serial dilutions in the medium to obtain the final concentrations of 625, 1250, 2500 and 5000 mg L^{-1} , respectively. Furthermore, a control sample containing unmodified lactoferrin was prepared, with additional concentrations of 20 000 and 10 000 mg L^{-1} . After treatment, the medium was removed and replaced with $100 \mu\text{L}$ of MTT solution (0.5 mg mL^{-1}) prepared in PBS. Plates were incubated for 2 h at 37°C to allow for formazan formation. After the incubation period, the MTT reagent was carefully removed, followed by the addition of $50 \mu\text{L}$ of absolute dimethyl sulfoxide (DMSO) to each well. The plates were gently agitated for 10 min. Absorbance readings were then taken at 570 nm and a reference wavelength of 630 nm, using a Varioscan™ LUX Multimode Microplate Reader. Viability percentages were calculated by comparing the treated cells to the untreated controls designated as 100% viable. All tests were conducted in five separate replicates. Additionally, observations of cell shape and density were made with an Olympus EP50 inverted phase-contrast microscope.

2.17.2. Measurement of cytotoxic effects through LDH activity. The cytotoxic effects were quantified by measuring lactate dehydrogenase (LDH) release using the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA), following the manufacturer's protocol. Each condition was analysed in quintuplicate. For analysis, $50 \mu\text{L}$ of culture supernatants collected after 48 h of incubation with the test substances from L929 murine fibroblasts, human epithelial colorectal adenocarcinoma Caco-2 cells, and hepatocellular carcinoma HepG2 cells were transferred to a new 96-well plate (Jet Biofil). The CytoTox96 reagent was freshly prepared by combining the assay buffer with the substrate mixture. The positive controls, representing 100% LDH release, were generated by treating cells with 0.8% Triton X-100 for 45 min. The negative

controls included untreated cells in the culture medium, while blanks contained the medium alone or the medium with the specified test substance, which were applied in order to eliminate background effects in the measurements. Subsequently, $50 \mu\text{L}$ of the reagent was added to each well, followed by incubation for 30 min at 21°C in the dark. The reaction was halted by adding $50 \mu\text{L}$ of the stop solution. LDH release, reflecting membrane damage, was measured from the absorbance at 490 nm using a Varioskan™ LUX Multimode Microplate Reader. The results are presented as mean values $\pm$ standard deviation (SD). Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test for multiple comparisons. All computations were carried out using GraphPad Prism 8.0.2 software (La Jolla, CA, USA). The levels of significance are represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For instances where cell viability surpassed the control values of the same concentration of LF, statistical significance was marked with a '#' symbol instead of an asterisk.

3. Results and discussion

3.1. Isotherm study

The binding behavior in the LF–Ru systems was investigated through the application of two isotherm models, namely the Langmuir and Freundlich models (Fig. 1A). A detailed description of the isotherm models and thermodynamic parameters used to analyze Ru(III) binding to LF is provided in the SI.

The calculated sorption parameters for both applied models are shown in Table 1.

The experimental sorption capacity ranged from 0.12 to 36.4 mg g^{-1} . The determined molar ratios of bound Ru to bLF in the formed LF–Ru complexes are shown in Table 2. The $q_{\text{eq}}/C_{\text{eq}}$ relationship was better fitted by the Langmuir model ($R^2 = 0.993$) than by the Freundlich model ($R^2 = 0.969$), indicating a saturation-like binding of Ru(III) to a limited number of accessible LF regions under the studied conditions.⁵⁴ The calculated maximum sorption capacity (q_{max}) was 56.9 mg g^{-1} , confirming a substantial affinity of Ru species for LF. The previously determined q_{max} of Fe(III) for LF was 36.3 mg g^{-1} .⁵⁵

Remarkably, R_L decreased as C_0 increased, consistent with the used relationship. The determined dimensionless equilibrium parameter ($R_L = 0.28\text{--}0.99$) indicates favorable LF–Ru formation. Although the experimental data were better fitted by the Langmuir model, a Freundlich contribution should not be excluded, suggesting that the system is not perfectly uniform across the entire loading range. The heterogeneity factor ($1/n < 1$) likewise supports favorable binding.⁵⁶ To further probe the high-loading behavior, the data were additionally examined using a modified BET-style representation. The resulting plot suggests that, after an initial saturation-like binding regime, a secondary accumulation step may occur at higher Ru precursor concentrations (Fig. 1B). In this context, the BET-type interpretation should be treated as a phenomenological description of high-loading behavior rather than as proof of classical multilayer adsorption on a porous solid. In particular, it may reflect sec-



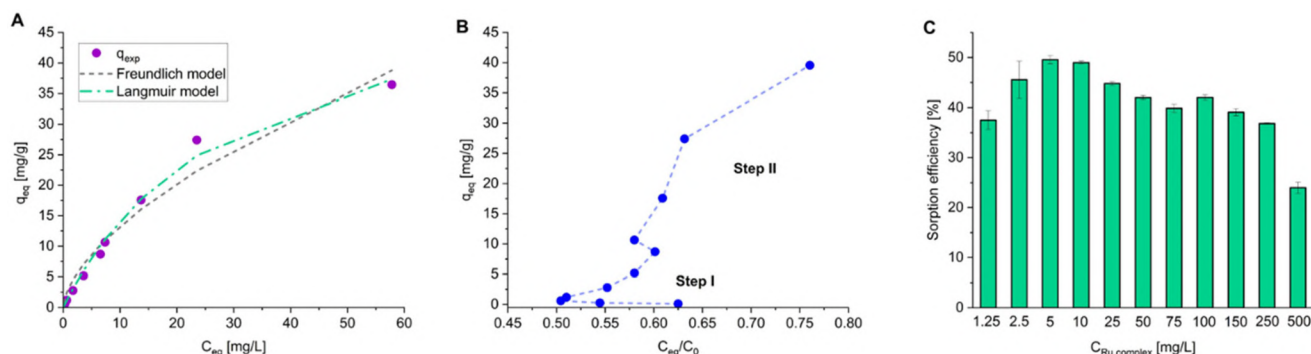


Fig. 1 (A) Isotherm of Ru(III) sorption onto LF, (B) modified BET isotherm, and (C) bar chart of the sorption efficiency of Ru(III) on LF.

Table 1 Sorption parameters for Freundlich and Langmuir models

Freundlich model				Langmuir model			
n	K_F [mg g ⁻¹]	S	R^2	q_{max} [mg g ⁻¹]	K_L [L mg ⁻¹]	S	R^2
1.63	3.24	45.8	0.969	56.9	0.033	10.3	0.993

ondary Ru accumulation or aggregation within the protein matrix instead of a strictly defined surface multilayer. This interpretation is more consistent with the TEM observations obtained for LF-500Ru, where Ru-rich domains were detected only at the highest loading. The calculated binding efficiency of Ru(III) on LF ranged from 24.0 to 49.6% (Fig. 1C). It increased from 37.5 to 49.0–49.6% as the initial Ru(III) complex concentration increased from 1.25 to 10 mg L⁻¹. Subsequently, the efficiency decreased from 44.8 to 24.0% over the 25–500 mg L⁻¹ range, supporting progressive saturation of the most accessible binding regions. The calculated change in Gibbs free energy (ΔG) ranged from -19.6 to -16.6 kJ mol⁻¹ across the tested concentrations, confirming that LF-Ru formation is spontaneous under the given conditions ($T = 310$ K). For comparison, the previously determined ΔG values for the LF-Ag system ranged from -25 to -11 kJ mol⁻¹.³⁷

3.2. Kinetic study

To evaluate the kinetics of LF-Ru binding, the two initial Ru(III) complex concentrations, namely 10 and 100 mg L⁻¹, were tested (Fig. 2).

A detailed description of the kinetic models used to evaluate Ru(III) binding to LF is provided in the SI. Thus, a zero-order kinetic model was applied to evaluate the changes in Ru

(III) concentration over time (C_t). For 10 mg L⁻¹ as the initial metal complex concentration, a fast metal binding to LF within 2 min ($k_0 = 0.48$ mg L⁻¹ min⁻¹) was observed, resulting in a decrease of the initial Ru(III) concentration (C_0) from 1.39 mg L⁻¹ to a C_t of 0.42 mg L⁻¹ (Fig. 2A). In the next step (5–30 min), a gradual increase in C_t from 0.55 to 0.93 mg L⁻¹ was observed. This phenomenon might be associated with structural changes in LF, leading to metal desorption and subsequent relocation of Ru(III) to more favorable binding sites. The obtained Weber–Morris plot indicated fast transport of Ru(III) from the solution to the protein surface, which is generally described as boundary layer diffusion and/or external mass transfer (Fig. 2B).⁵⁷ Subsequently, a sharp decrease in sorption capacity was observed, which might be caused by the release of weakly bound Ru(III). Finally, the formed LF-Ru system reached equilibrium after 30 min. The calculated sorption efficiency (% E) ranged from 29.2 to 69.7% over the analyzed time period (Fig. 2C). The results are comparable to a zero-order kinetic plot, showing the highest percentage of bound Ru(III) in 2 min, followed by gradual metal desorption and a decrease in sorption efficiency. After 30 min of incubation, the sorption efficiency remained nearly constant (29.2–35.3%), suggesting that the protein had reached its Ru(III)-binding equilibrium, possibly due to saturation of accessible binding sites. In the case of 100 mg L⁻¹ as the initial complex concentration, Ru(III) binding to LF was a one-step process which occurred rapidly ($k_0 = 3.14$ mg L⁻¹ min⁻¹) in 2 min (Fig. 2D). A minor desorption of Ru(III) occurred in the range of 2–20 min which corresponded to $C_t \sim 9$ –11 mg L⁻¹. The observed fluctuations might be caused by rearrangements in the LF structure followed by redistribution of the bound Ru(III). The Weber–Morris model confirmed fast binding of metal ions on the external surface (Fig. 2E). It was shown that intra-particle

Table 2 Molar ratios of $n(\text{Ru})/n(\text{LF})$ in the obtained LF-Ru complexes (mol Ru per mol LF)

$n(\text{Ru})/n(\text{LF})$										
LF-1.25Ru	LF-2.5Ru	LF-5Ru	LF-10Ru	LF-25Ru	LF-50Ru	LF-75Ru	LF-100Ru	LF-150Ru	LF-250Ru	LF-500Ru
0.10/1	0.22/1	0.48/1	0.94/1	2.21/1	4.10/1	6.89/1	8.43/1	13.91/1	21.70/1	28.85/1



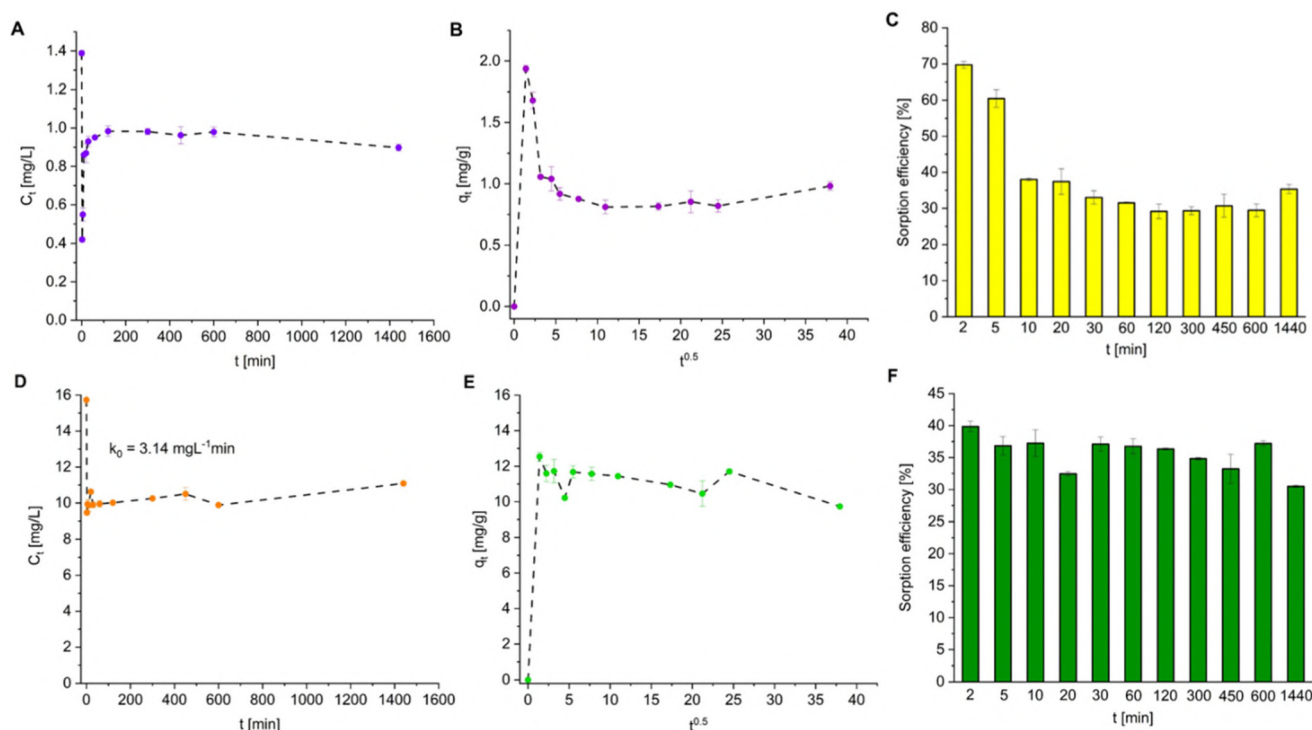


Fig. 2 (A and D) Zero-order kinetic model of LF–Ru binding, (B and E) Weber–Morris intra-particle diffusion model and (C and F) bar chart of the efficiency of Ru(III) sorption onto LF at the applied initial Ru(III) complex concentrations of (A–C) 10 mg L^{-1} and (D–F) 100 mg L^{-1} .

diffusion was not a key rate-limiting step; thus the Ru(III) ions initially bound on the protein surface underwent a brief redistribution before the system reached equilibrium. The calculated sorption efficiency was relatively constant over the whole time period and ranged from 30.5 to 39.9%, indicating greater stability and faster saturation of the LF–Ru complex at a higher initial concentration of the applied complex (Fig. 2F).⁵⁸

3.3. 3D fluorescence spectroscopy analysis

Fig. 3 presents the 3D spectra of LF and the LF–100Ru complex. All registered peaks are labelled in Table 3. Two main peaks at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 280/330 \text{ nm}$ and $\lambda_{\text{ex}}/\lambda_{\text{em}} = 225/327 \text{ nm}$ are observed in the 3D fluorescence spectra of LF which might be assigned to aromatic amino acids.

The maximum emission peak was slightly blue-shifted from 329 to 328 nm and from 327 to 324 nm after LF–Ru complex formation, which might be explained by less exposure of aromatic amino acid residues, especially Trp, to the polar solvent and rearrangement of their microenvironment to one that is more hydrophobic.⁵⁹ Such changes in polarity near Trp could provide more compact and stable protein conformation.⁶⁰ The additional peak arising at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 265/531 \text{ nm}$ was attributed to the quartz cuvette (Fig. S2). Despite this, the evident fluorescence quenching from 6219 to 3006 a.u. confirms the binding of Ru(III) to bLF. More detailed insights into the nature of the interactions between the fluorophore and quencher were obtained from the Stern–Volmer experiment presented in the next subsection.

3.4. Analysis of the LF–Ru quenching mechanism

The changes in LF fluorescence intensity were examined in a broad range of concentrations of the Ru(III) complex (quencher). Addition of the Ru(III) complex to the LF solution induced an effective decrease in protein fluorescence intensity (Fig. 4A).

Quenching mechanisms were analyzed with the application of the Stern–Volmer eqn (1):

$$F_0/F = 1 + K_{\text{SV}}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

where F and F_0 are the fluorescence intensities of LF with and without the added quencher, K_{SV} is the Stern–Volmer constant (M^{-1}), $[Q]$ is the concentration of the quencher (M), K_q is the quenching rate constant (M^{-1}), and τ_0 is the fluorescence time of the fluorophore without the quencher, which is typically equal to 10^{-8} s .

The evident quenching of fluorescence intensity confirms the binding of Ru(III) to bLF. Based on the Stern–Volmer plot, a linear relationship ($R^2 = 0.9895$) was observed at low Ru(III) concentrations, up to 0.2 mM , corresponding to a 16 : 1 molar ratio of metal to protein. The determined K_{SV} (Fig. 4B) was $(2.044 \pm 0.094) \times 10^4 \text{ M}^{-1}$ in the mentioned concentration range, which indicates a high availability of fluorophores to quencher molecules.⁶¹ For example, the calculated K_{SV} for the system of human transferrin (hTF) and the pentachloro complex of Ru(III) with a bidentate carbazole–pyridine carboxylic acid ligand was equal to $1.09 \times 10^5 \text{ M}^{-1}$.⁶² As observed, the plot curved upwards at higher metal concentrations. The



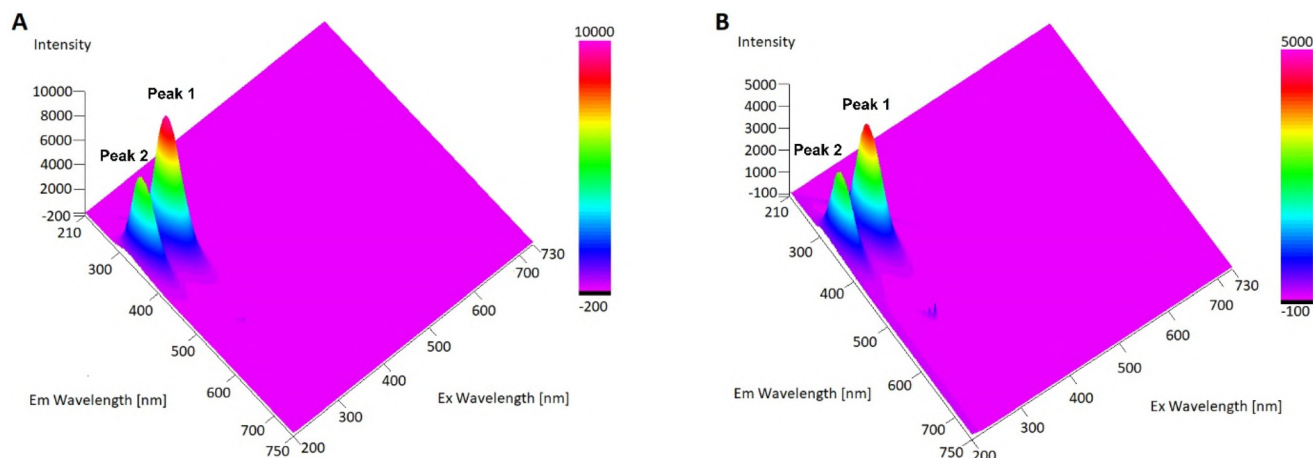


Fig. 3 3D spectra of (A) LF and (B) the LF-100Ru complex dissolved in deionized water at a concentration of 100 mg L⁻¹.

Table 3 Peaks observed on 3D fluorescence spectra of LF and the LF-100Ru complex (100 mg L⁻¹)

Sample	Peak 1			Peak 2		
	λ_{ex} [nm]	λ_{em} [nm]	Intensity [a.u.]	λ_{ex} [nm]	λ_{em} [nm]	Intensity [a.u.]
bLF	280	329	9634	225	327	6219
bLF-100Ru	280	328	4397	225	324	3006

positive curvature of the Stern-Volmer plot might be explained by the complicated nature of quenching effects. In general, the fluorescence quenching might be described as either a dynamic mechanism or a static mechanism. Dynamic quenching is characterized by collisional interactions between the biomolecule and quencher molecules in an excited state, while a static mechanism involves the formation of a non-fluorescent ground-state complex.⁶³ The static quenching phenomenon is commonly observed at high concentrations of the quencher.⁶⁴ Thus, we can suggest that the fluorophore-quencher complex forms at $[Q] \geq 0.2$ mM, whereas collision occurs at lower concentrations. Consequently, the obtained plot reflects the combination of both dynamic and static fluorescence quenching mechanisms.⁶⁵ Besides, the calculated K_q was equal to 2.044×10^{12} M⁻¹ which is much higher than the maximum value of K_q for dynamic quenching. For a more accurate determination of the quenching mechanism, the changes in K_q should be correlated with temperature.⁶³ Alternatively, the positive curvature might also be associated with the unequal accessibility of fluorophores to the quencher.⁶⁶ Indeed, the bLF molecule contains 13 Trp residues, each of which might be differently exposed to the Ru(III) complex.⁶⁷

For a more detailed analysis of the LF-Ru binding mechanism, the double logarithmic eqn (2) was applied and the relevant plot was designed (Fig. 4C):

$$\log[(F_0 - F)/F] = \log K_a + n \log [Q] \quad (2)$$

where K_a is the binding constant and n is the number of binding sites.

The double log plot of LF-Ru binding showed good linearity ($R^2 = 0.9958$) (Fig. 4D). The calculated K_a was $(1.789 \pm 0.0002) \times 10^4$ M⁻¹ and n was 0.998 ± 0.029 , indicating strong interactions in the LF-Ru system. Under the tested conditions, this result is best interpreted as one dominant fluorescence-reporting binding event rather than definitive proof that only one Ru-binding site exists in the LF molecule. Similar binding parameters were previously reported for the LF-gallic acid system ($T = 298$ K), where the determined K_a was 1.17×10^4 M⁻¹ and n was 1.08.⁶⁸ Likewise, Yan *et al.* indicated K_a and n values of $(1.190 \pm 0.012) \times 10^4$ M⁻¹ and 1.27 ± 0.02 , respectively, for the LF-ginsenoside (LF-Re) system at 298 K.⁶⁹ In contrast, the calculated K_a and n values for the apo-hTF/TM34 ruthenium system were $(37.44 \pm 1.27) \times 10^4$ M⁻¹ and 1.186, respectively.⁷⁰

3.5. Characterization of the LF and LF-Ru hydrodynamic size and zeta potential changes

DLS results revealed substantial changes in LF size depending on the medium used and the presence of the metal complex (Fig. 5A).

In the case of LF at pH 4, the presence of small particles of 7.5 and 43.8 nm as well as bigger aggregates of 5560 nm was observed; nevertheless, their contribution was much lower in comparison with LF dispersed in water. Remarkably, a notable decrease in the hydrodynamic diameter of LF after binding to the ruthenium complex was observed. The individual sharp peak at 24.3 nm indicates the formation of a monodisperse system. Based on this, ruthenium might be involved in stabilization of the LF structure, preventing protein aggregation. This suggestion was also confirmed by zeta potential measurements.

