*This study focuses on the design of cryoresistant buffalo cheese models, in which the lipid phase is volumetrically replaced by a Pickering emulsion gel based on a RuBisCO isolate derived from Lemna minor biomass. The core problem addressed is the limited upcycling potential of this phytoremediant into sustainable alternative proteins, caused by heavy metal hyperaccumulation during biomass harvesting in contaminated military and industrial biotopes. Results demonstrate that a thermodynamic detoxification protocol (acid shock at pH 3.0 and dialysis) reduced the initial concentrations of Pb (6.9 mg/kg) and Cd (0.6 mg/kg) to < 0.10 mg/kg (efficiencies of ≥ 98.5% and ≥ 83.3%, respectively) and Cu levels by ≥ 84.6%, ensuring the production of a decontaminated isolate. The resulting isolate underwent conformational engineering (a pH-shift from 11.0 to 7.0) to form nanoparticles that stabilized the initial Pickering emulsion. Following ionotropic gelation, the emulsion was utilized for the volumetric replacement of animal fat by up to 75%. Gel integration substantially enhanced the water-holding capacity of the system (up to 87%) and minimized cryo-syneresis (from 14% to 1.5%). During the Schreiber test (85°C), the hybrid model exhibited a transition from a viscoelastic melt to a thermostable gel with suppressed lipid exudation. These macroscopic effects are attributed to enhanced polymer hydration, Donnan osmotic swelling, and the spatial restriction of capillary water mobility. The observed structural transition and thermostability are likely driven by synergistic in situ interfacial cross-linking of the Pickering armor, disulfide bond formation, and thermally induced macromolecular consolidation indicative of late-stage Maillard reactions. The stability of this rheological test system confirms the efficacy of upcycling phytoremediants for non-food applications and the feasibility of extrapolating the protocol to standard food raw materials*

*Keywords: alternative proteins, biomass upcycling, heavy metal removal, multiphase rheology, phytoremediation*

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# ELUCIDATING MECHANISMS OF THERMODYNAMIC DETOXIFICATION AND CONFORMATIONAL ENGINEERING OF LEMNA MINOR RUBISCO: IN SITU PICKERING ARMOR CROSS-LINKING IN CRYORESISTANT HYBRID CHEESE MODELS

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## 1. Introduction

The global transformation of the bioeconomy against the backdrop of rapid demographic growth and depletion of traditional agricultural lands necessitates the transition to sustainable sources of macromolecular raw materials and the mitigation of the global resource shortage. Advancing this paradigm is anchored in proven circular economy strategies. They include both the design of hybrid multicomponent systems with an engineered texture [1] and the precision control over the physicochemical profile of multiphase dispersed systems using biopolymer nanocomposites [2, 3].

In this conceptual context, industrial upcycling of phytoremediating aquatic macrophyte biomass, in particular *Lemna minor* (duckweed), is considered one of the most thermodynamically effective strategies for generating alternative proteins. Chemical and electrochemical extractions yield duckweed protein isolates with a protein concentration above 80%. At the same time, induced conformational changes (transition from intermolecular to intramolecular β-structures and α-helices) significantly improve their functional properties compared to commercial analogs [4].

The high biotechnological potential of this biological system is due to the high rate of biomass accumulation and the dominant concentration of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme with a specific conformational architecture and pronounced amphiphilicity. In the extracts, RuBisCO is able to associate

with accompanying proteins (ATP synthase subunits, chlorophyll-binding proteins), forming intricate supramolecular assemblies (33–85 nm). They exhibit high surface activity, forming elastic interfacial films with an elastic modulus of about 45 mN/m and low interfacial tension (~15 mN/m) [5]. Although this protein holds substantial promise for the engineering of complex multiphase polymer colloids, its direct integration into the industry has not yet been implemented on a large scale [6]. Industrial scale-up is impeded by a number of micro- and macroscopic barriers, the resolution of which requires cross-disciplinary efforts at the interface of biogeochemistry and soft matter physics.

Therefore, elucidating the mechanisms of thermodynamic detoxification of such biomass and the conformational engineering of RuBisCO for stabilization of multiphase systems represents a critical research imperative. Given the existing regulatory barriers, adapting the architecture of the hybrid cheese model as a macroscopic rheological test system is a rational research approach. This allows for an objective assessment of the thermodynamic excess state of the isolate, fundamentally substantiating its potential for technical non-food upcycling.