All analyzed samples displayed a positive zeta potential under the established conditions (Fig. 5B). As expected, LF dispersed in water formed the least stable colloidal system ($\zeta = 9.3 \pm 0.5$ mV), since the pH of the given medium (pH 7) was near the



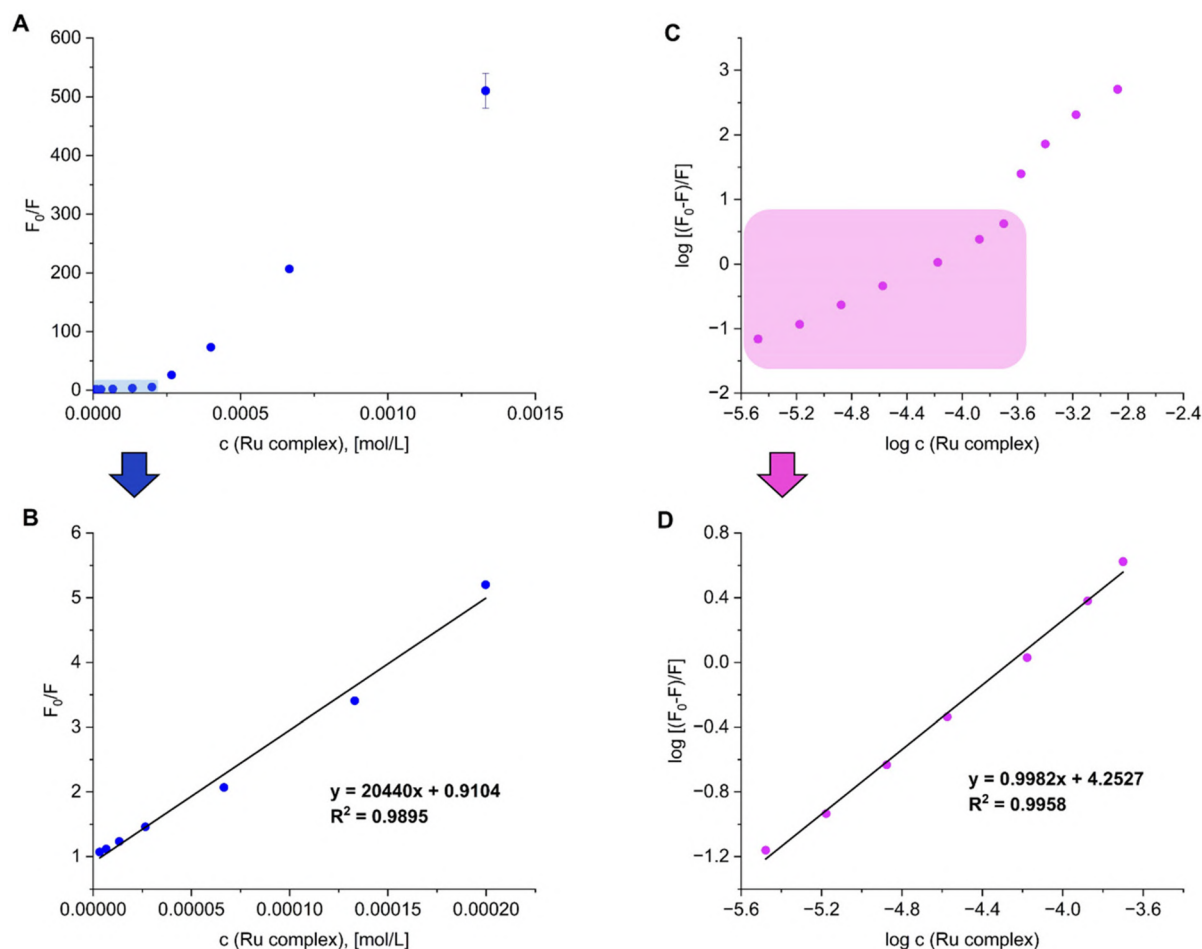


Fig. 4 (A) Stern–Volmer plots for fluorescence quenching in the presence of the ruthenium complex, (B) zoomed-in image of the selected region of Ru(III) complex concentration, (C) double Stern–Volmer plots for fluorescence quenching for the LF–Ru system analyzed at 298 K, and (D) zoomed-in image of the corresponding range of log c (Ru complex).

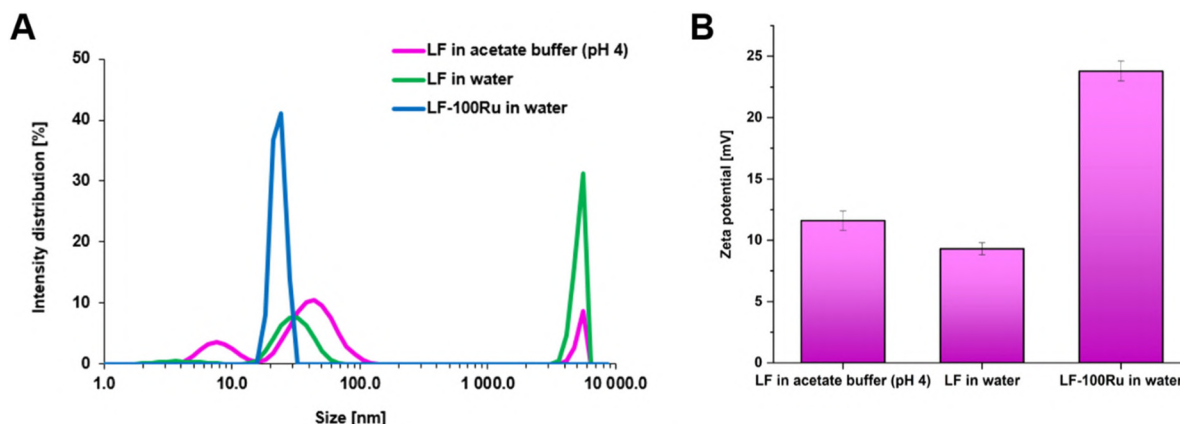


Fig. 5 (A) Particle size distribution and (B) zeta potential of LF and the LF–100Ru complex.

protein isoelectric point (pI). Previously, Pryshchepa *et al.* indicated that the pI of bLF was about 7.4 ± 0.2 .⁷¹ In contrast, LF dissolved at pH 4 exhibited a more positively charged surface; thus its zeta potential was $\zeta = 11.6 \pm 0.8$ mV. Similarly, Bokkhim *et al.*

showed that the ζ of native LF was +18 at pH 4, and it decreased to +10 mV at pH 7.⁷² A more acidic medium typically promotes the protonation of amino ($-\text{NH}_2$) functional groups, leading to increased zeta potential.⁷³ Samples with zeta potentials higher



than +20 mV and lower than −20 mV are described as stable.⁷⁴ Both LF samples, dispersed in water and in acetic buffer, formed fairly unstable colloids. The addition of the Ru(III) complex significantly affected the LF charge properties. Indeed, LF-Ru showed the highest zeta potential ($\zeta = 23.8 \pm 0.8$ mV) among all analyzed samples, thereby forming the most stable colloidal system. The evident increase in zeta potential could be related to conformational changes in the LF structure after Ru(III) binding that led to improved surface availability of positively charged amino acids, such as lysine, arginine, and histidine.⁷⁵ Besides, Ru(III) might be potentially involved in interaction with carboxylate groups ($-\text{COO}^-$), inducing the neutralization of negative charge in the LF molecule.⁷³

3.6. ATR-FTIR

ATR-FTIR analysis was performed to identify the protein functional groups involved in the binding with metal ions. Moreover, the observed shifting of amide bands provided information on the changes in the LF secondary structure. The obtained ATR-FTIR spectra of LF and the respective LF-Ru complexes are shown in Fig. 6.

The characteristic amide A band observed in the range of 3300–3100 cm^{-1} assigned to stretching vibrations of N–H and O–H of the LF functional groups was blue-shifted as the amount of bound Ru(III) in the protein increased.⁷⁶ These changes indicate that complexed Ru(III) might affect the strength of hydrogen bonding in the polypeptide backbone. In turn, a peak found at 3100–3050 cm^{-1} was attributed to stretching vibrations of N–H (amide B band). Similar to amide A, it was shifted to a shorter wavelength from 3061 to 3065 cm^{-1} . The characteristic bands observed in the ranges of 2965–2960, 2925–2920, and 2875–2870 cm^{-1} might be attributed to the stretching vibrations of asymmetric $-\text{CH}_3$, asymmetric $-\text{CH}_2$ and symmetric $-\text{CH}_2$, respectively.^{77,78}

The range of 1700–1600 cm^{-1} is characteristic of the amide I band and is commonly used for the determination of secondary structure content in the protein since it is considered to be less affected by amino acid side chain vibrations in contrast to amide II and amide III.^{79,80} It mainly corresponds to the stretching vibrations of C=O (80%) coupled with the C–N (20%) group in the peptide backbone whose peak position might differ depending on the characteristic secondary structure type, e.g. β -sheet (1640–1610 cm^{-1}), random coil (1650–1640 cm^{-1}), α -helix (1660–1650 cm^{-1}), or β -turn (1690–1660 cm^{-1}).⁸¹ Deconvolution of the amide I band allowed us to distinguish the contribution of each type of secondary structure (Fig. 7).

Indeed, β -sheet (50.3%) was the dominant type of secondary structure present in bLF, followed by β -turn (37.3%), α -helix/random coil (11.1%), and aggregated β -sheet (1.3%) (Fig. 8).

A significant increase in α -helix content after Ru(III) complexation indicated that the protein adopted a more stable and ordered conformation, particularly in the LF-100Ru sample, which is consistent with our fluorescence results. A similar tendency was previously observed by Chen *et al.*, resulting in LF complexation with lactose.⁸² On the other hand, Alhazmi *et al.* reported that the binding of metal ions, such as Fe(II), Ir(III), and Pt(IV), to BSA caused a visible decrease in α -helix content.⁸³ It was also indicated that the decrease in α -helix content resulted in AgNP immobilization onto BSA.⁸⁴ Interestingly, Hadden *et al.* reported minor differences in the secondary structure of iron unsaturated and saturated hLF.⁸⁵ Slight changes in the α -/ β -structure ratios observed for LF-1.25Ru and LF-100Ru indicate conformational rearrangements dependent on the amount of bound metal. Besides, loss of α -helix was observed in the LF-500Ru complex simultaneously with the increase of random coil structure. Thus, we can assume that excess bound Ru(III) might negatively affect the stability of the protein, leading to its partial unfolding.

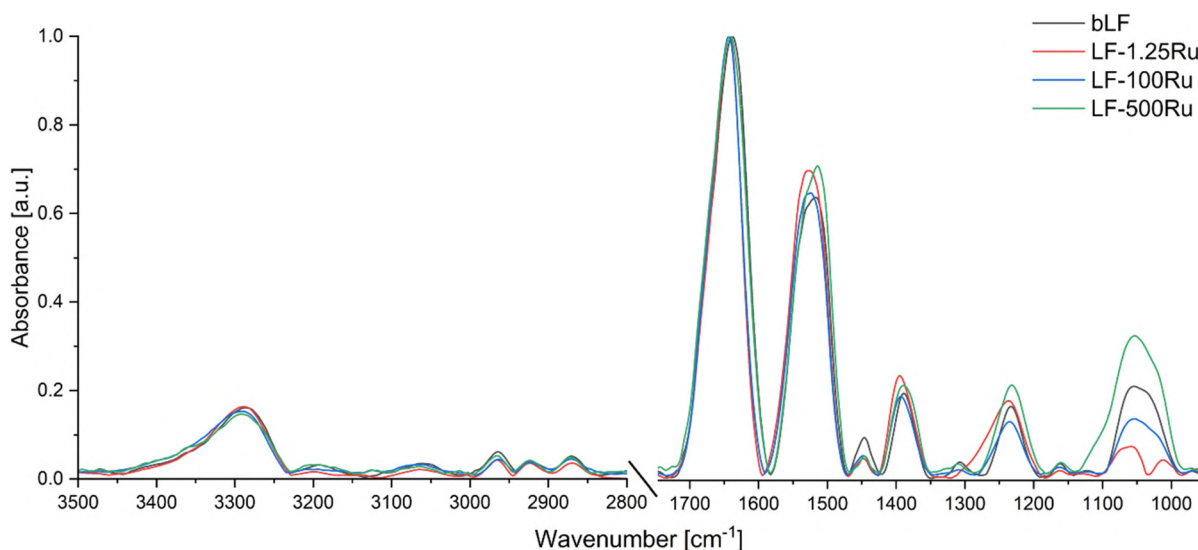


Fig. 6 ATR-FTIR spectra of LF and LF-Ru complexes.



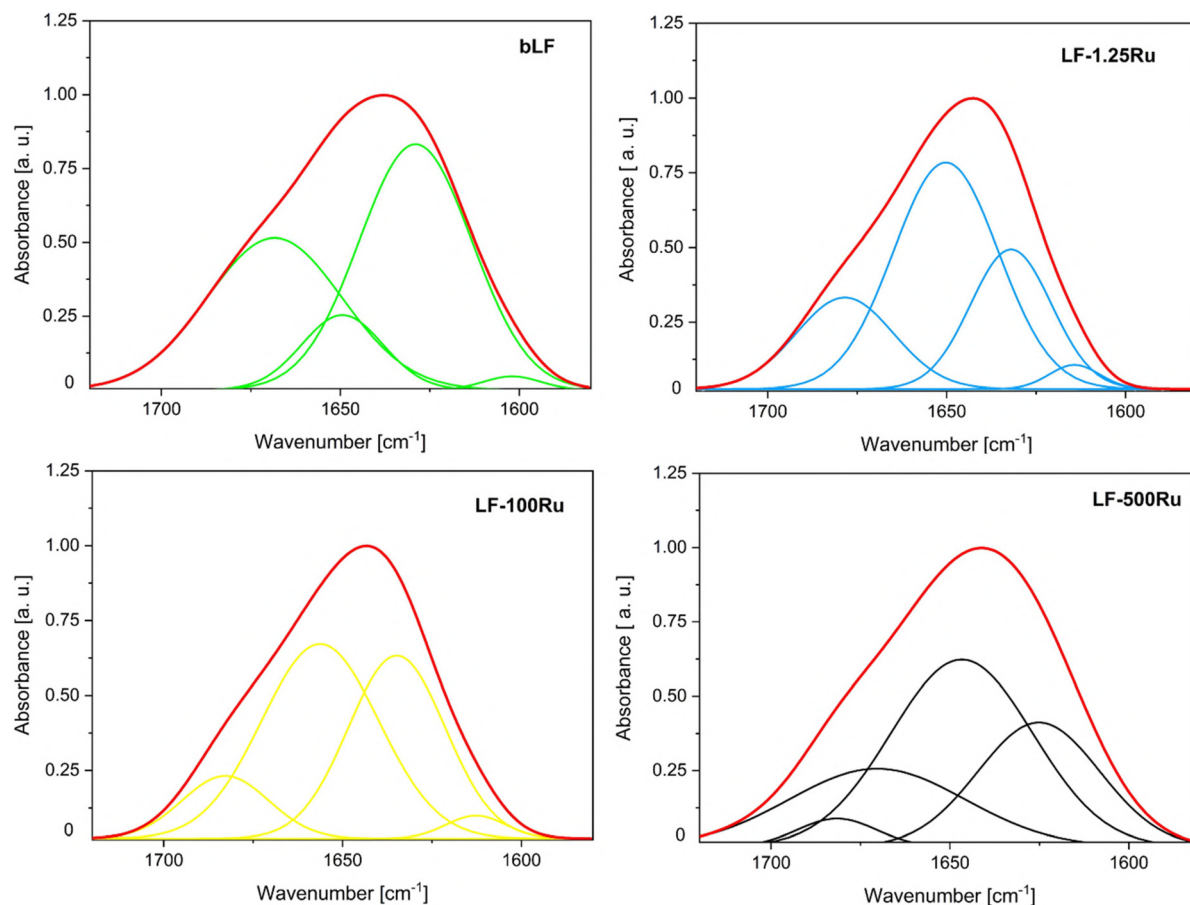


Fig. 7 Amide I band deconvolution results.

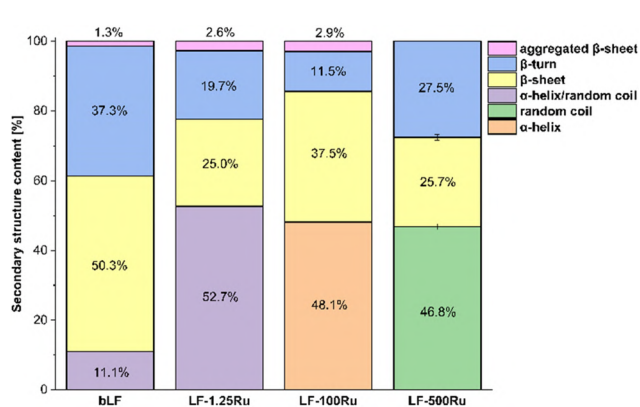


Fig. 8 Changes in the secondary structure composition of LF after its modification with Ru(III).

The amide II band was observed at $1580\text{--}1490\text{ cm}^{-1}$ and could be assigned to N-H bending coupled with C-N stretching vibrations.⁸⁶ A blue shift from 1517 to 1526 and 1524 cm^{-1} and a red-shift from 1517 to 1515 cm^{-1} were observed in the case of LF-1.25Ru, LF-100Ru, and LF-500Ru, respectively, confirming the conformational changes in the LF induced by binding with the Ru(III) complex. Although the given region is

characteristic of a β -sheet structure, it might additionally overlap with the tyrosine C-C ring stretching vibration.^{85,87,88} Similarly, peak shifts were observed in the $1240\text{--}1230\text{ cm}^{-1}$ and $1165\text{--}1160\text{ cm}^{-1}$ ranges, which are also characteristic of the tyrosine side chain.⁸⁹ Based on this, we can suggest that tyrosine might be potentially involved in the binding with Ru(III). In turn, the peaks observed at $1450\text{--}1440$ and $1400\text{--}1380\text{ cm}^{-1}$ could arise from the $-\text{CH}_3$ asymmetric and symmetric bending vibrations of the amino acid side chains, respectively.⁹⁰ Despite this, the range $1400\text{--}1380\text{ cm}^{-1}$ might be attributed to aspartic acid side chain vibrations. Thus, the blue shift of the following absorption band from 1389 to 1395 and 1393 cm^{-1} for LF-1.25Ru and LF-100Ru, respectively, might be a result of the interaction between Ru(III) and the carboxyl group of aspartic acid.⁸⁹ The characteristic region of the amide III band is shown at $1350\text{--}1200\text{ cm}^{-1}$ which mainly includes N-H bending and C-N stretching vibrations.^{91,92} The peaks appearing at $1310\text{--}1305\text{ cm}^{-1}$ and $1240\text{--}1230\text{ cm}^{-1}$ confirm the presence of α -helix and β -sheet structures, respectively, in LF and LF-Ru samples.⁹¹ In turn, the range $1200\text{--}950\text{ cm}^{-1}$ is considered a carbohydrate fingerprint and the appeared peaks might be attributed to the carbohydrate residues (C-O, C-C stretching and C-O-H deformation vibrations) of LF as a glycosylated protein.^{78,93,94} The peak



Table 4 Comparison of the secondary structure content between experimental and theoretical data

Type of secondary structure		Theoretical							Experimental		
		1BLF	4OQO	1	2	3	4	5	LF	LF-1.25Ru	LF-100Ru
α -Helix	Helix 1 regular	17.4	17.8	17.4	17.9	17.4	17.4	17.4	16.1	16.7	17.3
	Helix 2 distorted	12.2	12.1	12.2	11.4	12.2	12.2	12.2	0.0	0.0	0.0
Antiparallel β -sheet	Anti 1 left-twisted	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.1	13.6	11.8
	Anti 2-relaxed	1.7	1.2	1.9	1.9	1.2	1.2	1.7	23.7	25.8	24.3
	Anti 3 right-twisted	10.4	10.7	10.6	9.4	10.6	10.6	10.3	1.6	2.6	1.9
Parallel β -sheet		5.5	6.0	4.9	5.4	5.3	5.3	5.3	0.0	0.0	0.0
β -Turn		18.3	15.6	18.7	17.4	17.5	17.5	17.5	13.3	11.1	12.4
Others (random coil)		34.5	36.6	34.3	36.6	35.8	35.8	35.6	31.3	30.2	32.3

This analysis was performed using the BestSel algorithm, where 1 = glide_His253_holo_1BLF, 2 = glide_His595_apo_4OQO, 3 = glide_His595_holo_1BLF, 4 = metadock_His253_holo_1BLF, and 5 = metadock_His595_apo_4OQO.

shifting in the following region after Ru(III) complexation might be caused by the difference in accessibility of glycans resulting in LF conformational changes. Besides, the peak found at 1054 cm^{-1} might be attributed to the tryptophan side chain (C=C or C-N stretching and C-H bending vibrations).⁹⁵ As shown, Ru(III) complexation caused shifts in the peak positions, which may be directly associated with conformational modifications in the bLF structure.

3.7. Synchrotron radiation circular dichroism (SRCD)

The following technique was used for studying the changes in bLF secondary structure resulting in Ru(III) binding. The application of the BestSel algorithm allowed for the calculation of secondary structure content from theoretical models. A comparison of the results obtained for the theoretical structures and the experimental data is shown in Table 4 and SRCD spectra are shown in Fig. 9.

The secondary structure element contents calculated for the apo- and holo-forms based on their crystal structures showed only minor differences. Likewise, superposition of the crystal structures indicates that iron ions did not cause significant changes in the spatial organization of bLF (Fig. 10).

In the next step, analysis of the models with docked Ru(III) was carried out. Metal ions did not induce structural changes in the LF structure. As observed, the secondary structure content of the crystallographic and experimental structures of LF showed differences. This might be caused by the high flexibility of LF (random-coil content >30%). On the other hand, the experimental data show that metal complexation does not cause significant changes in the secondary structure of LF with increasing concentrations of Ru(III). This behavior indicates that Ru(III) binds to the LF molecule in a stable manner, without disturbing its secondary structure. As shown, the β -structure was a predominant secondary structure in all analyzed samples with contents ranging from 38 to 42%. The total β -structure percentage for LF-1.25 and LF-100Ru samples was in good agreement with FTIR results; nevertheless, the content of individual structures differed. Remarkably, Ru(III) complexation with LF promoted only a minor increase in α -helix

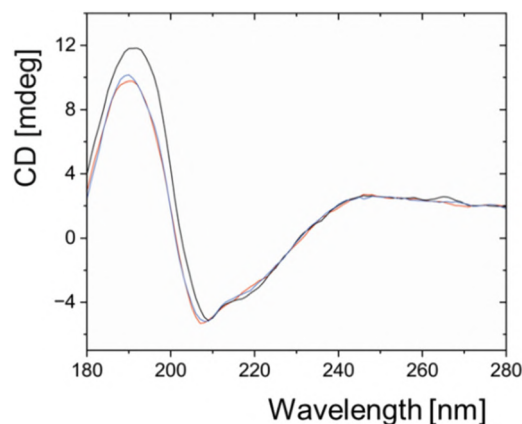


Fig. 9 SRCD spectra of the analyzed samples (black – native LF, red – LF-1.25Ru, blue – LF-100Ru).

content which is not consistent with previous FTIR results of amide I band deconvolution.

The differences in secondary structure content obtained from FTIR and SRCD analyses can be attributed to the specific sensitivities and inherent limitations of each technique. As mentioned previously, CD spectroscopy is considered a more reliable method for quantifying α -helical structures, whereas FTIR spectroscopy is generally more sensitive to β -sheet structures.⁹⁶ For example, some amino acid side chains, particularly aspartic acid and glutamic acid, may contribute slightly to peptide bond vibrations in the amide I region (1700–1600 cm^{-1}), potentially affecting FTIR band assignments.⁹⁷ On the other hand, a major limitation of CD analysis arises from spectral overlap between peptide backbone signals and contributions from aromatic and sulfur-containing amino acid side chains.⁹⁸ Therefore, some authors recommend performing CD measurements at wavelengths below 178 nm to minimize such interference and improve secondary structure discrimination.⁹⁹

3.8. Small angle X-ray scattering

SAXS is a powerful method for studying the structure and oligomerization of proteins in solution and enables calculations



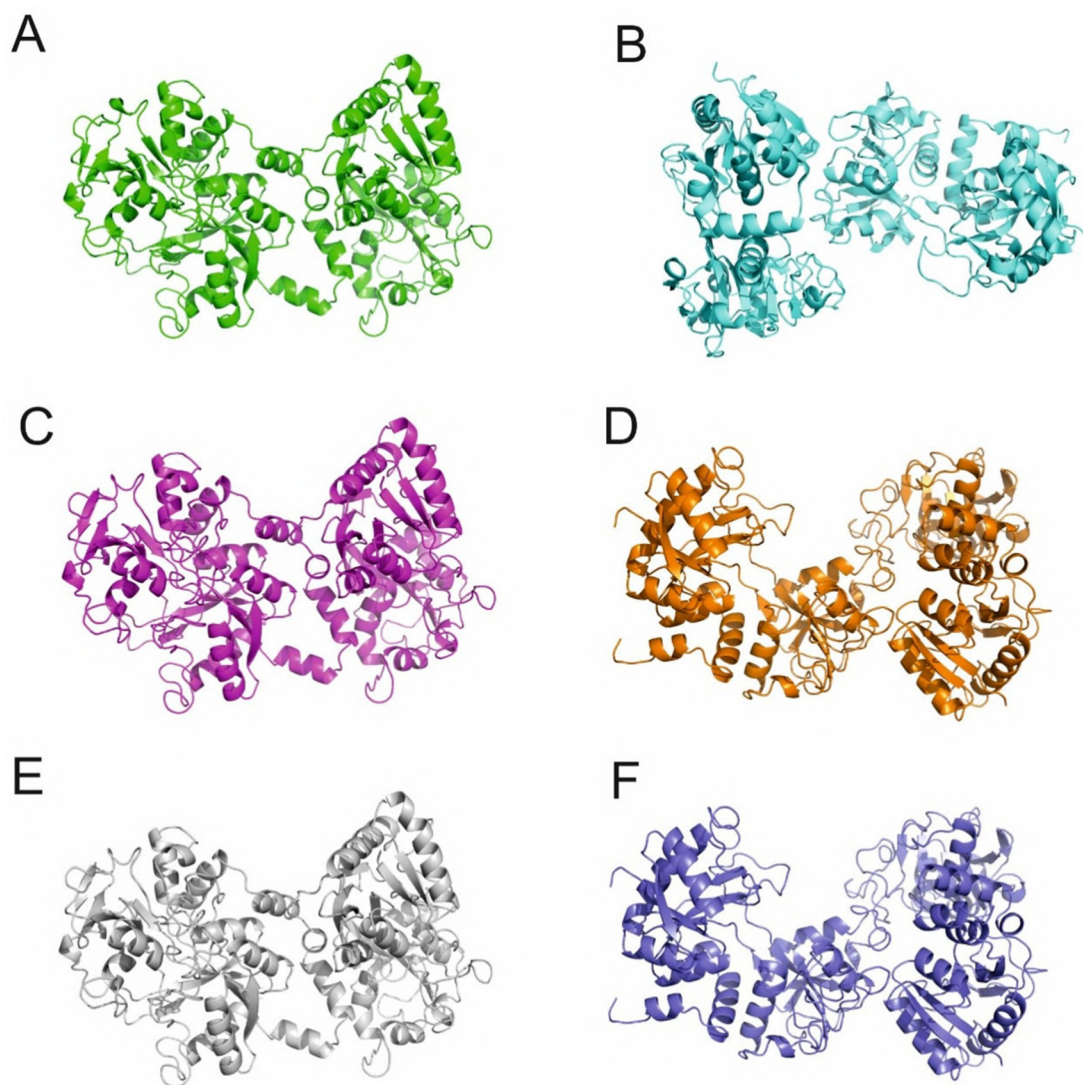


Fig. 10 Comparison of the spatial structures of the analyzed crystal structures and models with docked ruthenium ions (A – 1BLF, B – 4OQO, C – glide_His253_holo_1BLF, D – glide_His595_apo_4OQO, E – metaldock_His253_holo_1BLF, F – metaldock_His595_apo_4OQO).

Table 5 Comparison of structural parameters for crystal structures and analyzed models with docked ruthenium ions, where 1 = glide_His253_holo_1BLF, 2 = glide_His595_apo_4OQO, 3 = glide_His595_holo_1BLF, 4 = metaldock_His253_holo_1BLF, and 5 = metaldock_His595_apo_4OQO

Structural parameters	1BLF	4OQO	1	2	3	4	5
R_g [nm]	3.65	3.68	3.66	3.65	3.65	3.65	3.65
D_{max} [nm]	9.88	10.3	10.2	9.95	9.96	9.96	9.96
Volume [$\text{\AA}^3$]	1.2149×10^5	1.2386×10^5	1.2517×10^5	1.2517×10^5	1.2517×10^5	1.2517×10^5	1.2517×10^5

of structural parameters for crystal structures or theoretical structures. Structural parameters for the analyzed models and experimental data are summarized in Tables 5 and 6, respectively.

The formation of aggregates may be related to changes in the protein surface charge. To assess the influence of the medium, measurements were performed in both water and phosphate buffer. The linearity of the Guinier region con-

firmed that the analyzed LF solutions were monodisperse under the respective conditions. Based on SAXS data, LF existed predominantly as a dimer in water but as a monomer in phosphate buffer.

The radius of gyration (R_g) of the monomer calculated using CRY SOL is 3.6 nm. The R_g value determined for LF in phosphate buffer was also approximately 3.6 nm, whereas for LF analyzed in water it was about 6 nm. The maximum particle



Table 6 SAXS data collection and structural parameters for LF and LF–Ru complexes

Structural parameters	Sample			
	LF	LF–1.25Ru	LF–100Ru	LF–500Ru
R_g (from $p(r)$) (nm)	5.33	6.06	6.54	5.60
R_g (from Guinier) (nm)	5.17	5.89	6.75	5.78
D_{max} (nm)	17.28	18.18	19.55	19.51

diameter (D_{max}) likewise supported dimerization of LF in water, and the calculated volume led to the same conclusion. Analysis of the pair-distance distribution function and the *ab initio* models indicated an elongated dimer, suggesting association of the monomers along their longitudinal axes. Together, these results show that the ionic strength of the solution strongly influences LF oligomerization: LF behaves predominantly as a monomer in phosphate buffer but as a dimer in water.

Ru(III) interacted with LF and caused measurable changes in its structural parameters. However, only the two lowest precursor concentrations led to an increase in particle size (R_g and D_{max}), whereas the highest concentration decreased the apparent dimer size. This effect is likely related to the higher ionic strength of the solution. Kratky plots provide qualitative information on protein flexibility and unfolding (Fig. 11). The analysis indicates that LF is a highly flexible protein and that Ru(III) modification does not markedly alter the dynamics of the LF polypeptide chain. Within the resolution of the SAXS experiment, ruthenium binding therefore does not appear to disrupt the global LF structure.

3.9. Microscopy study

3.9.1. LM and SEM-EDS. According to the LM images, the LF structure is represented by large irregular plates (Fig. 12A). On the other hand, the surface of LF–Ru complexes became more crushed and granular, resulting in lyophilization (Fig. 12D, G and J). Besides, characteristic changes in the color of the LF–Ru complex with the measurable metal concentration were observed (Fig. 12J). According to the SEM

images, the morphology of the LF surface might be described as smooth-plated (Fig. 12B). In turn, the structure of the LF–Ru complexes was characterized by a higher fragmentation degree and increased roughness (Fig. 12E, H and K). The energy-dispersive X-ray spectra indicated carbon (C), nitrogen (N), oxygen (O), and sulfur (S) in all analyzed samples, which are known as key elements that form the protein structure (Fig. 12C, F, I and L). The previously determined weight ratio of C:N:O:S for LF was 54:18:24:1.4, which is consistent with the obtained results.²⁹ Remarkably, EDS results confirmed an abundance of ruthenium (Ru) element in LF–500Ru (Fig. 12L) samples whose content in the analyzed area was 2.48%. Moreover, the low amounts of iron (Fe), in the range of 0.11–0.18%, observed in all analyzed samples might be explained by the natural ability of LF to chelate Fe(III) ions; thus the initial iron saturation degree was 17% in non-modified LF.¹⁰⁰

3.9.2. TEM imaging. The obtained TEM images indicated significant changes in the LF microstructure with the increase in bound Ru(III) (Fig. 13). The remarkable increase in C percentage in all samples may be associated with the use of a carbon grid for the analysis. As observed, the LF–1.25Ru sample was mainly characterized by an amorphous protein matrix (Fig. 13A–C). Ruthenium was not detected in the analyzed region, which may be attributed to its low content and to the limited quantitative reliability of EDS in such nanometer-scale regions. The darker domains observed in LF–100Ru may correspond to local Ru-rich regions (Fig. 13E–G). At this stage, these observations should be interpreted cautiously as evidence of heterogeneous Ru accumulation rather than definitive proof of nucleation centers. Analogously to the SEM-EDS results, Ru was detected in LF–500Ru samples (Fig. 13L) at 0.36%. According to the TEM images of LF–500Ru, spherical heterogeneous metal nanoparticles were present (Fig. 13I–K). The particle size ranged from 5 to 10 nm. Lattice fringes visible in the magnified area were consistent with nanostructured crystalline RuO₂, for which the measured interplanar distance (*d*-spacing) was 3.06 Å. This *d*-spacing is characteristic of rutile-type RuO₂ and corresponds to the (110) plane.¹⁰¹

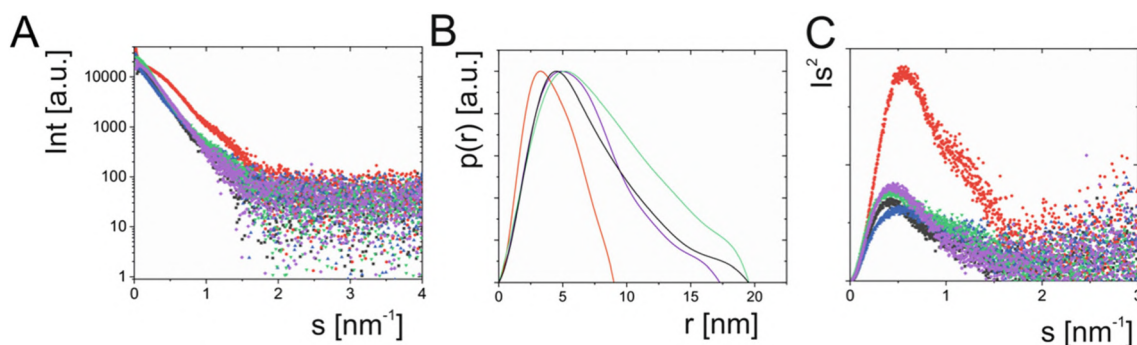


Fig. 11 SAXS data of LF and LF–Ru complexes: (A) experimental X-ray scattering data plotted as a function of the scattering angle for LF, (B) pair-distance distribution function, and (C) Kratky plot for the analyzed samples, where LF – black line, LF in phosphate buffer – red line, LF–1.25Ru – blue line, LF–100Ru – green line, LF–500Ru – violet line.



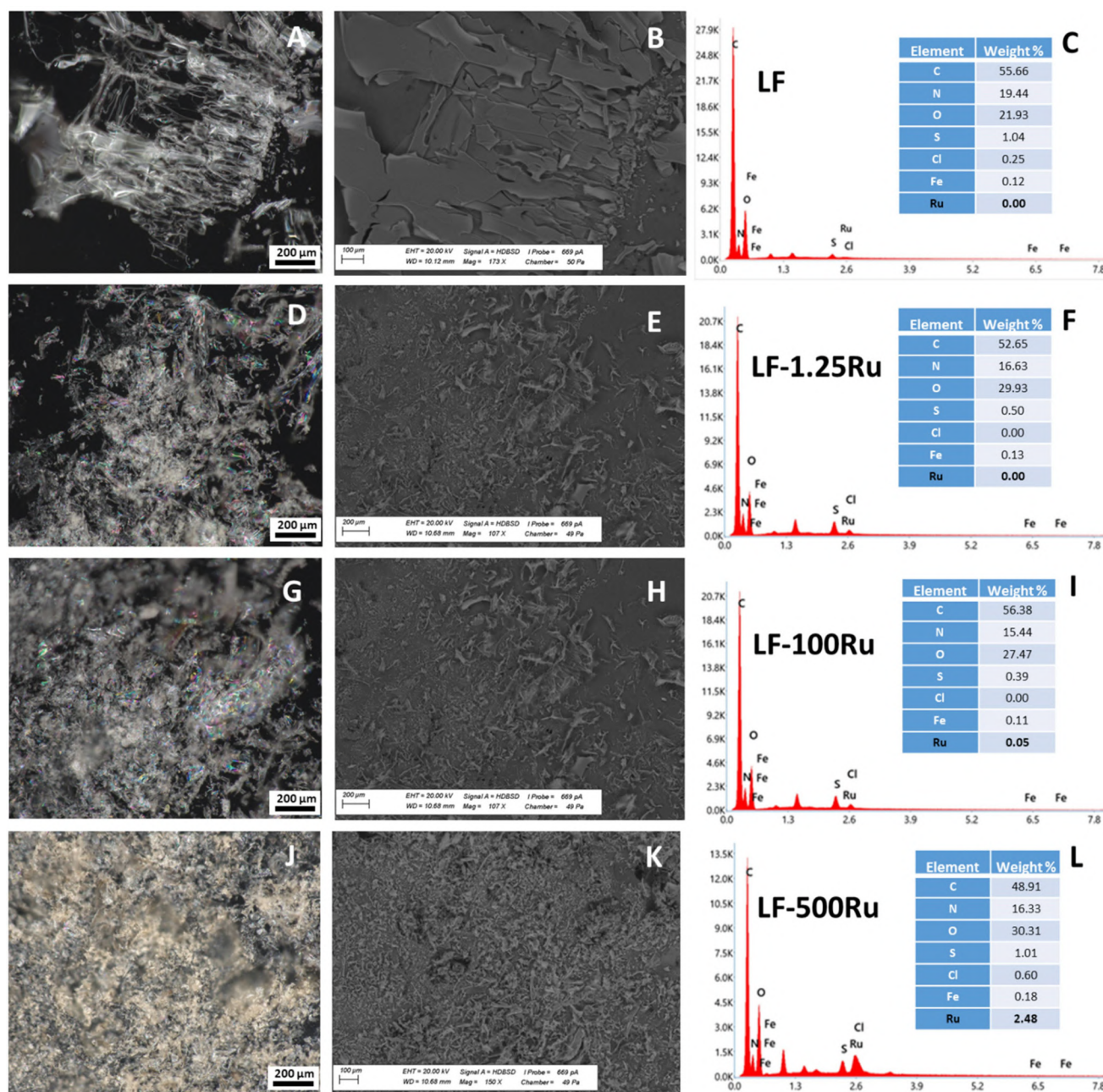


Fig. 12 Images of (A, D, G and J) LM, (B, E, H and K) SEM, and (C, F, I and L) EDS spectra recorded for (A–C) LF, (D–F) LF–1.25Ru, (G–I) LF–100Ru, and (J–L) LF–500Ru.

Protein-assisted synthesis of metal nanoparticles is widely recognized as a green synthetic approach. For example, He *et al.* reported BSA-assisted synthesis of RuO_2 nanoparticles with a mean size of about 7 nm,¹⁰² whereas RuO_2 nanoparticles obtained by green synthesis from *Murraya koenigii* leaves were found in the range of 5–12 nm.¹⁰³ Interestingly, Kaur *et al.* suggested that arginine, lysine, and tyrosine may participate in electron-transfer processes during metal-ion reduction,¹⁰⁴ and dissolved oxygen can in turn promote oxidation, leading to metal-oxide nanoparticle formation.¹⁰⁵ However, in the present system, TEM evidence for RuO_2 -like NPs was obtained only for

LF–500Ru, corresponding to the highest Ru loading. Therefore, any link between LF residues, charge-transfer processes, and nanoparticle growth should be treated as a possible high-loading scenario rather than as a general mechanism of LF–Ru complex formation. The present data do not allow us to conclude that nanoparticle formation contributes to the biological response of LF–1.25Ru or LF–100Ru.

3.10. Desorption study

As indicated, no significant Ru release from LF–100Ru was observed under simulated intestinal conditions over 3 h,



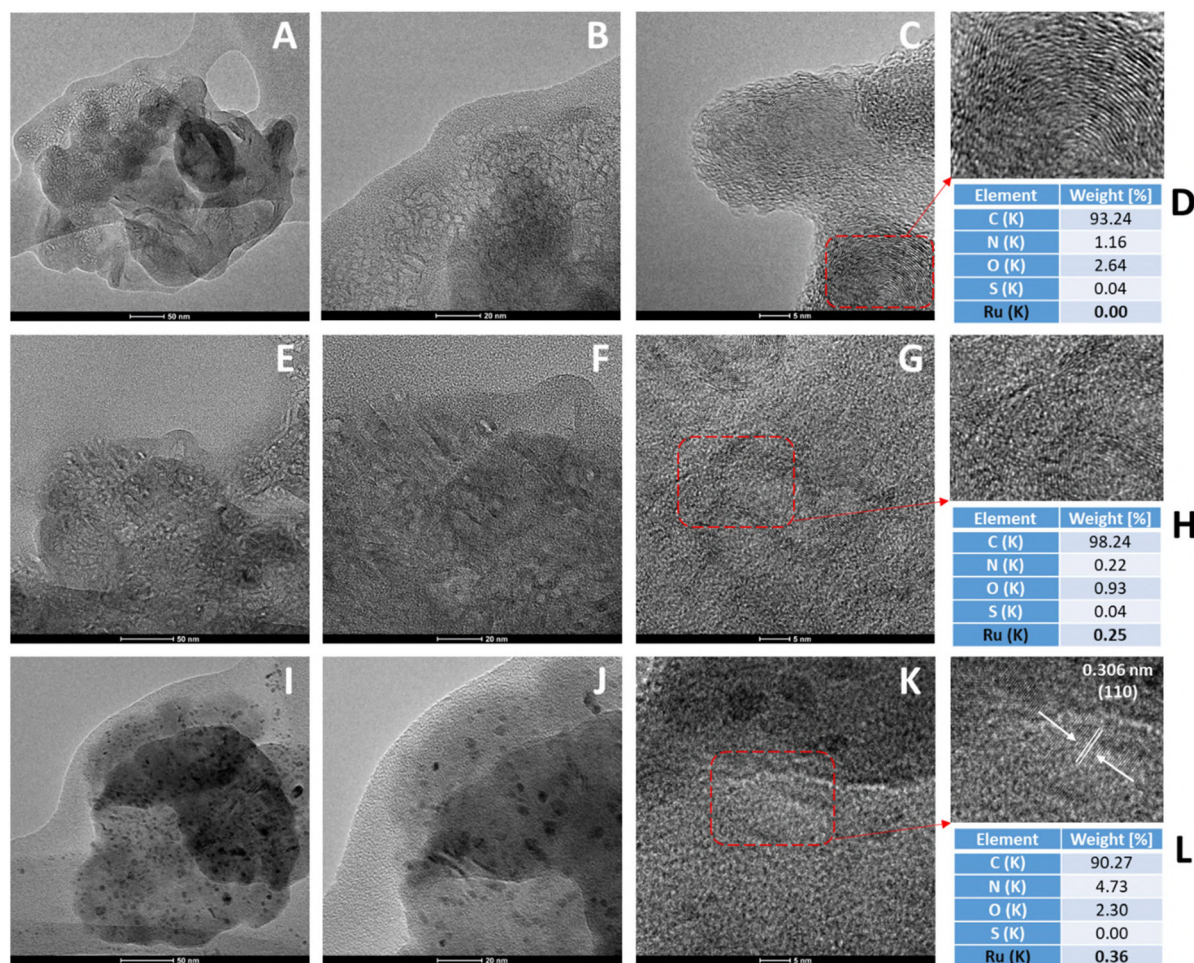


Fig. 13 TEM images of (A–C) LF-1.25Ru, (E–G) LF-100Ru, and (I–K) LF-500Ru and the elemental composition based on EDS results for (D) LF-1.25Ru, (H) LF-100Ru, and (L) LF-500Ru.

whereas metal loss under simulated gastric conditions without enzymes did not exceed 1.5% over the same time. Indeed, Marques *et al.* observed a similar trend in iron release from LF incubated at pH 2 and 7.4 within short time intervals.⁹⁵ The evident metal desorption was noticed only after 16 h of incubation at pH 2. Previously, Majka *et al.* confirmed the high sensitivity of LF iron saturation to pH conditions. Thus, the iron content in bLF decreased to about 1% after 24 h of dialysis in citrate buffer at pH 2.¹⁰⁶ Besides, Fe(III) release from bLF incubated in 0.1 M hydrochloric acid (pH 1.2) and phosphate buffer (pH 6.8) for over 2 h accounted for about 58 and 1.1% of the metal, respectively.⁵⁵ Based on the desorption study results, a strong binding affinity between bLF and Ru(III) in the formed complex can be assumed, which is consistent with the isotherm and fluorescence study findings. Previously, Raimondi *et al.* related low metal desorption to an effectively irreversible binding process.⁵⁷ From a practical perspective, the high stability of LF-100Ru in physiological fluids may positively influence the potential bioavailability of the synthesized complex.

3.11. SDS-PAGE study

SDS-PAGE results indicated a minor difference in electrophoretic mobility between LF and LF-100Ru. The molecular masses of LF and LF-Ru separated under non-reducing conditions were found to be 80 and 77 kDa, respectively (Fig. 14A).

In contrast, the sizes of LF and LF-100Ru were about 90 and 89 kDa under reducing conditions. Previously, a similar phenomenon during the separation of native LF and the LF-Fe complex was noted.⁵⁵ The authors related the changes in LF electrophoretic mobility under different modes to the loss of the biomolecule's metal binding properties after the addition of a reducing agent. Additionally, high molecular weight aggregates at $M_w > 150$ kDa were noted in all samples in both reducing and non-reducing modes, which indicated that their association was not regulated *via* disulfide covalent bonds.¹⁰⁷

The results of pepsin digestion kinetics confirmed a high sensitivity of both samples, LF and the LF complex, to gastric conditions (Fig. 14B). Remarkably, LF-Ru was more stable than LF at the initial stage of digestion (1 min). However, the



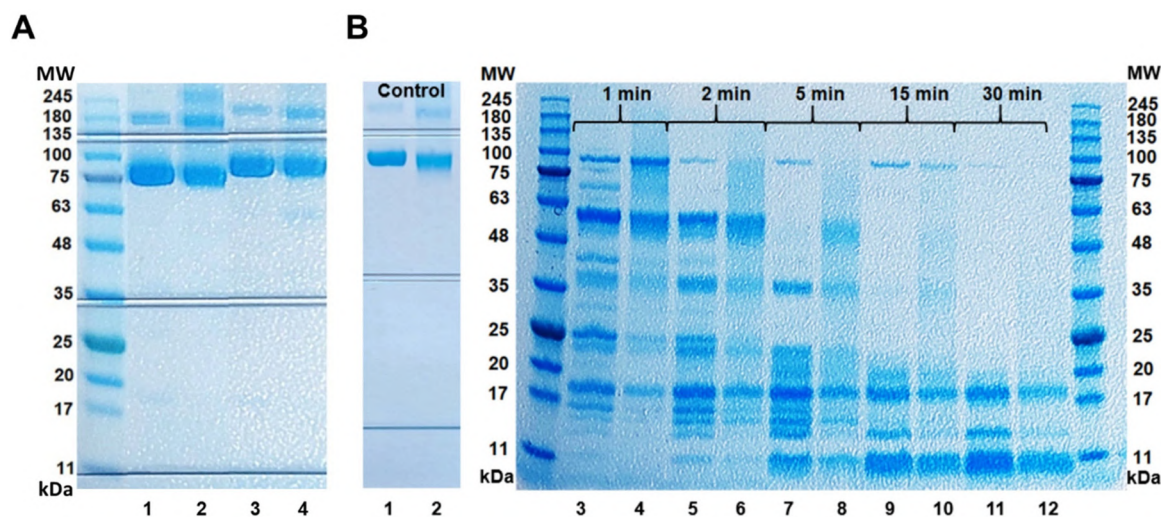


Fig. 14 (A) SDS-PAGE mass profiling of (1, 3) LF and (2, 4) the LF-100Ru complex performed under (1, 2) non-reducing and (3, 4) reducing conditions; (B) pepsin digestion kinetics of (3, 5, 7, 9, and 11) LF and (4, 6, 8, 10, and 12) the LF-100Ru complex performed after 1, 2, 5, 15, and 30 min of substrate incubation with the enzyme; control samples of (1) LF and (2) LF-100Ru.

LF structure showed a higher resistance to pepsin in the later stages (2–30 min). Previously, Pryshchepa *et al.* observed the complete digestion of LF and the LF-Ag complex after 30 min of sample incubation with the enzyme.³⁷ Taking into account the results of the control digestion, the LF-Ru structure exhibits significantly greater sensitivity to SGF containing pepsin than to SGF alone. Although the desorption study results confirmed a strong binding affinity in the formed LF-Ru complexes, the high digestibility of LF-Ru under gastric conditions minimizes its delivery efficiency to the target place (intestine). Numerous previous studies have demonstrated the potential of protein hydrolysates to act as carriers for various metal ions, including Fe(II), Ca(II), and Zn(II), resulting in enhanced bioaccessibility and bioavailability.¹⁰⁸ The binding affinity of a particular peptide for a metal is mainly influenced by its amino acid sequence, molecular mass, and steric effects.¹⁰⁸ Regarding Ru(III), several amino acids, including histidine (His), cysteine (Cys), lysine (Lys), aspartic acid (Asp), and glutamic acid (Glu), were mentioned as the most significant ligands.^{109,110} Despite this, other residues, *e.g.* tyrosine (Tyr), phenylalanine (Phe), glycine (Gly), alanine (Ala), and leucine (Leu) residues, might be successfully involved in the binding of Ru(III). Thus, it can be assumed that efficient peptic digestion does not necessarily result in metal release, as Ru(III) may remain coordinated to peptide fragments generated during digestion. Conversely, the loss of the LF primary structure during proteolysis may impair recognition by specific cellular receptors, potentially reducing metal uptake.¹¹¹ To preserve the integrity of the LF-Ru system and facilitate its controlled, site-specific release in the intestine, the application of an appropriate encapsulation approach may be required. Previously, Kilic *et al.* tested bovine serum albumin-tannic acid (BSA-TA) microcapsules in order to enhance the LF bioavailability.¹¹² The proposed delivery system effectively pro-

tected LF against gastric degradation, thus enabling protein release in the small intestine. Moreover, Niu *et al.* showed that LF encapsulated in a pectin-based colloidal system retained approximately $47 \pm 1.4\%$ of its native structure after 30 min of *in vitro* gastric digestion, demonstrating a significantly higher degree of protection compared to non-encapsulated LF, for which only $7 \pm 1.1\%$ of the native structure remained.¹¹³

3.12. Molecular docking in the LF-Ru complex

3.12.1. Ligand complex – geometry optimization and single-point calculation. Geometry optimization of the $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex performed using the Schrödinger Suite produced Ru-Cl bond lengths ranging from 2.27 to 2.35 Å, consistent with reported experimental values.¹¹⁴ The electrostatic potential (ESP) atomic charge on the Ru atom was +0.92, and the complex exhibited a near-octahedral geometry with bond angles of 94–96°. For comparison, geometry optimization performed using ORCA within the MetalDock workflow resulted in slightly longer Ru-Cl bond lengths (2.34–2.43 Å) and a lower Ru atomic charge (+0.52), likely due to differences in the applied basis sets. Nevertheless, the complex geometry remained close to octahedral with bond angles ranging from 94 to 98°.

3.12.2. Molecular docking – H253 site (holo-structure, PDB ID: 1BLF). The potential binding of the Ru(III) complex to bLF was investigated using molecular docking. Prior to docking, the coordinated water molecule was removed from the $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex, as it is expected to dissociate in aqueous biological environments. The DFT-optimized $[\text{RuCl}_5]^{2-}$ complex was treated as rigid during docking to preserve its geometry (Fig. S3).

Rigid docking using Glide produced a docking score of $-8.708 \text{ kcal mol}^{-1}$, with an initial Ru-Ne (His253) distance of 3.76 Å. After local minimization of the histidine residue, this



distance decreased to 2.37 Å, indicating the formation of a coordination bond between Ru and His253 (Fig. 15A and B). The chloride ligands formed salt bridges with positively charged residues (K210, K301, R121) and halogen bonds with Y92, Y192, K210, and K301. The Ru center also interacted with D297 and formed π -cation interactions with Y92 and Y192, which are known to coordinate Fe³⁺ in the native complex. The coordination bond preserved an octahedral geometry with angles of 88.5–92.7°. Distance-constrained docking shortened the Ru–N bond to 2.26 Å but significantly worsened the docking score (–2.006 kcal mol^{–1}). Similarly, induced-fit docking produced a less favorable pose (–4.104 kcal mol^{–1}) with a Ru–His distance exceeding 4 Å, indicating that rigid docking provided the most favorable configuration.

To confirm the docking pose, the MetalDock workflow was applied. A comparable orientation was obtained, with an initial Ru–Ne distance of 2.48 Å, which decreased to 2.61 Å after histidine minimization (Fig. 15C and D). The estimated binding free energy was –6.83 kcal mol^{–1}, and the interaction pattern closely resembled the Glide result, although one coordination angle (78.4°) slightly deviated from ideal octahedral geometry.

3.12.3. Molecular docking – H595 site (holo-structure, PDB ID: 1BLF). Docking at the alternative His595 site was also evaluated. This region forms a sterically constrained environment, and most Glide poses positioned the complex 5–7 Å from the Ne atom of His595. The closest pose showed a Ru–Ne distance of 3.24 Å with a docking score of –2.411 kcal mol^{–1}, suggesting that coordination bond formation is unlikely (Fig. 15E and F).

Similarly, MetalDock docking at this site failed to produce a stable coordination geometry, with the Ru atom remaining approximately 4 Å from the His595 Ne atom.

3.12.4. Molecular docking – H595 site (apo-structure, PDB ID: 4OQO). Docking was also performed using the apo-bLF structure. In this case, the solvent-exposed C-lobe provided greater spatial accessibility around His595, facilitating ligand binding. The best Glide pose showed a Ru–Ne distance of 2.6 Å with a docking score of –3.803 kcal mol^{–1} and the coordination bond geometry featured angles close to 90° (Fig. 15G). MetalDock produced an even closer pose with a Ru–Ne distance of 2.46 Å and an estimated binding free energy of –7.02 kcal mol^{–1}. This configuration was additionally stabilized by interactions with nearby residues, including D395 (Fig. 15H).

3.13. Molecular dynamics

Classical MD simulations were performed to elucidate the interaction mechanism between the [RuCl₅]^{2–} complex and three structural configurations of bovine lactoferrin (bLF). The equilibrated structures obtained after 30 ns of production simulation are shown in Fig. 16. The initial configurations for each system were derived from the molecular docking results to ensure realistic starting orientations of the metal complex within or near the protein binding regions. Throughout the simulation, the [RuCl₅]^{2–} complex exhibited a stable association with several amino acid residues of bLF through short-range non-covalent interactions, including hydrogen bonding, halogen contacts, and van der Waals forces. In the apo-bLF

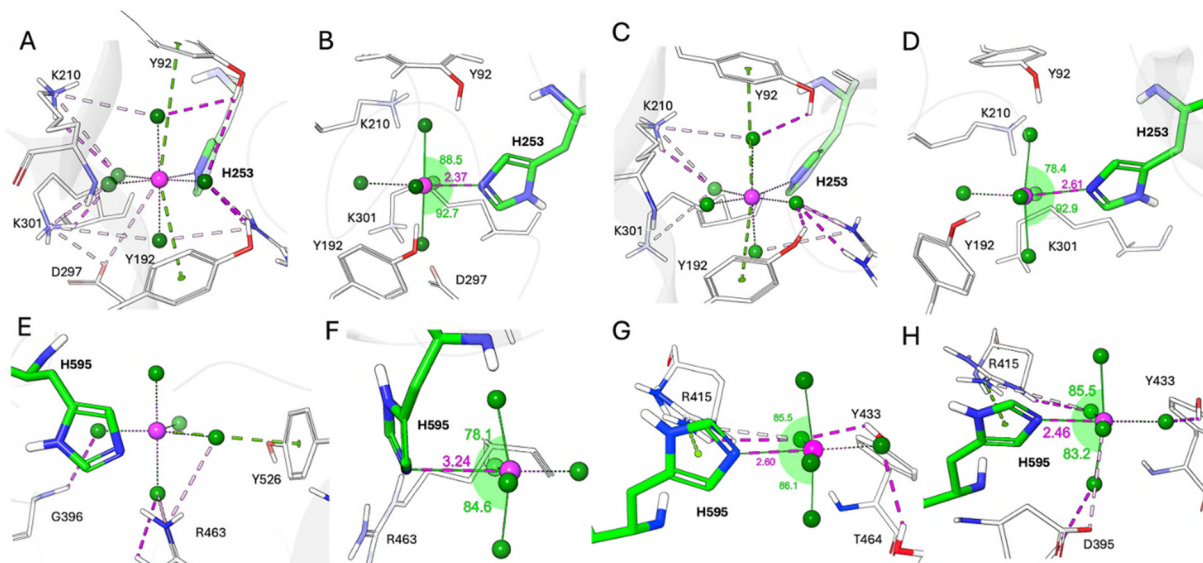


Fig. 15 Molecular docking of the Ru(III) complex to bLF H253 and H595 sites. (A and B) Glide docking to holo-bLF site H253 (PDB ID 1BLF): (A) Ru complex interactions with the residues surrounding the coordination site H253, (B) distance and geometry of the coordination bond to H253 (non-covalent interactions are omitted for clarity); (C and D) MetalDock docking to holo-bLF site H253 (PDB ID 1BLF): (C) Ru complex interactions with the residues surrounding H253, (D) distance and geometry of the coordination bond to H253; (E and F) glide docking to holo-bLF site H595 (PDB ID 1BLF): (E) Ru complex interactions with the residues surrounding the coordination site H595, (F) distance and geometry of the coordination bond to H595; (G and H) docking to apo-bLF site H595 (PDB ID 4OQO): Ru complex interactions and coordination bond obtained using (G) glide and (H) MetalDock. Salt bridges in light pink and halogen bonds in magenta; π -cation interactions in green.



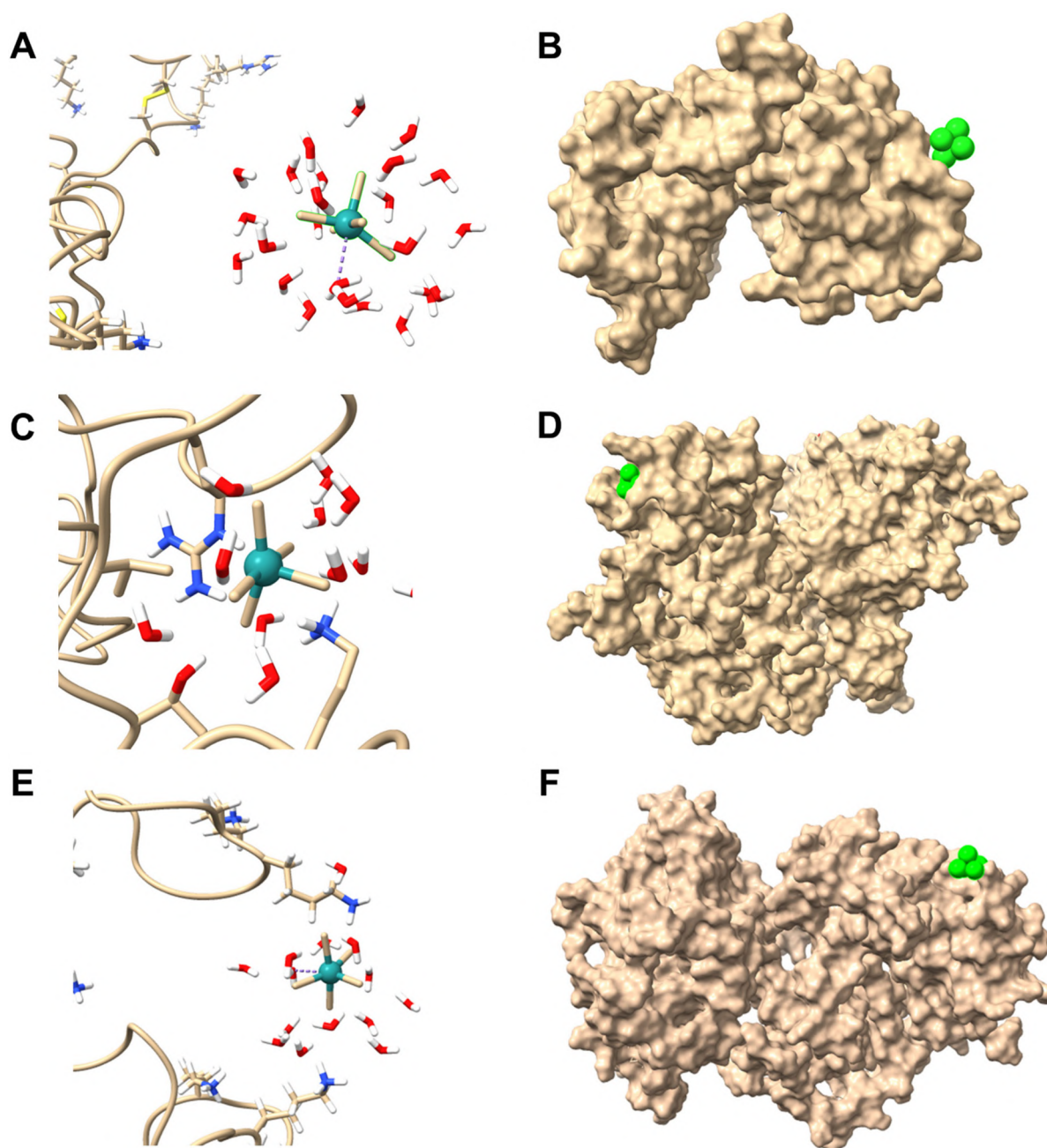


Fig. 16 MD snapshot of BLF with the metal complex after the production run of (A and B) Apo_blf, Holo_blf at His253 (C and D), and His595 (E and F), respectively. The metal complex is shown in (B), (D), and (F) as lime spheres.

system, the complex remained near the protein surface, stabilized by surrounding water molecules that formed a well-defined solvation shell. The closest interaction distances were measured as 3.18 Å between the Ru atom and Cys335 (HB2) and 2.06 Å between Arg349 (HE) and the Cl5 atom of the complex after 28 ns of simulation. By the end of 30 ns, the complex maintained solvation but shifted slightly away from the protein surface, indicating dynamic yet non-dissociative behavior. For the 1BLF-His253 configuration, the metal complex localized near the His253 site, forming comparatively

stronger and more stable interactions with several residues: Lys28-HZ2 (2.10 Å), Arg39-HE (2.15 Å), and Thr35-HG1 (2.16 Å). In contrast, in the 1BLF-His595 configuration, the complex stabilized closer to the protein surface with the shortest distances observed for Lys27-HZ2 (1.93 Å) and Lys100-HZ1 (4.53 Å). These observations suggest that arginine and lysine residues play dominant roles in anchoring the complex through short polar contacts, followed by contributions from cysteine and threonine residues. The chlorine atoms of the complex were particularly involved in halogen bonding and



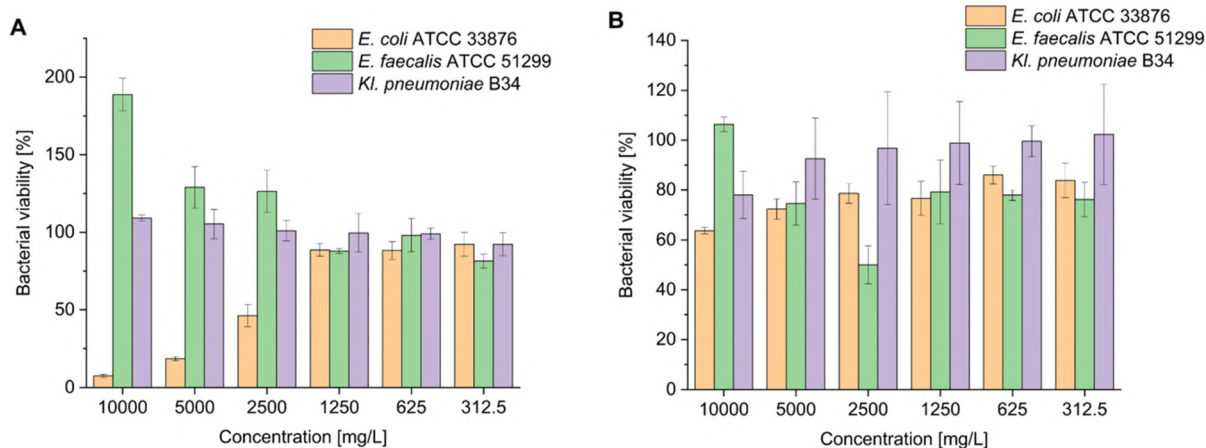


Fig. 17 Changes in bacterial viability $\pm$ SD after treatment with (A) LF and (B) the LF-100Ru complex.

weak dispersion interactions with the side-chain donor atoms of these residues. Collectively, these results indicate that both polar and weakly hydrophobic residues cooperatively stabilize the metal complex on the bLF surface, in good agreement with the molecular docking predictions.

The structural stability and conformational behavior of bovine lactoferrin (bLF) upon complexation with the metal complex were evaluated through root mean square deviation (RMSD) analysis, as shown in Fig. S4. RMSD quantifies the average displacement of atomic positions relative to the reference structure and serves as a sensitive indicator of backbone stability and equilibration during molecular dynamics simulations. In all three systems, namely apo-bLF, His253-bound, and His595-bound, an initial rise in RMSD was observed during the first few nanoseconds, corresponding to the relaxation of the protein from its docked conformation toward an energetically favorable state. The apo-bLF configuration showed large fluctuations throughout the simulation, indicating higher conformational flexibility in the absence of the metal complex. In contrast, both holo-systems showed a more gradual RMSD stabilization after approximately 20 ns, reflecting that the protein-metal complex systems had reached equilibrium without any major structural distortion. Among the two holo-configurations, the His253-bound complex maintained the lowest and most stable RMSD plateau suggesting that the coordination of the $[\text{RuCl}_5]^{2-}$ anion at this site enhances the rigidity of the surrounding structural domain and promotes overall backbone stability. The equilibrated His595-bound complex exhibited a slightly higher RMSD plateau with moderate fluctuations, implying greater local flexibility near the binding interface. The absence of continuous RMSD drift beyond 20 ns confirms that none of the systems experienced large-scale unfolding or denaturation under the simulated conditions. Collectively, the RMSD profiles demonstrate that the Ru(III) complexation exerts a stabilizing influence on bLF, particularly at the His253 binding site, likely through persistent polar and halogen-mediated interactions that restrict large-amplitude backbone motions.

Furthermore, to complement the MD analysis, density functional theory (DFT) calculations were performed on model complexes formed between the $[\text{RuCl}_5]^{2-}$ species and representative amino acid functional groups. These calculations are provided in the SI and were designed to study the intrinsic donor preferences of the Ru(III) center toward N-, O-, and S-donor atoms of individual amino acids (Figs. S5 and S6).

3.14. Antibacterial activity of LF and LF-100Ru

The ability of both LF and LF-100Ru to affect the growth of the selected bacterial strains, namely, *E. coli* ATCC 33876, *E. faecalis* ATCC 51299, and *Kl. pneumoniae* B34, was evaluated over a wide concentration range of 312.5–10 000 mg L^{-1} using a resazurin-based assay. This concentration range corresponds to 3.30–105.4 mg L^{-1} of Ru in LF-100Ru. According to the obtained results, both LF and the LF-100Ru complex affected the viability of the analyzed bacterial strains (Fig. 17). It was observed that the viability of *E. coli* notably decreased from 92 to 8% as the protein concentration increased. Previously, Biernbaum *et al.* determined that the MIC values of pure LF and raw LF found in bovine milk for *E. coli* were 3500 and 14 000 mg L^{-1} , respectively.¹¹⁵ Moreover, it was indicated that LF-Zn and LF-Cu complexes were characterized by higher antibacterial activity against *E. coli* than native and apo-LF.^{116,117} The authors assumed that the greater antibacterial potential of metal-modified LF complexes might be associated with their ability to interact with bacterial cell surface, thereby resulting in its damage (bactericidal effect). On the other hand, LF-Mn complexes exhibited moderate antibacterial activity towards *E. coli* and were less effective than native and apo-LF but more effective than holo-LF.¹¹⁸ The enhanced activity of apo-LF can be attributed to its bacteriostatic mechanism, characterized by iron sequestration and the consequent inhibition of bacterial growth. Based on our results, treatment with LF-100Ru at the highest concentration resulted in a reduction of *E. coli* viability to only 64%. Thus, we can assume that Ru(III) bound to LF may negatively affect the protein's inhibitory activity, either due to the potential occupation of iron-binding sites by the



incorporated metal ions or weaker interactions with the bacterial cell membrane. As observed, supplementation with LF promoted the growth of *E. faecalis*; thus, its viability was positively correlated with the protein concentration and increased two-fold at 10 000 mg L⁻¹ of LF. In the case of LF-100Ru, the viability of *E. faecalis* ranged from 50 to 107%, depending on the applied complex concentration. Remarkably, the highest inhibitory potential of LF-100Ru was observed at a concentration of 2500 mg L⁻¹. Previously, it was confirmed that *E. faecalis* strains were highly resistant to LF and the protein supplementation may facilitate bacterial growth.¹¹⁹ Previously, Garbe *et al.* explained this phenomenon with the ability of *E. faecalis* to use glycans found in the LF structure as nutrients.¹²⁰ In contrast, Milanowski *et al.* indicated that the MIC value of LF-Ag for *E. faecalis* was 1250 mg L⁻¹ which might be associated with the formation of a nanocomplex characterized by a high antibacterial efficiency.^{121,122} Previous studies indicated that Ru-based complexes exhibited a much higher antibacterial effect toward Gram-positive species than towards Gram-negative bacteria.¹²³ For instance, ruthenium incorporation significantly enhanced the antimicrobial activity of shikimate cross-linked chitosan composites against *S. aureus*.⁴ Indeed, no significant antibacterial effect of LF was observed against *K. pneumoniae*, whose viability remained relatively constant over the entire concentration range. On the other hand, treatment with LF-100Ru at a concentration of 10 000 mg L⁻¹ caused a decrease in bacterial viability to 78%. Remarkably, Murata *et al.* reported that LF at a concentration of 8000 mg L⁻¹ inhibited the growth of *K. pneumoniae* by 50%.¹²⁴ In contrast, Kutila *et al.* reported that the antimicrobial activity of LF exhibited strain-dependent variability against *K. pneumoniae*.¹²⁵ It is worth noting that the tested concentration range primarily allows for the evaluation of biocompatibility and interaction trends rather than therapeutic antibacterial efficacy. For instance, the determined MIC values of eravacycline, the popular synthetic antibiotic, against clinical isolates of *E. coli*, *E. faecalis*, and *K. pneumoniae* were 0.5, 0.25, and 2 mg L⁻¹, respectively.¹²⁶ Furthermore, the MIC values of a series of carbene-Ru(II) complexes against *E. coli* ranged from 800 to 1000 mg L⁻¹, whereas the MIC of ampicillin was 3.12 mg L⁻¹.¹²⁷ Overall, these findings indicate that Ru(III) coordination modifies the biological response of LF in a strain-dependent manner.

In relation to the obtained digestion results, it is worth noting that LF-derived peptides, including LF1-11, lactoferricin, and lactoferrampin, exhibit strong antibacterial activity and may be more effective against bacteria than intact LF.¹²⁸ The reported MIC values for LF and lactoferricin against *Staphylococcus aureus* were 500 and 8 mg L⁻¹, respectively.¹²⁴ These N-terminal peptides are highly basic (pI ~ 9.7–11.7) and interact strongly with negatively charged lipopolysaccharides (LPS), leading to membrane destabilization and subsequent bacterial damage.¹²⁸ The desorption study results confirmed the strong binding of Ru(III) to the protein; therefore, it can be assumed that Ru(III) bound to LF fragments may influence their local charge distribution, potentially modulating the

strength of peptide-membrane interactions. Despite this, previous structural modelling and MIC studies have demonstrated that the relative positioning of cationic residues flanking the β -strand in lactoferricin, as well as their orientation with respect to hydrophobic tryptophan residues, determines the efficiency of membrane interaction and disruption.¹²⁹ On the other hand, Tomita *et al.* reported that the addition of iron (0.1 M FeSO₄) had no effect on the antibacterial activity of pepsin-derived LF peptides against *E. coli*, confirming their iron-independent (bactericidal) mechanism of action, in contrast to intact LF.^{130,131} In contrast, calcium and magnesium ions at concentrations higher than 0.1 M significantly reduced the activity of lactoferricin by interfering with peptide-membrane interactions.¹³² Since the present study does not directly address peptide-level interactions with Ru(III), this aspect remains unclear and could represent an interesting direction for future investigation.

3.15. Cytotoxicity of LF-Ru complexes and their impact on cell viability

To evaluate the effects of the three LF-Ru complexes tested at four different concentrations (625, 1250, 2500 and 5000 mg L⁻¹) on cell viability and membrane integrity, two complementary assays were performed using L929 fibroblasts, HepG2 hepatic cells and Caco-2 intestinal epithelial cells. The MTT and LDH assays were performed to evaluate the biological response of the cells to LF-Ru treatment. The MTT assay (Fig. 18A–C) assesses metabolic activity as an indicator of cell viability, while the LDH assay (Fig. 19A–C) measures membrane integrity, providing complementary information on potential cytotoxic effects. The calculated concentrations of Ru in the respective LF-Ru complexes are shown in Table 7.

For the L929 cell line (Fig. 18A), non-modified LF exhibited cytotoxic effects at 20 000 and 10 000 mg L⁻¹, as evidenced by a significant reduction in cell viability to approximately 50 and 52%, respectively. Therefore, subsequent studies of LF-Ru complexes began at 5000 mg L⁻¹, a concentration at which LF alone showed only borderline cytotoxicity, with viability around 70%. At 2500 mg L⁻¹ and below, LF no longer exhibited toxic effects, and a progressive increase in cell proliferation was observed as the concentration decreased. The LF-Ru complexes did not show clear cytotoxicity toward L929 cells under most conditions. However, a decrease in cell viability was observed for LF-100Ru at 625 mg L⁻¹ and for LF-1.25Ru at 1250 mg L⁻¹, with viability reaching 72 and 74%, respectively; these results were accompanied by relatively high standard deviations. Notably, at 5000 mg L⁻¹, all three LF-Ru complexes significantly enhanced cell proliferation compared with LF, suggesting that Ru incorporation may reduce the adverse effect of high LF concentrations in this model. The LDH assay results for L929 (Fig. 19A) confirmed the cytotoxicity of high-dose LF, showing a dose-dependent increase in LDH release at 20 000, 10 000, and 5000 mg L⁻¹, indicative of membrane damage and loss of integrity. In contrast, lower LF concentrations (<1250 mg L⁻¹) showed LDH levels comparable to the cellular control. Among the LF-Ru complexes, LF-500Ru



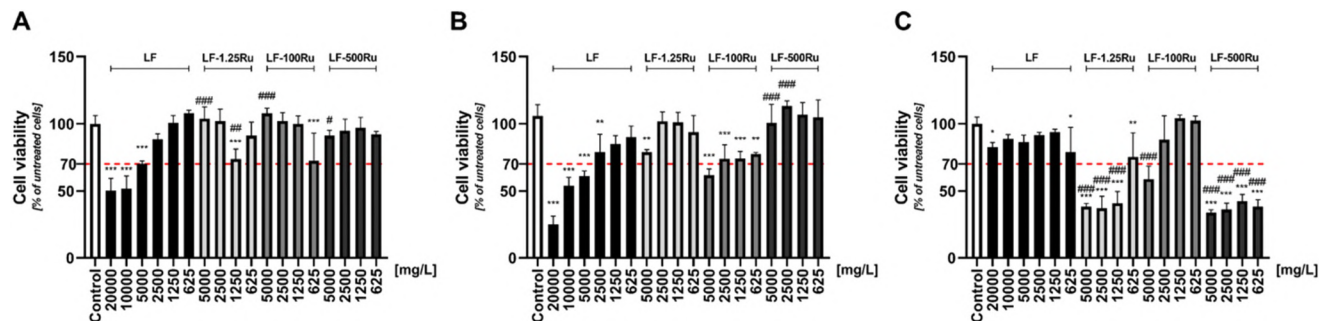


Fig. 18 Viability of (A) L929 fibroblasts, (B) HepG2 cells, and (C) Caco-2 cells following 48 h of incubation with LF, LF-1.25Ru, LF-100Ru, and LF-500Ru was assessed relative to untreated cells (defined as 100%). Results are presented as the mean $\pm$ SEM from five independent replicates. Statistical significance is denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Comparisons against the LF control are indicated by pound signs (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). A dashed line represents the established cytotoxicity threshold.

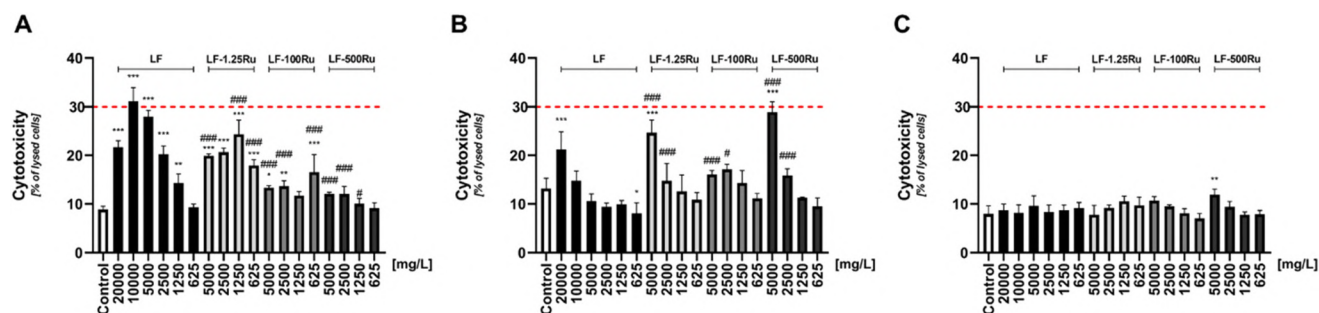


Fig. 19 LDH release from (A) L929 fibroblasts, (B) HepG2 cells, and (C) Caco-2 cells after 48 h of exposure to LF, LF-1.25Ru, LF-100Ru, and LF-500Ru was measured as an indicator of membrane integrity and cytotoxicity, and expressed relative to untreated controls (set as 100%). Data are reported as mean $\pm$ SEM from five independent experiments. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Comparisons against the LF-treated group are marked with pound signs (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). The cytotoxicity threshold is denoted by a dashed horizontal line.

Table 7 Ruthenium concentration in the tested LF-Ru complexes

Concentration of the added LF-Ru complex [mg L ⁻¹]	LF-1.25Ru		LF-100Ru		LF-500Ru	
	Ruthenium concentration					
	Ru [mg L ⁻¹]	Ru [μM]	Ru [mg L ⁻¹]	Ru [μM]	Ru [mg L ⁻¹]	Ru [μM]
5000	0.62	6.12	52.7	521.2	175.8	1739.6
2500	0.31	3.06	26.3	260.6	87.9	869.8
1250	0.15	1.53	13.2	130.3	44.0	434.9
625	0.08	0.77	6.6	65.2	22.0	217.5

induced only minimal LDH release across all tested concentrations, aligning with the MTT results. Similarly, LF-100Ru displayed low LDH activity (~11–17%). LF-1.25Ru consistently elevated LDH release (~18–25%) across all tested concentrations and therefore showed the highest membrane-damaging tendency among the analyzed formulations, although the overall cytotoxicity remained below 30%.

HepG2 cells (Fig. 18B) exhibited a response to LF similar to that of L929 cells. Concentrations of 20 000, 10 000, and 5000 mg L⁻¹ led to a marked decline in viability, to approxi-

mately 25, 54, and 61%, respectively. In contrast, LF-500Ru showed a potential stimulatory effect on HepG2 cells at all tested concentrations, with viability at or above 100% and no clear indication of cytotoxicity. While these values did not differ significantly from the cellular control, significant improvements relative to LF-treated cells were observed at 5000 and 2500 mg L⁻¹, suggesting a more favorable response profile. LF-100Ru at 5000 mg L⁻¹ showed cytotoxicity comparable to that of LF, with viability around 61%, whereas lower concentrations (625–2500 mg L⁻¹) did not show overt cytotoxicity but remained close to the threshold level. LF-1.25Ru did not display clear cytotoxicity; the 5000 mg L⁻¹ dose was borderline, while lower concentrations did not differ significantly from either control. The LDH assay results for HepG2 (Fig. 19B) supported the MTT findings. LF induced a strong, dose-dependent increase in LDH release, with the highest membrane disruption observed at 20 000 mg L⁻¹, reaching approximately 21%. All three LF-Ru complexes showed low LDH levels at 625–1250 mg L⁻¹, consistent with good cellular tolerance. At 5000 mg L⁻¹, elevated LDH activity was recorded for LF-500Ru (~29%), LF-1.25Ru (~25%), and LF-100Ru (~16%). Despite this increase, the MTT data indicated



enhanced cell viability in the presence of LF-500Ru and LF-1.25Ru, suggesting that the observed LDH release may reflect transient stress responses rather than classical cytolytic effects.

In Caco-2 cells (Fig. 18C), LF exhibited no cytotoxic activity across the entire concentration range, highlighting its good biocompatibility with intestinal epithelial cells. By contrast, LF-500Ru induced a significant reduction in viability (approximately 34–39%) across all tested concentrations (625–5000 mg L⁻¹), without a clear dose–response trend. LF-100Ru was cytotoxic only at 5000 mg L⁻¹, while lower concentrations were well tolerated. The LF-1.25Ru complex reduced cell viability at concentrations ranging from 5000 to 1250 mg L⁻¹, demonstrating high cytotoxicity (approximately 37–41%). The concentration of 625 mg L⁻¹ was considered borderline, with cell viability reaching 75%. Interestingly, LDH measurements in Caco-2 cells (Fig. 19C) revealed low levels of enzyme release across all conditions, regardless of compound or concentration. LDH values remained comparable to the cellular control, indicating preserved membrane integrity and the absence of acute cell damage. This apparent discrepancy with MTT results – where a reduction in metabolic activity was observed, particularly for LF-500Ru – suggests a non-cytolytic mechanism of action. The data indicate that these complexes may exert cytostatic effects, inhibiting proliferation or metabolic function (*e.g.*, *via* cell cycle arrest or mild stress induction), without disrupting membrane structures or inducing necrotic cell death.

The results obtained from the MTT and LDH assays provide important insight into the cellular response to LF and LF-Ru complexes across different models. While LF induced cytotoxic effects in L929 and HepG2 cells at higher concentrations, it did not affect the viability of Caco-2 cells, which is consistent with the known compatibility of LF with intestinal epithelial models. For example, Matsuzaki *et al.* demonstrated that intact bovine LF was actively taken up and released by Caco-2 enterocytes without compromising cell integrity.¹³³ Zhao *et al.* likewise showed that bLF enhanced Caco-2 viability in a concentration-dependent manner at 50–200 mg L⁻¹ and improved transepithelial electrical resistance (TEER) together with tight-junction protein expression.¹³⁴ LF-Ru complexes displayed cell-line- and loading-dependent effects rather than a uniform toxicity pattern. LF-500Ru enhanced viability in HepG2 and L929 cells under several conditions, but the same formulation reduced metabolic activity in Caco-2 cells, indicating that its response is context dependent. The most intriguing observations were made in the Caco-2 model, where decreases in metabolic activity occurred together with minimal LDH release. This pattern suggests a predominantly non-cytolytic effect, more consistent with cytostatic or metabolic modulation than with acute membrane damage. In the present study, LF-100Ru was relatively well tolerated by normal L929 fibroblasts, while a decrease in viability was observed for HepG2 and Caco-2 cancer cells at 5000 mg L⁻¹ (corresponding to 521.2 μM Ru). These findings indicate a differential cellular response under the tested conditions rather than pharmacological selectivity. For comparison, cisplatin has been reported to induce approximately 90% loss of colorectal cancer cell via-

bility (HT29 and SW480) at 10 μM, whereas KP1019 reduces cell viability by about 50% (IC₅₀) at 50–80 μM, depending on the cell line.¹³⁵ Similarly, the IC₅₀ values of a series of Ru(II) polypyridyl complexes and cisplatin against HepG2 cells were 17.7–25.3 μM and 11.5 μM, respectively.¹³⁶ Notably, the corresponding free ligands did not exhibit cytotoxicity. Such observations highlight that Ru-based systems often exhibit biological effects at comparatively higher concentrations than platinum drugs. In contrast, although NAMI-A demonstrated significant antimetastatic activity, it did not decrease the viability of mammary adenocarcinoma (TS/A), oral carcinoma (KB), or melanoma (B16-F10) cell lines even at 100 μM,¹³⁷ and its reported IC₅₀ against HT-29 cells was 339 μM.¹³⁸ Accordingly, the assays performed in this study were intended primarily to evaluate biocompatibility and preliminary cellular responses rather than therapeutic efficacy.

Cellular morphology was evaluated after 48 h of incubation using an inverted phase-contrast microscope. All images are shown in the SI. Representative images of L929 fibroblasts (Fig. S7), HepG2 hepatocytes (Fig. S8), and Caco-2 epithelial cells (Fig. S9) are presented after exposure to all three LF-Ru complexes at 5000 and 625 mg L⁻¹ together with the respective controls.

The comparative analysis of cell morphology was consistent with the results of the MTT and LDH assays, further confirming the observed cytotoxic or biocompatible effects of the tested compounds. Microscopic analysis of L929 fibroblasts revealed a typical spindle-shaped morphology and uniform adherence, consistent with healthy cell architecture. No signs of cytotoxic damage were observed. The LF-500Ru complex was suspended directly in the culture medium, which explains the presence of dark particulate matter visible in all its micrographs.

Phase-contrast microscopy of HepG2 cells revealed morphology consistent with the biochemical assays. In cultures treated with LF-500Ru, both tested concentrations (5000 and 625 mg L⁻¹) preserved normal epithelial features: the cells remained polygonal, tightly packed, and adherent, indicating good cytocompatibility. This was particularly evident at 625 mg L⁻¹, where the image was not disturbed by visible particles of the complex. Treatment with LF-100Ru at 5000 mg L⁻¹ led to visible morphological alterations, including cell rounding, partial detachment, and decreased confluence, consistent with cytotoxicity; however, at 625 mg L⁻¹, most cells maintained near-normal morphology. For LF-1.25Ru, cells exposed to 5000 mg L⁻¹ showed minor morphological changes, such as reduced density and occasional rounding, whereas at 625 mg L⁻¹, the monolayer structure remained largely intact.

In the control and LF-treated cells at both 5000 and 625 mg L⁻¹, Caco-2 cells maintained a characteristic cobblestone-like morphology and dense monolayer structure, indicative of high viability and the absence of overt cytotoxicity. Microscopic evaluation of Caco-2 cells after treatment with LF-Ru complexes revealed changes that were generally consistent with the viability and LDH results, although not all biochemical effects were mirrored by marked structural damage. Although the



MTT assay showed reduced metabolic activity for LF–Ru complexes at 5000 mg L⁻¹, phase-contrast microscopy revealed only minor morphological changes at both tested concentrations (5000 and 625 mg L⁻¹). The cells displayed reduced density but largely retained their epithelial cobblestone morphology, with only minor signs of reduced confluence and focal detachment. These results suggest that metabolic activity was impaired, as confirmed by the MTT assay, whereas overall membrane integrity and basic cell architecture remained relatively preserved, consistent with the low LDH release observed in these samples.

4. Conclusions

In the present work, bLF was successfully modified with Ru(III) ions and the resulting LF–Ru systems were characterized using complementary experimental and computational methods. Batch sorption experiments revealed saturation-like binding behavior, with binding efficiencies ranging from 24.0 to 49.6%. Thermodynamic analysis indicated favorable and spontaneous LF–Ru formation ($\Delta G < 0$). Kinetic studies showed that most Ru(III) uptake occurred rapidly within approximately 2 min and was followed by only minor redistribution before equilibrium was reached. Fluorescence quenching confirmed strong interactions in the LF–Ru system; however, the $n \approx 1$ result should be interpreted as one dominant fluorescence-reporting binding event rather than proof that only one Ru-binding site exists in lactoferrin. Desorption experiments demonstrated only minor Ru release from LF–100Ru in SGF and SIF without enzymes, indicating high stability of this formulation under the tested conditions. At the same time, pepsin digestion showed rapid loss of the intact LF scaffold in SGF, suggesting that although Ru may remain associated with digestion fragments, receptor-mediated delivery of intact LF–Ru could be limited without additional formulation strategies. ATR-FTIR suggested loading-dependent conformational rearrangements, whereas SRCD and SAXS indicated that the overall secondary structure and solution flexibility of LF were largely preserved. Molecular docking and MD simulations identified His253 in holo-bLF as the most favorable modeled binding region and supported stable association of the [RuCl₅]²⁻ species through polar and halogen-mediated contacts. DFT calculations on simplified residue models were used only as qualitative descriptors of donor preference and do not define the actual coordination environment in folded LF. TEM data further suggest that Ru-rich domains and RuO₂-like nanoparticles may arise only at the highest loading (LF–500Ru), indicating a secondary high-loading phenomenon rather than a general mechanism of LF–Ru formation. Biologically, antibacterial activity was evaluated for LF and LF–100Ru, whereas desorption was studied for LF–100Ru. Selected LF–Ru formulations produced differential cellular responses *in vitro*: LF–100Ru was relatively well tolerated by L929 fibroblasts but reduced metabolic activity in HepG2 and Caco-2 cells at 5000 mg L⁻¹, while the Caco-2 response was largely

non-cytolytic according to the LDH data. Together, these results show that lactoferrin can act as an active coordination scaffold for Ru(III), but the final biological outcome depends strongly on Ru loading and on whether the system remains a protein-bound complex or proceeds to secondary Ru accumulation at high precursor concentrations.

Author contributions

The manuscript was written through contributions of all authors. T. D.-T. was responsible for the conception and design of the study, conducted the experiments, and analyzed the data; M. E. and K. R. contributed to *in vitro* experiment and corresponding data analysis; P. P. contributed to the study design and provided guidance during the research; G. T. contributed to LM, SEM-EDS, and TEM-EDS analysis and corresponding data analysis; K. S., O. P., and L. S. contributed to SAXS and SRCD measurements and corresponding data analysis; R. v. E. assisted in revising the manuscript; K. M., W. G., and A. K. contributed to molecular docking experiment and corresponding data analysis; R. S. and P. K. J. contributed to MD and DFT study and corresponding data analysis. T. D.-T. and P. P. drafted the manuscript and all authors participated in discussion and revision. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

All data supporting this study are included in the main text and the Supplementary Information (SI). The Supplementary Information file contains additional details on the isotherm and kinetic analyses of Ru(III) binding to lactoferrin, supplementary 3D fluorescence spectra, molecular modelling results from MD and DFT studies, and additional cell-imaging data related to cytotoxicity and cell viability. Supplementary Information is available. See DOI: <https://doi.org/10.1039/d6qi00255b>.

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Supplementary information

Lactoferrin as an Active Coordination Scaffold for Ruthenium(III)

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• Isotherm study

Description of the isotherm models and thermodynamic parameters used to analyze Ru(III) binding to LF

The nature of LF-Ru binding was analyzed by application of two most common isotherm models, such as Langmuir and Freundlich models. The Langmuir model describes the monolayer sorption on the homogeneous sites which are characterized by the equal binding affinity. The model might be expressed as following relationship (1):

$$q_{eq} = (q_{max}K_L C_e)/(1 + K_L C_e) \quad (1)$$

where q_{eq} is the amount of bounded Ru(III) per LF [mg/g], q_{max} is the maximum amount of bounded Ru(III) per LF [mg/g], C_e is the equilibrium concentration of Ru(III) [mg/L], K_L is the Langmuir constant [L/mg].

In order to estimate the sorption favorability the dimensionless equilibrium parameter (R_L) might be calculated as follows (2):

$$R_L = 1/(1+K_L C_0) \quad (2)$$

where C_0 is the initial Ru(III) concentration [mg/L]. Based on the R_L value, the sorption process might be defined as: 1) irreversible ($R_L = 0$); 2) favorable ($0 < R_L < 1$); 3) linear ($R_L = 1$), and 4) unfavorable ($R_L > 1$)¹.

In turn, Freundlich model is relevant mathematical model characterizing the multilayer sorption onto heterogeneous surface which might be presented by the following relationship (3):

$$q_{eq} = K_F C_e^{1/n} \quad (3)$$

where K_F is the Freundlich constant [mg/g] and n is the intensity of sorption.

The sorption efficiency of metal binding onto LF was calculated as follows (4):

$$\%E = 100 \times (C_0 - C_{eq})/C_0 \quad (4)$$

The spontaneity of LF-Ru complex formation was assessed by calculating of the change of Gibbs free energy (ΔG) expressed as (5):

$$\Delta G = -RT \ln K_D \quad (5)$$

where R is the gas constant which is equal to 8.314 J/(K·mol), T is the temperature used for LF-Ru synthesis (310 K), K_D is the distribution coefficient [L/g] which is calculated as the following ratio (6):

$$K_D = q_{eq}/C_{eq} \quad (6)$$

• Kinetic study

Description of the kinetic models applied to Ru(III) binding to LF

In order to describe the kinetics of LF-Ru formation, zero-order kinetic model was applied. The following model represent a system where the rate of reaction is not affected by the concentration of reagents (7):

$$C_t = C_0 - k_0 t \quad (7)$$

where C_t is the concentration of Ru(III) at time, C_0 is the initial concentration of Ru(III) at $t = 0$ min [mg/L], k_0 is the zero-order kinetic constant [mg/(L·min)], and t is the time of reaction [min].

Despite it, the application of Weber-Morris model (8) provided the information if the rate of LF-Ru complex formation is limited by intra-particle diffusion step²:

$$q_t = k_{id} t^{0.5} + I \quad (8)$$

where q_t is the sorption capacity [mg/g] of Ru(III) onto LF at time, k_{id} is the intra-particle diffusion rate constant [mg/(g·√min)], I is a constant indicating the thickness of the boundary layer [mg/g].

- **3-D fluorescence spectroscopy analysis**

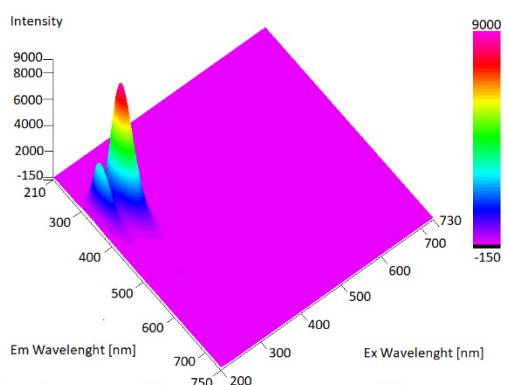


Figure S1. 3D-spectra of bLF dissolved in acetic buffer (pH 4) at the concentration of 100 mg/L.

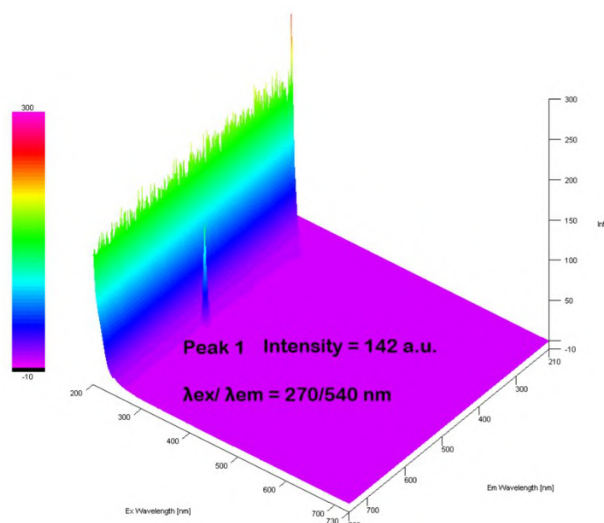


Figure S2. 3D-spectra of empty quartz cuvette.

- **Molecular docking in LF-Ru complex**

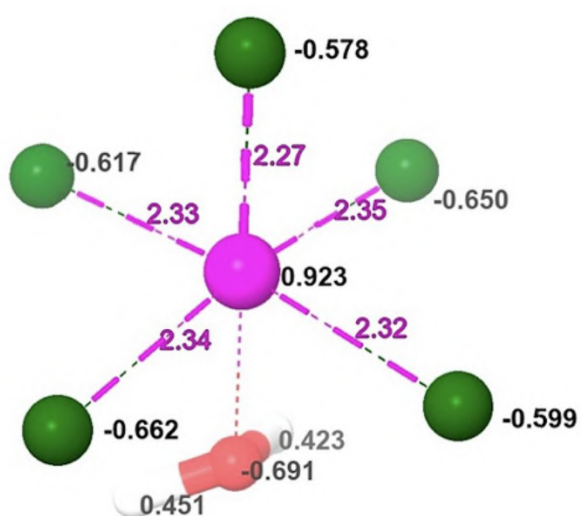


Figure S3. DFT-optimized structure of the $[\text{RuCl}_5(\text{H}_2\text{O})]^{2-}$ complex showing Ru-ligand bond lengths (Å, magenta) and electrostatic potential-derived atomic charges (black).

- **Molecular Dynamics**

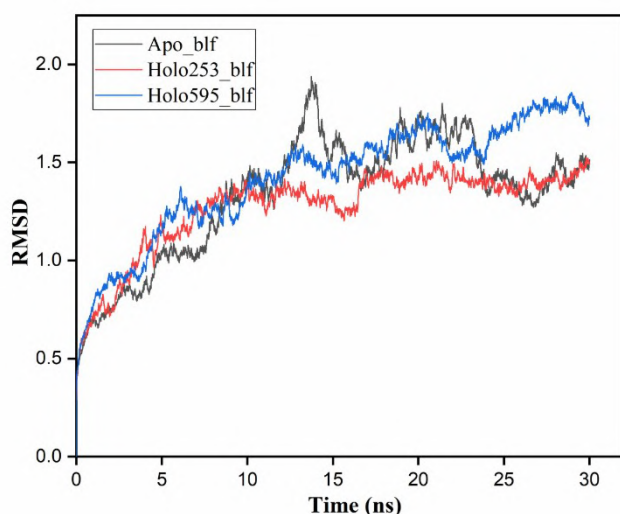


Figure S4. The RMSD of **bLF** systems with and without metal complex.

DFT study

To complement the molecular dynamics (MD) simulations, density functional theory (DFT) calculations were carried out to explore the electronic interactions between the $[\text{RuCl}_5]^{2-}$ complex and representative amino-acid functional groups. Whereas MD describes how the complex approaches and remains associated with the protein over time, the DFT calculations were intended only to provide a simplified picture of how the Ru(III) center may interact chemically with typical donor atoms present on the protein surface. Geometry optimizations were first performed for the isolated metal complex and for individual amino acids. These optimized structures were then used to construct model systems in which the $[\text{RuCl}_5]^{2-}$ species interacts with selected residues suggested by the MD simulations (threonine, cysteine, lysine, and arginine). For each amino acid-complex pair, the candidate configurations were fully relaxed and the lowest-energy structure was taken as the preferred interaction geometry. The resulting optimized structures are shown in Figure S5.

The adsorption energy (E_{ad}) which quantifies the strength of the interaction, was calculated using the following relation ³ (9):

$$E_{\text{ad}} = E_{\text{complex}} - (E_{\text{metal-complex}} + E_{\text{amino-acid}}) \quad (9)$$

where $E_{\text{metal-complex}}$ and $E_{\text{amino-acid}}$ are the total energies of the isolated metal complex and amino acids, whereas E_{complex} is total energy of metal-complex adsorbed amino acids, respectively.

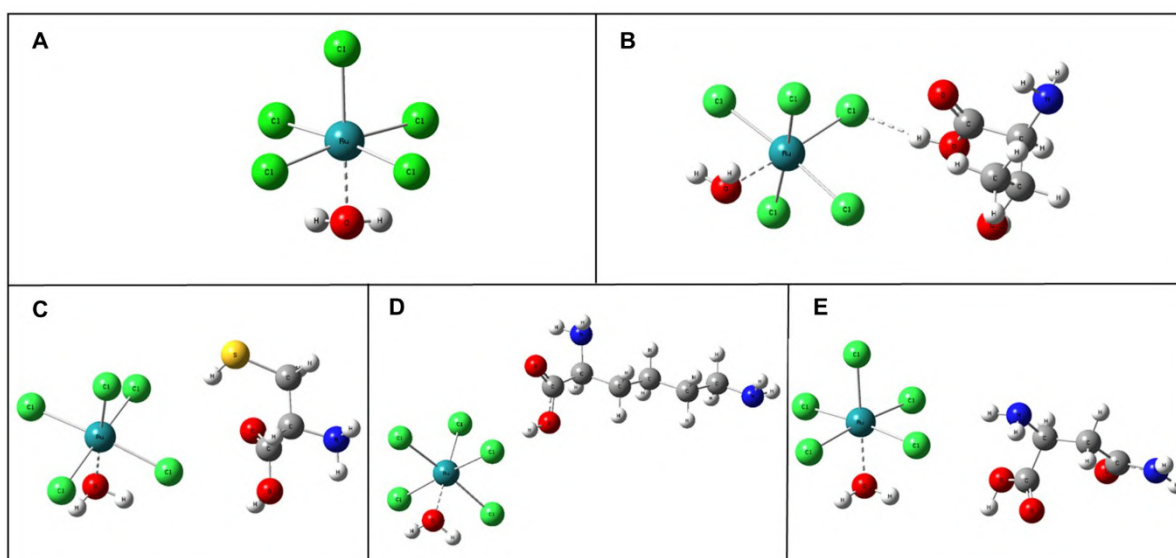


Figure S5. The geometric optimized structure of (A) metal complex, interaction of metal complex with (B) threonine, (C) cysteine, (D) lysine, and (E) arginine.

A more negative adsorption energy implies a stronger and more exergonic interaction, reflecting a greater intrinsic affinity between the metal complex and the isolated amino-acid model ⁴. For threonine, the nearest contact occurs between a chlorine atom of the complex and a hydrogen atom from the hydroxyl group at a distance of 2.18 Å, with an adsorption energy of -4.95 eV. Similarly, cysteine, lysine, and arginine display minimum approach distances of 2.63 Å, 3.07 Å, and 2.65 Å with respective adsorption energies of -4.39 eV, -5.03 eV, and -5.33 eV. Among these simplified models, lysine and arginine show the most negative adsorption energies, consistent with comparatively strong electrostatic attraction between their donor groups and the negatively charged metal complex. These values should be interpreted as qualitative donor-affinity trends rather than as direct descriptors of the folded-protein coordination environment.

The interaction trends obtained from the DFT calculations are broadly consistent with the classical MD simulation results, supporting the general donor preferences observed in the trajectory analysis. Minor quantitative differences in adsorption energies and distances can be attributed to methodological differences between the two computational approaches. It should be emphasized that the amino acids were treated as isolated molecules and therefore do not represent the full environment of the folded protein, where steric constraints, neighboring residues, long-range electrostatics, solvent competition, and cooperative effects can substantially influence binding. Hence, the DFT results are not used to define the actual binding site in lactoferrin. Instead, they are interpreted only as qualitative indicators of which donor groups are chemically capable of stabilizing the complex. The specific residue contacts discussed in the main text are derived primarily from the MD trajectory analysis.

In molecular systems, the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) are often used as frontier-orbital descriptors of possible charge redistribution, chemical

reactivity, and redox behavior ^{5,6}. Figure S6 presents the HOMO, LUMO, and Mulliken charge distributions for the isolated metal complex and for the simplified amino-acid adducts.

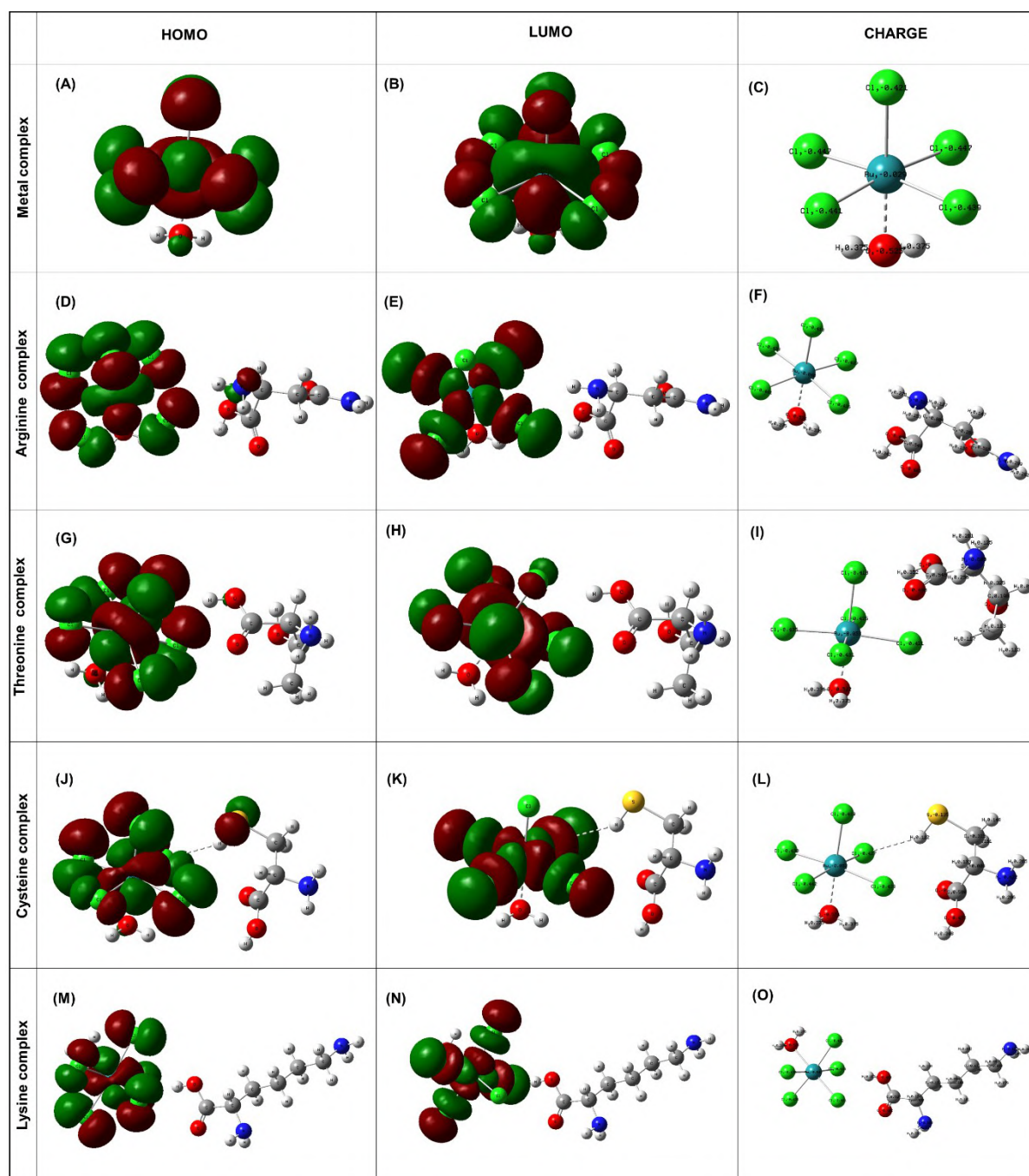


Figure S6. Schematic representation of HOMO, LUMO, and charge redistribution upon interaction of the metal complex with specific amino acid residues of **bLF**.

In these model systems, the orbital distributions change after complex formation, indicating that interaction with N-, O-, and S-donor groups can alter the electronic structure of the Ru species. The largest orbital rearrangements are observed for arginine and cysteine, whereas lysine and threonine show more moderate changes. Mulliken charge analysis likewise indicates residue-dependent redistribution of electron density after adduct formation. These observations are consistent with partial electronic communication between the metal complex and the isolated donor groups. However, because these

calculations were carried out on simplified residue models in the absence of the full protein matrix, solvent competition, and neighboring residues, they should not be interpreted as direct evidence for the extent or direction of charge transfer in folded lactoferrin. Instead, they provide only a qualitative indication that coordination to different donor types can perturb the electronic structure of the Ru complex to different extents.

- **Cytotoxicity of LF-Ru complexes and their impact on cell viability**

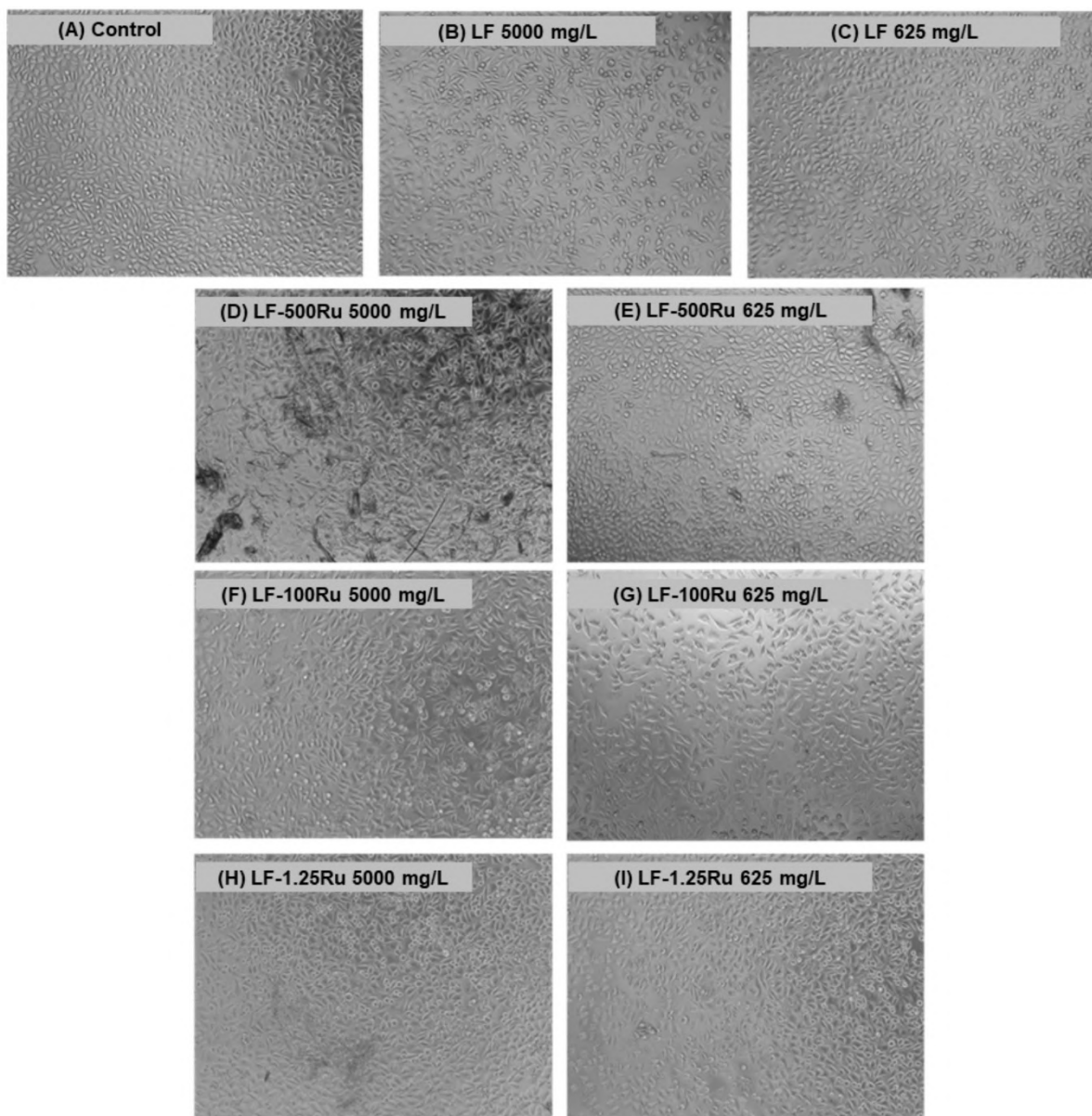


Figure S7. Images of L929 fibroblasts growing on the (A) control, (B-C) LF, (D-E) LF-500Ru, (F-G) LF-100Ru and (H-I) LF-1.25Ru for 2 days. Detailed information regarding the type and concentration of the samples is provided in the figure.

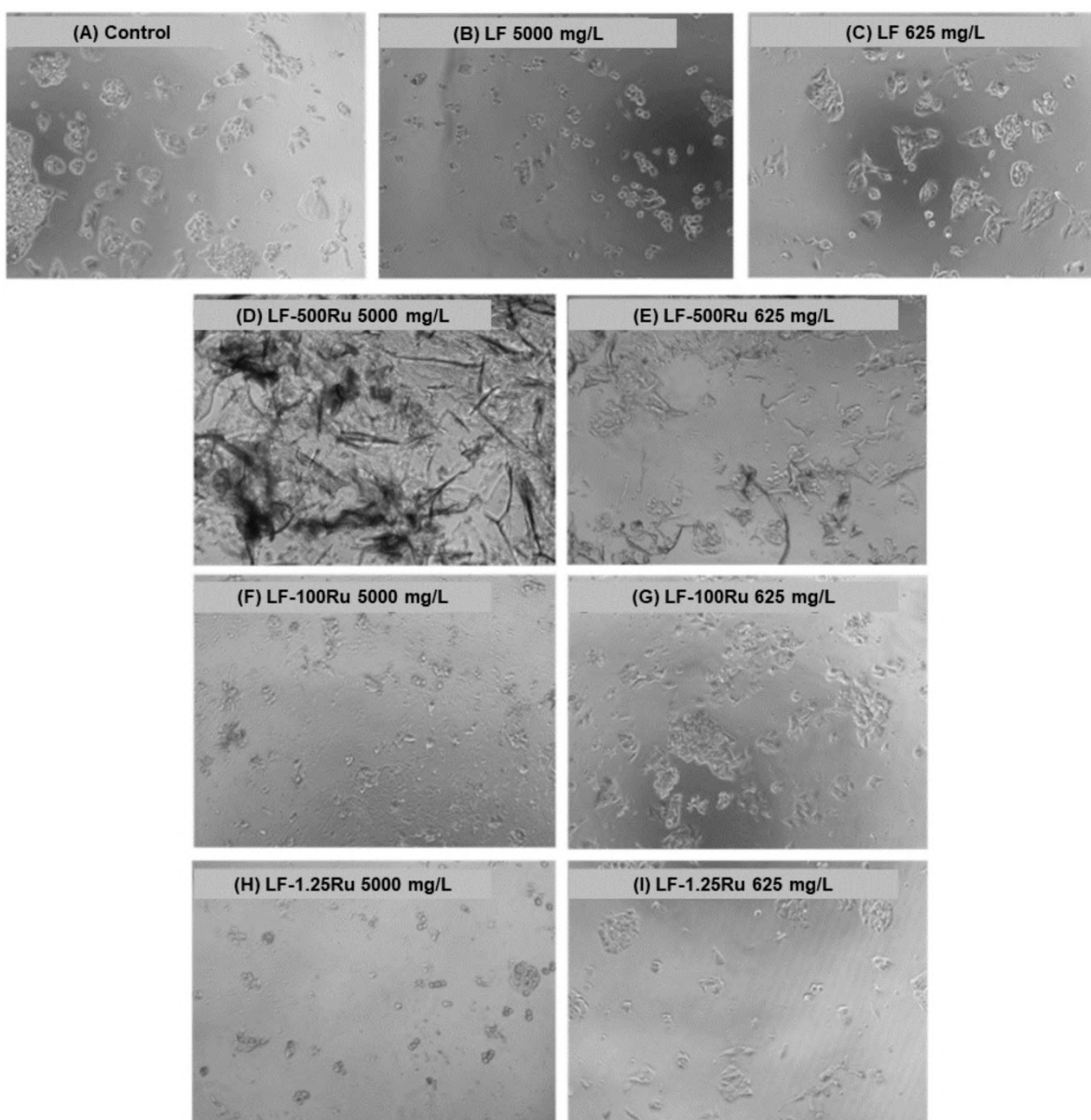


Figure S8. Images of HepG2 growing on the (A) control, (B-C) LF, (D-E) LF-500Ru, (F-G) LF-100Ru and (H-I) LF-1.25Ru for 2 days. Detailed information regarding the type and concentration of the samples is provided in the figure.

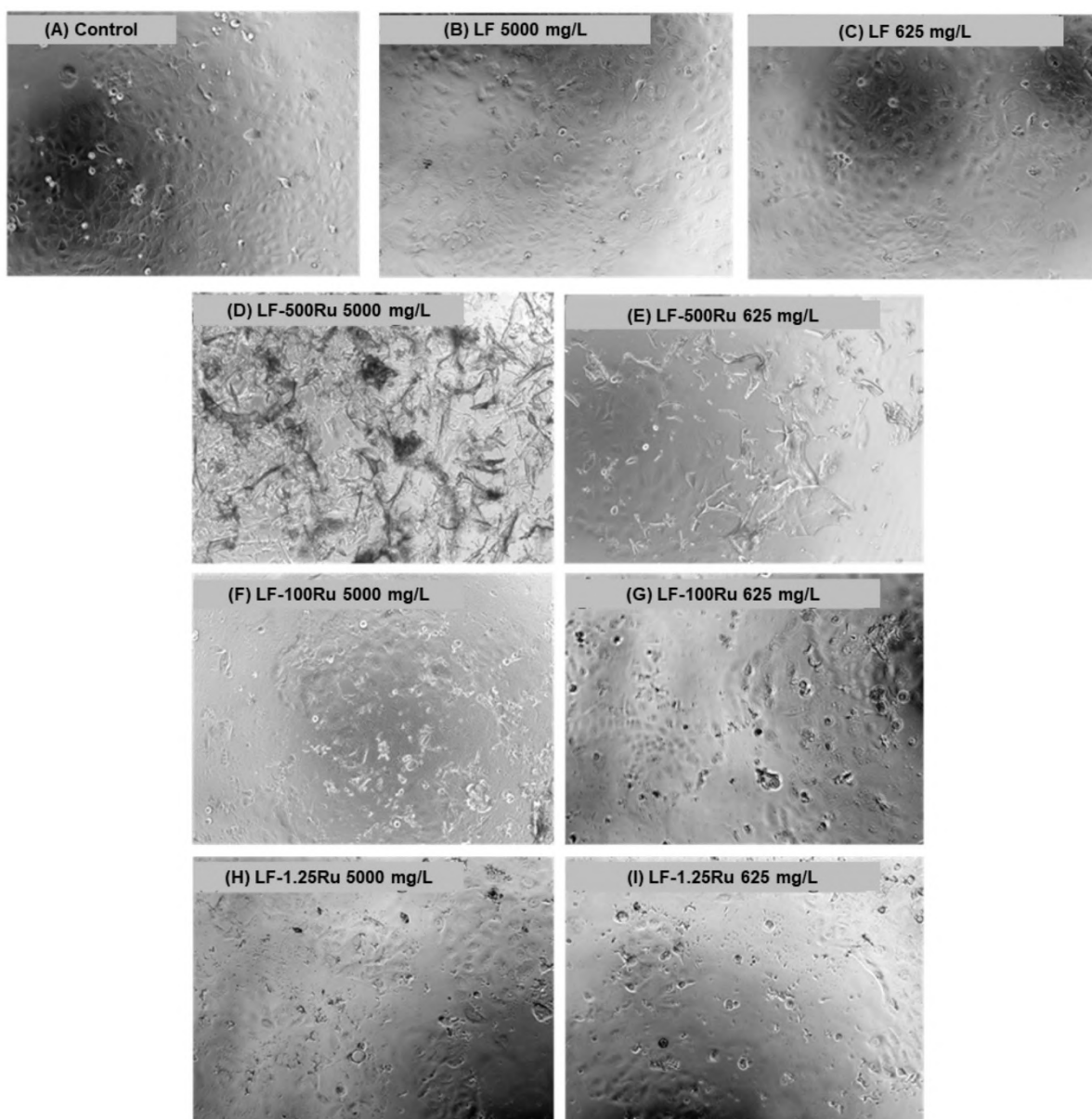


Figure S9. Images of Caco-2 cells growing on the (A) control, (B-C) LF, (D-E) LF-500Ru, (F-G) LF-100Ru and (H-I) LF-1.25Ru for 2 days. Detailed information regarding the type and concentration of the samples is provided in the figure.

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Podsumowanie i wnioski

Prace wchodzące w skład rozprawy doktorskiej obejmują zagadnienia związane z kompleksową analizą biokoloidów pochodzenia mleczarskiego oraz oceną możliwości ich funkcjonalnej modyfikacji. W pracy [P1] przeprowadzono modyfikację kazeiny jonami srebra oraz scharakteryzowano otrzymane układy kazeina-srebro pod względem ich właściwości fizykochemicznych i aktywności biologicznej. W pracy [P2] usystematyzowano aktualny stan wiedzy dotyczący laktoferyny bydlęcej, ze szczególnym uwzględnieniem jej struktury, metod izolacji, technik analitycznych oraz właściwości biologicznych. Praca [P3] dotyczyła wpływu ultrafiltracji na właściwości fizykochemiczne laktoferyny bydlęcej oraz znaczenia tego procesu w przygotowaniu próbek do analiz instrumentalnych. W pracy [P4] oceniono wpływ *Lactiplantibacillus plantarum* na skład śmietanki, w tym na zawartość witaminy D3 oraz profil kwasów tłuszczowych. Z kolei praca [P5] poświęcona została badaniu molekularnych mechanizmów wiązania rutenu(III) przez laktoferynę bydlęcą oraz charakterystyce zmian strukturalnych i efektów biologicznych towarzyszących tworzeniu układów LF-Ru.

Rozprawę można interpretować jako odpowiedź na dwa równoległe procesy zachodzące we współczesnym mleczarstwie. Pierwszy z nich ma charakter rynkowy i polega na przechodzeniu od modelu opartego na produkcji masowej do modelu wysokiej waloryzacji surowca, w którym o konkurencyjności decyduje zdolność do wydzielania, stabilizacji oraz funkcjonalizacji wyspecjalizowanych frakcji mleka. Drugi ma charakter naukowy i dotyczy zmiany sposobu myślenia o biokoloidach mlecznych: zamiast traktować je wyłącznie jako składniki żywności, postrzega się je jako programowalne układy molekularne zdolne do transportu, koordynacji metali oraz kształtowania aktywności biologicznej. W tym sensie przedstawione wyniki wpisują się w aktualne tendencje rozwoju sektora, w którym granice między technologią żywności, nutraceutyką i biomateriałami stają się coraz mniej wyraźne [1–5,9–17,28–30].

Biorąc pod uwagę wyniki przedstawione w publikacjach wchodzących w skład rozprawy doktorskiej można zrobić następujące wnioski:

- W pracy [P1] wykazano, że kompleks amoniakalny srebra ($[\text{Ag}(\text{NH}_3)_2]^+$) stanowi stabilny prekursor do modyfikacji kazeiny w warunkach alkalicznych, który również zapewnił stabilność koloidalną białka. Sorpcja srebra na kazeinie ma charakter niejednorodny oraz wieloetapowy, na co wskazuje lepsze dopasowanie danych eksperymentalnych do modelu Freundlicha. Potwierdzono również, że kazeina jest skuteczną matrycą do immobilizacji jonów $\text{Ag}(\text{I})$, a jej odsalanie prowadzi do istotnego zwiększenia maksymalnej pojemności

sorpcyjnej ($q_{\max}$) z 157,1 do 258,2 mg/g, podkreślając istotne znaczenie soli micelarnych w mechanizmie wiązania metalu.

- Związanie metalu prowadzi do zmian w organizacji miceli kazeinowych, obejmujących wzrost ich rozmiaru hydrodynamicznego oraz modyfikację konformacji białka. Analiza ATR-FTIR wykazała, że wiązanie Ag(I) przez kazeinę może zachodzić zarówno z udziałem powierzchniowych grup funkcyjnych reszt kwasu asparaginowego (Asp) oraz kwasu glutaminowego (Glu), jak i micelarnych reszt fosfoserynowych.
- Stwierdzono, że w trakcie modyfikacji kazeiny srebrem dochodzi do powstawania nanocząstek srebra (AgNPs), co potwierdzono w analizach mikroskopowych STEM. Uzyskane wyniki wskazują, że kazeina może stanowić matrycę sprzyjającą formowaniu nanostruktur srebra. Więc, można wywnioskować że wiązanie Ag(I) z kazeiną przebiega dwuetapowo i obejmuje początkowe wiązanie jonów metalu przez grupy funkcyjne białka, a następnie ich częściową bioredukcję.
- Zaobserwowano, że kompleksy srebro-kazeina (Ag-CN) wykazują aktywność przeciwbakteryjną wobec bakterii Gram-dodatnich (*Staphylococcus aureus*) oraz Gram-ujemnych (*Klebsiella pneumoniae*), przy jednocześnie niższej cytotoksyczności wobec komórek fibroblastów mysich L929 niż wolne jony srebra. Otrzymane wyniki wskazują, że białkowe układy nośnikowe typu Ag-CN mogą ograniczać niepożądane działanie wolnego metalu co może stanowić podstawę do projektowania efektywnych preparatów antyseptycznych o kontrolowanych właściwościach.
- W pracy [P3] wykazano, że ultrafiltracja (UF) jest użytecznym oraz nieniszczącym etapem przygotowania próbek laktoferyny bydlęcej do dalszych analiz. Obecność niskocząsteczkowych zanieczyszczeń w preparatach LF, takich jak sole i peptydy, może być związana zarówno z wykorzystaniem wysoko stężonych roztworów buforowych w procesie izolacji białka, jak i z jego długotrwałym przechowywaniem w niewłaściwych warunkach. Jak wykazano, proces UF nie powodował istotnych zmian masy cząsteczkowej białka, lecz wpływał na morfologię jego powierzchni co również może być wynikiem zamrażania oraz następnie przeprowadzonej liofilizacji próbki. Stwierdzono, że przed ultrafiltracją obecność soli oraz innych niskocząsteczkowych zanieczyszczeń prowadziła do supresji sygnału laktoferyny, obniżając jakość analiz spektrometrycznych oraz utrudniając prawidłową identyfikację białka. Zastosowanie ultrafiltracji, poprzez usunięcie soli i innych niskocząsteczkowych zanieczyszczeń, doprowadziło do wzrostu intensywności sygnału pochodzącego od LF oraz poprawy jakości jej identyfikacji w

analizie MALDI-TOF/MS, co wyrażono wzrostem pokrycia sekwencji z 52,7% do 61,6% oraz pokrycia intensywności z 84,5% do 89,2%. Wynik ten ma istotne znaczenie metodologiczne, ponieważ zwiększa reprezentatywność próbki, poprawia powtarzalność oznaczeń oraz podnosi wiarygodność uzyskiwanych danych. Ma to istotne znaczenie dla dalszych badań nad aktywnością biologiczną LF, ponieważ umożliwia bardziej precyzyjną ocenę zależności między jej strukturą a funkcją biologiczną, w tym również mechanizmów warunkujących swoiste interakcje z receptorami komórkowymi.

- Po raz pierwszy oceniono wpływ dodanego szczepu *Lactiplantibacillus plantarum* B/00401 na skuteczność wzbogacania śmietanki w witaminę D3, przy czym efektywność procesu była uzależniona od warunków inkubacji, zwłaszcza ekspozycji na światło, mieszania, czasu inkubacji, stosunku śmietanki do bakterii oraz formy inokulum. Najkorzystniejsze wyniki uzyskano dla liofilizatu *L. plantarum* B/00401, co potwierdza znaczenie formy technologicznej szczepu dla jego aktywności metabolicznej. Przewagą tego rozwiązania nad tradycyjną fortyfikacją jest naturalna biosynteza witaminy D3, której towarzyszy jednocześnie podwyższenie wartości odżywczej produktu. Stwierdzono, że śmietanka stanowi obiecującą matrycę do wzbogacania w witaminę D3, a zastosowanie szczepu *Lactiplantibacillus plantarum* B/00401 jest obiecującym sposobem biofortyfikacji produktów mleczarskich.
- Wykazano ponadto, że suplementacja śmietanki szczepem *L. plantarum* B/00401 prowadzi do modyfikacji profilu kwasów tłuszczowych, obejmującej spadek udziału nasyconych kwasów tłuszczowych (SFA) oraz wzrost udziału jednonienasyconych kwasów tłuszczowych (MUFA). Uzyskane zmiany mogą zwiększać wartość prozdrowotną produktu, choć równocześnie wymagają uwzględnienia potencjalnego wpływu na trwałość oraz zmianę okresu przydatności produktu końcowego.
- Na podstawie badań izotermicznych przedstawionych w pracy [P5] wykazano, że tworzenie układów LF-Ru przebiega spontanicznie oraz z wysokim powinowactwem laktoferyny do Ru(III). Lepsze dopasowanie danych eksperymentalnych do modelu Langmuira wskazuje, że dominującym mechanizmem wiązania metalu jest nasyceniowe obsadzanie ograniczonej liczby dostępnych miejsc wiązania w strukturze białka.
- Z wyników badań spektroskopowych, t.j. spektrofлуorymetria, DLS, ATR-FTIR, SRCD wynika, że oddziaływanie Ru(III) z LF prowadzi do zmian w jej organizacji strukturalnej, które przejawiały się wygaszaniem fluorescencji, niewielkim przesunięciem maksimów emisji, zmianą średnicy hydrodynamicznej, wzrostem potencjału zeta, a także lokalnymi

modyfikacjami w obrębie struktury drugorzędowej. Jednocześnie, wyniki SAXS potwierdziły, że kompleksowanie Ru(III) przez LF prowadzi do zmian rozmiaru oraz organizacji przestrzennej układu w roztworze, jednak bez istotnej destabilizacji globalnej struktury białka co z kolei też było potwierdzone metodami obliczeniowymi.

- Z badań desorpcji wynika, że otrzymane układy LF-Ru wykazują ograniczone uwalnianie rutenu w symulowanych płynach żołądkowym oraz jelitowym, niezawierających enzymów trawiennych, co potwierdza wysokie powinowactwo metalu do białka w utworzonych kompleksach. Wskazuje to na potencjalną przydatność LF jako nośnika jonów rutenu. Z drugiej jednak strony analiza SDS-PAGE po trawieniu pepsyną wykazała całkowitą degradację kompleksów w czasie 30 minut, co podkreśla potrzebę ich dodatkowej enkapsulacji w celu ochrony integralności układu w warunkach fizjologicznych oraz umożliwienia jego efektywnego dostarczenia do miejsca docelowego.
- Stwierdzono, że kompleksowanie Ru(III) przez LF modyfikuje jej odpowiedź biologiczną w sposób zależny od badanego modelu komórkowego. LF-Ru wykazywał niższą tolerancję w komórkach nowotworowych HepG2 i Caco-2 niż w prawidłowych fibroblastach L929, co sugeruje zróżnicowaną odpowiedź komórkową oraz wskazuje na potencjalną przydatność układów LF-Ru w dalszych badaniach biomedycznych.

Na tej podstawie można stwierdzić, że moje badania tworzą nową perspektywę wykorzystania surowców mlecznych. Kazeina została ukazana jako wielofunkcyjna matryca koordynacyjna oraz biomateriałowa, zdolna do tworzenia układów antyseptycznych o potencjale wdrożeniowym. Laktoferyna została przedstawiona jako składnik premium, którego znaczenie nie ogranicza się do funkcji odżywczej, lecz obejmuje rolę aktywnego rusztowania dla kompleksów metali i zaawansowanych systemów celowanego transportu. Śmietanka natomiast została potraktowana jako reaktywna matryca biofortyfikacji, w której procesy metaboliczne i koloidalne mogą być sterowane w celu uzyskania produktu o podwyższonej wartości zdrowotnej. Łącznie wyniki te wzmacniają tezę, że przyszłość mleczarstwa będzie opierała się nie tylko na przetwarzaniu mleka, lecz na świadomym projektowaniu funkcjonalności jego frakcji [12–17,28–30].

Streszczenie

W niniejszej pracy opracowano metodologię kompleksowej analizy prostych układów koloidalnych pochodzenia mleczarskiego, obejmujących białka mleka, tj. kazeinę i laktoferynę, oraz bardziej złożonych układów, takich jak śmietanka. Badane układy były poddane ukierunkowanej modyfikacji w celu kształtowania ich właściwości funkcjonalnych oraz poszerzenia możliwości aplikacyjnych. Podjęty kierunek badań jest istotny, ponieważ pozwala on rozwiązywać problemy związane z ograniczoną funkcjonalnością naturalnych składników mleka, projektowaniem żywności wzbogacanej, poprawą jakości analiz dotyczących białek oraz opracowywaniem nowych układów nośnikowych i bioaktywnych o potencjale aplikacyjnym dla przemysłu mleczarskiego oraz obszaru biomedycznego.

W ramach pracy opracowano metodę naturalnego wzbogacania śmietanki w witaminę D3 z wykorzystaniem *Lactiplantibacillus plantarum*. Wykazano, że skuteczność biosyntezy witaminy D3 zależy od warunków prowadzenia procesu, a najkorzystniejsze wyniki uzyskano przy zastosowaniu liofilizowanej formy bakterii, w warunkach bez mieszania i przy ekspozycji na światło. Stwierdzono również, że zastosowanie bakterii wpływa na profil kwasów tłuszczowych śmietanki, prowadząc do spadku udziału nasyconych kwasów tłuszczowych (SFA) oraz wzrostu udziału jednonienasyconych kwasów tłuszczowych (MUFA), co może sprzyjać poprawie właściwości prozdrowotnych produktu.

Istotnym elementem badań była także charakterystyka fizykochemiczna laktoferyny bydlęcej (bLF). Wykazano, że stabilność tego układu białkowego zależy od pH oraz siły jonowej, a obecność niskocząsteczkowych zanieczyszczeń, takich jak sole i peptydy, może pogarszać jakość analiz. Zastosowanie ultrafiltracji (UF) umożliwiło skuteczne oczyszczenie próbki bez istotnego wpływu na masę cząsteczkową białka, a jednocześnie poprawiło reprezentatywność próbki oraz wiarygodność wyników identyfikacji uzyskiwanych metodą MALDI-TOF/MS. Wyniki te potwierdziły znaczenie odpowiedniego przygotowania próbek w dalszych badaniach nad właściwościami fizykochemicznymi oraz biologicznymi LF.

Modyfikacja białek mleka jonami metali stanowi obiecującą platformę do projektowania układów bioaktywnych o ulepszonej lub rozszerzonej funkcjonalności. W niniejszej pracy przeprowadzono modyfikację kazeiny (CN) jonami srebra Ag(I), a także funkcjonalizację LF jonami rutenu Ru(III). Na podstawie badań izotermicznych wykazano, że sorpcja Ag(I) przez kazeinę ma charakter heterogeniczny oraz wieloetapowy, a przeprowadzone odsalanie białka zwiększa jego zdolność do wiązania srebra. Wykazano również, że związanie Ag(I) prowadzi do zmian w organizacji miceli kazeinowych oraz konformacji białka. Wyniki badań

spektroskopowych oraz mikroskopowych wskazują, że proces ten obejmuje początkowe wiązanie Ag(I) przez grupy funkcyjne kazeiny, a następnie częściową bioredukcję prowadzącą do powstawania nanocząstek srebra oraz tworzenia nanokompleksów Ag-CN. Otrzymane układy Ag-CN wykazywały aktywność przeciwbakteryjną przy jednocześnie niższej cytotoksyczności niż wolne jony srebra, co wskazuje na ich potencjał w projektowaniu biokompatybilnych preparatów antyseptycznych.

Z kolei wyniki badań izotermicznych oraz kinetycznych wskazują że tworzenie się kompleksów LF-Ru przebiega samorzutnie i w krótkim czasie, a mechanizm wiązania Ru(III) może być określony jako nasyceniowy, polegający na stopniowym zajmowaniu ograniczonej liczby dostępnych miejsc wiązania. Wyniki badań spektroskopowych, SAXS oraz analiz obliczeniowych potwierdziły, że obecność Ru(III) wywołuje zmiany w organizacji strukturalnej oraz właściwościach fizykochemicznych LF, nie prowadząc jednak do istotnego zaburzenia jej globalnej stabilności. Otrzymane kompleksy LF-Ru charakteryzowały się ograniczonym uwalnianiem rutenu, natomiast ich szybka degradacja pod wpływem pepsyny wskazuje na potrzebę zastosowania dodatkowej enkapsulacji w celu zachowania integralności układu w warunkach fizjologicznych. Jednocześnie wykazano, że związanie Ru(III) wpływa na odpowiedź biologiczną LF, zmniejszając żywotność komórek nowotworowych przy jednocześnie większej tolerancji ze strony fibroblastów, co potwierdza potencjał biomedyczny tych układów.

W ujęciu aplikacyjnym praca ta pokazuje, że mleko oraz jego frakcje mogą być traktowane jako surowce platformowe dla nowoczesnego mleczarstwa wysokiej wartości dodanej. Zaproponowane podejście łączy analizę rynku z pogłębionym chemizmem oddziaływań i wskazuje, że kazeina, laktoferyna oraz śmietanka mogą pełnić role wykraczające poza ich klasyczne funkcje żywieniowe: odpowiednio nośnika antyseptycznego, aktywnego rusztowania koordynacyjnego oraz matrycy naturalnej biofortyfikacji. Tym samym rozprawa wzmacnia koncepcję przetwórstwa mleczarskiego opartego na selektywnym odzysku frakcji, precyzyjnym projektowaniu ich właściwości i kierowaniu ich do zastosowań funkcjonalnych, nutraceutycznych oraz biomateriałowych.

Abstract

In this work, a methodology was developed for the comprehensive analysis of simple dairy-derived colloidal systems, including milk proteins such as casein and lactoferrin, as well as more complex systems such as cream. The investigated systems were subjected to targeted modification in order to tailor their functional properties and broaden their application potential. This research direction is important because it helps address problems related to the limited functionality of natural milk components, the design of fortified foods, the improvement of protein-related analytical quality, and the development of new carrier and bioactive systems with application potential in the dairy industry and the biomedical field.

As part of this work, a method for the natural enrichment of cream with vitamin D3 using *Lactiplantibacillus plantarum* was developed. It was demonstrated that the efficiency of vitamin D3 biosynthesis depends on the process conditions, and the most favorable results were obtained when the lyophilized form of the bacteria was used under non-mixing conditions and light exposure. It was also found that the application of the bacteria affected the fatty acid profile of the cream, leading to a decrease in the proportion of saturated fatty acids (SFA) and an increase in the proportion of monounsaturated fatty acids (MUFA), which may contribute to improving the health-promoting properties of the product.

An important part of the study also involved the physicochemical characterization of bovine lactoferrin (bLF). It was shown that the stability of this protein system depends on pH and ionic strength, and that the presence of low-molecular-weight impurities, such as salts and peptides, may deteriorate the quality of the analyses. The application of ultrafiltration (UF) enabled effective purification of the sample without significantly affecting the molecular mass of the protein, while at the same time improving sample representativeness and the reliability of identification results obtained by MALDI-TOF/MS. These findings confirmed the importance of proper sample preparation for further studies on the physicochemical and biological properties of LF.

The modification of milk proteins with metal ions constitutes a promising platform for designing bioactive systems with improved or expanded functionality. In this work, casein (CN) was modified with silver ions Ag(I), and LF was functionalized with ruthenium ions Ru(III). Based on isothermal studies, it was shown that the sorption of Ag(I) by casein is heterogeneous and multi-step in nature, and that protein desalting increases its ability to bind silver. It was also demonstrated that Ag(I) binding leads to changes in the organization of casein micelles and in protein conformation. Spectroscopic and microscopic results indicated that this process

involves the initial binding of Ag(I) by functional groups of casein, followed by partial bioreduction leading to the formation of silver nanoparticles and Ag-CN nanocomplexes. The resulting Ag-CN systems exhibited antibacterial activity together with lower cytotoxicity than free silver ions, indicating their potential for the design of biocompatible antiseptic formulations.

In turn, the results of isothermal and kinetic studies indicated that the formation of LF-Ru complexes proceeds spontaneously and within a short time, and that the Ru(III) binding mechanism may be described as saturation-like, involving the gradual occupation of a limited number of accessible binding sites. The results of spectroscopic studies, SAXS, and computational analyses confirmed that the presence of Ru(III) induces changes in the structural organization and physicochemical properties of LF, without causing significant disruption of its global stability. The obtained LF-Ru complexes were characterized by limited ruthenium release, whereas their rapid degradation under the action of pepsin indicated the need for additional encapsulation in order to preserve system integrity under physiological conditions. At the same time, it was demonstrated that Ru(III) binding affects the biological response of LF, decreasing the viability of cancer cells while being better tolerated by fibroblasts, which confirms the biomedical potential of these systems.

From an application-oriented perspective, this work demonstrates that milk and its fractions can be regarded as platform raw materials for modern high-value dairy processing. The proposed approach combines market-oriented analysis with an in-depth understanding of interaction chemistry and shows that casein, lactoferrin, and cream can perform roles that go beyond their classical nutritional functions, namely as an antiseptic carrier, an active coordination scaffold, and a matrix for natural biofortification, respectively. Thus, the dissertation reinforces the concept of dairy processing based on the selective recovery of fractions, the precise design of their properties, and their targeted use in functional, nutraceutical, and biomaterial applications.

Dorobek naukowy

• Publikacje naukowe

Publikacje naukowe wchodzące w skład rozprawy doktorskiej

1. **Tetiana Dyrda-Terniuk**, Oleksandra Pryshchepa, Katarzyna Rafińska, Mateusz Kolankowski, Adrian Gołębiowski, Michał Gloc, Renata Dobrucka, Krzysztof Kurzydłowski, Paweł Pomastowski. Immobilization of Silver Ions onto Casein. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **2023**, 667, 131390.
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2. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties. *Journal of Agricultural and Food Chemistry*, **2023**, 71, 20500–20531.
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1. **Tetiana Dyrda-Terniuk**, Mateusz Sugajski, Oleksandra Pryshchepa, Joanna Śliwiak, Magdalena Buszewska-Forajta, Paweł Pomastowski, Bogusław Buszewski. The Study of Protein–Cyclitol Interactions. *International Journal of Molecular Sciences*, **2022**, 23, 2940. IF = 4.9; 140 pkt. MNiSW doi.org/10.3390/ijms23062940

• Zgłoszenie patentowe

Paweł Pomastowski, **Tetiana Dyrda-Terniuk**. *Sposób syntezy kompleksów na bazie laktoferyny bydlęcej i rutenu(III) oraz zastosowanie kompleksów laktoferyny bydlęcej jako środka przeciwdrobnoustrojowego do zwalczania bakterii Gram-ujemnych Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa, bakterii Gram-dodatnich Staphylococcus aureus albo grzybów Candida albicans, Candida krusei*, 2024, **nr zgłoszenia: P.448969**

• Konferencje naukowe

Aktywny udział w konferencjach naukowych

1. **Tetiana Dyrda-Terniuk**, Adrian Gołębiowski, Oleksandra Pryshchepa, Katarzyna, Rafińska, Magdalena Buszewska-Forajta, Paweł Pomastowski, Bogusław Buszewski. 10th Nordic Plasma Conference on Plasma Spectrochemistry (Loen, Norwegia, 12.06.2022-15.06.2022) – „Studying the mechanism of silver ions binding to casein” (poster).
2. **Tetiana Dyrda-Terniuk**, Mateusz Sugajski, Oleksandra Pryshchepa, Joanna Śliwiak, Magdalena Buszewska-Forajta, Paweł Pomastowski, Bogusław Buszewski. XI Polska Konferencja Chemii Analitycznej, PoKoChA 2022 (Łódź, Polska, 19.06.2022-23.06.2022) – „Investigation of D-sorbitol and D-(–)-quinic acid interactions with Bovine Serum Albumin” (poster).
3. **Tetiana Dyrda-Terniuk**, Adrian Gołębiowski, Oleksandra Pryshchepa, Katarzyna, Rafińska, Magdalena Buszewska-Forajta, Paweł Pomastowski, Bogusław Buszewski. 18th International Students Conference “Modern Analytical Chemistry” (Praga, Czechy, 15.09.2022-16.09.2022) – „Analytical methodology for studying the binding mechanism between silver ions and casein” (prezentacja ustna).
4. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. ChemBiotIC Chemistry & Biotechnology International Conference (Wrocław, Polska, 22.06.2023-23.06.2023) – „General characteristic of bovine lactoferrin: molecular mass, isoelectric point, level of glycosylation” (poster on-line).
5. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. 27th International Symposium on Separation Sciences (Kluż-Napoca, Rumunia, 24.09.2023-27.09.2023) – „Studying the influence of ultrafiltration on the physicochemical properties of bovine lactoferrin” (poster).

6. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. 11th Nordic Plasma Conference on Plasma Spectrochemistry (Loen, Norwegia, 09.06.2024-13.06.2024) – „The study of lactoferrin-ruthenium complexes: Insight into the binding mechanism” (poster).

7. **Tetiana Dyrda-Terniuk**, Viorica Railean, Aleksandra Bogumiła Florkiewicz, Justyna Walczak-Skierska, Mateusz Kolankowski, Joanna Rudnicka, Dorota Białczak, Paweł Pomastowski. 28th International Symposium on Separation Sciences (Messina, Włochy, 22.09.2024-25.09.2024) – „The application of freeze-dried *Lactobacillus plantarum* as an innovative approach for cream enrichment in Vitamin D3 and fatty acid profile modification” (prezentacja ustna).

3 miejsce w konkursie “Who Wants to be an INTERNATIONAL SEPARATION SCIENCE STAR ISSS2024”

8. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. 33rd Annual Conference Biomaterials in Medicine and Veterinary Medicine (Rytro, Polska, 10.10.2024-13.10.2024) – „The Comprehensive Study on Lactoferrin-Ruthenium Complexes: Methodology of Preparation, Binding Mechanism, and Biological Properties” (poster).

9. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. V Ogólnopolskie Forum Chemii Nieorganicznej (Warszawa, Polska, 20.03.2025-22.03.2025) – „Synthesis and analysis of the mechanism of lactoferrin-ruthenium complex formation (LF-Ru)” (poster).

10. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. 34th Annual Conference Biomaterials in Medicine and Veterinary Medicine (Rytro, Polska, 09.10.2025-12.10.2025) – „The Comprehensive Study on Lactoferrin-Ruthenium Complexes: Methodology of Preparation, Binding Mechanism, and Biological Properties” (poster).

Współautorstwo

1. **Tetiana Dyrda-Terniuk**, Paweł Pomastowski. 33rd Annual Conference Biomaterials in Medicine and Veterinary Medicine (Rytro, Polska, 10.10.2024-13.10.2024) – „Understanding the effect of ultrafiltration on the physicochemical and structural aspects of bovine lactoferrin”.

2. **Tetiana Dyrda-Terniuk**, Oleksandra Pryshchepa, Kinga Robotnik, Paweł Pomastowski. 11th Nordic Plasma Conference on Plasma Spectrochemistry (Loen, Norwegia, 09.06.2024-13.06.2024) – „Mechanistic understanding and biomedical implications of d-electron metal-protein interactions in dairy proteins”.

3. Oleksandra Pryshchepa, **Tetiana Dyrda-Terniuk**, Kamil Szpotkowski, Łukasz Skowroński, Paweł Pomastowski. XVII International Conferences on Lactoferrin: structure, function and applications (Mazatlán, Meksyk, 09.11.2025-13.11.2025) – „High-yield batch isolation of

lactoferrin using magnetically recoverable core - shell $\text{Fe}_3\text{O}_4/\text{SiO}_2$ sorbents and ruthenium(III) - lactoferrin complexes for theranostics”.

4. Dorota Białczak, **Tetiana Dyrda-Terniuk**, Viorica Railean, Aleksandra B. Florkiewicz, Justyna Walczak-Skierska, Joanna Rudnicka, Paweł Pomastowski. International Scientific Conference on Neurodegenerative Disorders and the Gut-Brain Axis (Toruń, Polska, 08.04.2025-09.04.2025) – „The impact of *Lactiplantibacillus plantarum* on the cream composition: insight into changes of vitamin D3 content and fatty acid composition”.

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• **Udział w szkoleniach**

- Udział w szkoleniach z obsługi aparatury oraz analiz ICP-OES (Pro-Environment Polska).

• **Udział w seminariach**

- Udział w seminarium pt. „Biophysical characterization of proteins” (Warszawa, 05.12.2023-06.12.2023) organizowane przez A.P. Instruments Sp. z o. o.

• **Współorganizacja konferencji**

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- Współorganizacja Międzynarodowej Konferencji Naukowej „Choroby neurodegeneracyjne a oś mózgowo-jelitowa” (Toruń, Polska, 8-9 kwietnia 2025).

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- Wykonawca w projekcie NUTRITECH (FENG.01.01-IP.01-001/24) pt. „Opracowanie ulepszonej technologii otrzymywania soku na bazie kondensatów litu, biologicznie aktywnych ekstraktów owocowych oraz wydłużonym terminie przydatności do spożycia” finansowanego przez Naukowy Centrum Badań i Rozwoju (listopad 2025-czerwiec 2026).

Oświadczenia współautorów



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Niniejszym oświadczam że jako autor przedłożonej rozprawy doktorskiej mój wkład w powstanie poniższych artykułów:

[P1] Tetiana Dyrda-Terniuk, Oleksandra Pryshchepa, Katarzyna Rafińska, Mateusz Kolankowski, Adrian Gołębiowski, Michał Gloc, Renata Dobrucka, Krzysztof Kurzydłowski, Paweł Pomastowski. Immobilization of Silver Ions onto Casein. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **2023**, 667, 131390.

polegał na współudziale w opracowaniu koncepcji badań, realizacji badań eksperymentalnych dotyczących otrzymywania i charakterystyki układów kazeina-srebro, analizie i interpretacji wyników oraz współudziale w przygotowaniu i redakcji manuskryptu.

[P2] Tetiana Dyrda-Terniuk, Paweł Pomastowski. The Multifaceted Roles of Bovine Lactoferrin: Molecular Structure, Isolation Methods, Analytical Characteristics, and Biological Properties. *Journal of Agricultural and Food Chemistry*, **2023**, 71, 20500–20531.

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[P3] Tetiana Dyrda-Terniuk, Paweł Pomastowski. Impact of Ultrafiltration on the Physicochemical Properties of Bovine Lactoferrin: Insights into Molecular Mass, Surface Morphology, and Elemental Composition. *Journal of Dairy Science*, **2024**, 107, 10280–10298.

polegał na współudziale w opracowaniu koncepcji badań, realizacji badań eksperymentalnych dotyczących wpływu ultrafiltracji na właściwości fizykochemiczne laktoferyny, analizie i interpretacji wyników oraz współudziale w przygotowaniu i redakcji manuskryptu.

[P4] Tetiana Dyrda-Terniuk, Viorica Railean, Aleksandra Florkiewicz, Justyna Walczak-Skierska, Mateusz Kolankowski, Joanna Rudnicka, Dorota Białczak, Paweł Pomastowski. The Impact of *Lactiplantibacillus Plantarum* on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition. *International Dairy Journal*, **2025**, 161, 106118.

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polegał na współudziale w opracowaniu koncepcji badań, realizacji badań eksperymentalnych dotyczących tworzenia i charakterystyki kompleksów laktoferyna-ruten, analizie i interpretacji wyników oraz współudziale w przygotowaniu i redakcji manuskryptu.

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[P4] Tetiana Dyrda-Terniuk, Viorica Railean, Aleksandra Florkiewicz, Justyna Walczak-Skierska, Mateusz Kolankowski, Joanna Rudnicka, Dorota Białczak, **Paweł Pomastowski**. The Impact of Lactiplantibacillus Plantarum on the Cream Composition: Insight into Changes of Vitamin D3 Content and Fatty Acid Composition. *International Dairy Journal*, 2025, 161, 106118.

[P5] Tetiana Dyrda-Terniuk, Michalina Ehlert, Kacper Roszak, Grzegorz Trykowski, Kamil Szpotkowski, Łukasz Skowroński, Oleksandra Pryshchepa, Rudi van Eldik, Katarzyna Mizgalska, Wayne Guida, Aleksandra Karolak, Riddhi Sainda, Prafulla K Jha, **Paweł Pomastowski**. Lactoferrin as an Active Coordination Scaffold for Ruthenium(III). *Inorganic Chemistry Frontiers* (manuskrypt zaakceptowany dnia 15.03.2026)

wchodzących w skład rozprawy doktorskiej mgr. Tetiany Dyrda-Terniuk, oświadczam że mój wkład polegał na pełnieniu nadzoru merytorycznego, współudziale w opracowaniu koncepcji badań, pomocy w planowaniu eksperymentów, interpretacji i dyskusji wyników oraz redagowaniu i korekcie manuskryptów. Wyniki prac [P2], [P3] oraz [P5] są bezpośrednim rezultatem kierowanego przeze mnie projektu pt. „Opracowanie preparatywnej metody izolacji biologicznej aktywnej laktoferyny” finansowanego przez Narodowy Centrum Badań i Rozwoju w ramach programu LIDER XIII (LIDER13/0292/2022).



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dr Aleksandra Pryshchepa

Toruń, 07.04.2026

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[P5] Tetiana Dyrda-Terniuk, Michalina Ehlert, Kacper Roszak, Grzegorz Trykowski, Kamil Szpotkowski, Łukasz Skowroński, **Aleksandra Pryshchepa**, Rudi van Eldik, Katarzyna Mizgalska, Wayne Guida, Aleksandra Karolak, Riddhi Sainda, Prafulla K Jha, Paweł Pomastowski. Lactoferrin as an Active Coordination Scaffold for Ruthenium(III). *Inorganic Chemistry Frontiers* (manuskrypt zaakceptowany dnia 15.03.2026)

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wchodzącej w skład rozprawy doktorskiej mgr. Tetiany Dyrda-Terniuk, oświadczam że mój wkład polegał na współudziale w opracowaniu koncepcji badań oraz redagowaniu manuskryptu.

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which is part of the doctoral dissertation of Tetiana Dyrda-Terniuk, M.Sc., I declare that my contribution consisted of participating in the development of the research concept.

Yours sincerely,



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wchodzącej w skład rozprawy doktorskiej mgr Tetiany Dyrdy-Terniuk, oświadczam, że mój wkład w jej powstanie polegał na zaplanowaniu i przeprowadzeniu analiz metodami mikroskopii korelacyjnej LM-SEM-EDS, TEM-STEM-EDS oraz redagowaniu manuskryptu.



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Dr. Aleksandra Karolak

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As part of the PhD thesis of Ms. Tetiana Dyrda-Terniuk, MSc, my contribution involved performing molecular docking studies and editing the manuscript.

Sincerely,

A handwritten signature in black ink, appearing to read "Aleksandra Karolak". The signature is fluid and cursive, with the first name "Aleksandra" being more prominent than the last name "Karolak".

Professor Wayne C. Guida
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CHE 205
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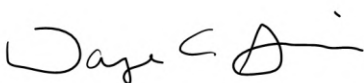
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STATEMENT

As a co-author of the following publication:

[P5] Tetiana Dyrda-Terniuk, Michalina Ehlert, Kacper Roszak, Grzegorz Trykowski, Kamil Szpotkowski, Łukasz Skowroński, Oleksandra Pryshchepa, Rudi van Eldik, Katarzyna Mizgalska, Wayne Guida, Aleksandra Karolak, Riddhi Sainda, **Prafulla K Jha**, Paweł Pomastowski. Lactoferrin as an Active Coordination Scaffold for Ruthenium(III). *Inorganic Chemistry Frontiers* (manuscript was accepted 15.03.2026)

As part of the PhD thesis of Ms. Tetiana Dyrda-Terniuk, MSc, my contribution involved performing MD and DFT calculations and editing the manuscript.

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