## 2. Literature review and problem statement

The key limiting factor that complicates the direct integration of native duckweed biomass into food chains is its critical ecotoxicological profile, which is significantly exacerbated by the accumulation of these toxicants in contaminated military and industrial biotopes. Functioning as a natural bioaccumulator, *L. minor* exhibits a profound capacity for heavy metal (Pb, Cd, Cu) hyperaccumulation. This process is governed at the transcriptomic level by the intense overexpression of the glutathione S-transferase (GST) gene family (in particular, *LmGSTF*3). Such adaptation enhances antioxidant defense and photoprotection mechanisms, and modulates the glycolytic metabolism of the macrophyte, allowing it to survive and accumulate contaminants in tissues at concentrations significantly exceeding the established EU regulatory thresholds [7]. Traditional industrial methods of aqueous-salt extraction or isoelectric precipitation are inherently ineffective since transition metal ions form stable coordination bonds with electron-donating oxygen and nitrogen centers of polypeptide chains. Fundamental molecular dynamics simulations (200-ns MD using the CHARMM force field) show that the binding of metal ions (in particular $Cd^{2+}$ and $Pb^{2+}$) to globular proteins induces a profound destabilization of their native conformation. This causes significant fluctuations in the root mean square deviation (RMSD) and changes in the accessible surface area (SASA) in critical regions such as EF-loops and H-strands [8]. Endogenous polysaccharides pose an additional barrier to decontamination, as they are capable of forming strong polyelectrolyte complexes with proteins and heavy metals, complicating their desorption [9].

Given these challenges, the dissociation of the described stable complexes exceeds the efficacy of classical methods and requires rigorous thermodynamic intervention. Demetallation should rely on competitive protonation of ligands: acid shock and a high proton gradient enable the chemical displacement of contaminants from their centers [4]. However, selective copper removal faces a fundamental chemical constraint: the specific reduction of Cu(II) ions to Cu(I) induces the formation of stable covalent thiol complexes. Studies using X-ray absorption spectroscopy (XAS) demonstrate that the binding of copper in high-affinity centers (ATCUN motifs) induces allosteric shifts. This exposes reactive disulfide bonds and initiates thiol-mediated electron transfer. According to the theory of hard and soft acids and bases (HSAB), this interaction creates a redox trap, forming coordination nodes that are thermodynamically resistant to proton substitution even during deep dialysis [10].

However, even if redox traps are overcome and deep demetallation of the isolate is achieved, the next fundamental challenge is its functional integration into multicomponent systems. This requires colloidal stabilization of such hybrid architectures, in particular rheologically complex buffalo cheese models, where the key task is the volumetric replacement of the high-melting native lipid phase. The technological viability of volumetrically replacing the lipid phase with alternative proteins has been previously validated through the construction of traditional protein-lipid matrices, which exhibit high structural homogeneity and pronounced kinetic stability [11]. Therefore, the use of classical lipid-water emulsions with standard surfactants is technologically inefficient. Under the influence of high ionic strength of the polymer network, such dispersions quickly degrade due to Ostwald ripening and coalescence [12]. An effective macroscopic solution is the construction of Pickering emulsion gels; however, the native conformation of RuBisCO is characterized by insufficient interfacial rigidity to form a strong barrier shell around lipid droplets. Its activation requires precise conformational engineering of macromolecules (via controlled pH-shift), which induces a directed intermolecular hydrophobic assembly of unfolded chains. Such a shift triggers the unfolding of the protein and a decrease in its surface hydrophobicity with subsequent coassembly into soluble aggregates, which optimizes their interfacial functionality [13]. As a result, rigid, structurally reassembled protein nanoparticles are generated, whose high surface activity and dense spatial packing establish a strong mechanical barrier that ensures prolonged colloidal stability of the dispersed phase [14].

Despite the high stability of isolated Pickering emulsions, their macroscopic integration into composite polymer systems reveals a disruption of rheological behavior under intense thermomechanical gradients. Upon heating to 85°C, the polymer architecture of classical analogs collapses, transforming the system into a fluid viscoelastic melt, accompanied by the exudation of free lipids from the polymer matrix [15]. Conversely, under deep freezing conditions (–18°C), hybrid systems undergo microstructural destruction due to the freezing of free capillary water, the macrocrystals of which mechanically rupture the polymer framework. SAXS and IR spectroscopy data confirm that the main cause of such destruction is not protein denaturation but an alteration in the molecular network of water and water-solid interactions. After thawing, this leads to irreversible cryo-syneresis [16].

Preventing such degradation requires designing robust barrier mechanisms, which is conceptually consistent with the principles of developing nanocomposite biopolymer systems capable of synergistically preserving the native microstructure and providing strong spatial stabilization. For hybrid cheese models, this necessitates synergistic control of the water state: initiating processes in which Donnan osmotic swelling of ionotropic chains dramatically increases water-holding capacity. This process is based on the reactive electrochemical-mechanical theory, according to which a high

concentration of charges on the chains accumulates counterions, generating osmotic pressure that thermodynamically stretches the polymer network [17]. The formation of such a gel architecture (in particular, according to the "egg-box" model for alginates, where calcium ions form mirror-symmetric binding zones) fixes the ionotropic framework [18].

This process induces a pronounced enthalpic effect of hydration, which thermodynamically precludes ice nucleation by bound water. In addition, the specific conformational flexibility of amphiphilic polypeptides acts as a powerful ice recrystallization inhibitor (IRI), preventing thermodynamic crystal growth [19]. At the same time, the residual capillary moisture is governed by the Gibbs-Thomson nanoconfinement effect. Thermodynamic studies in porous media reveal that the depression of the freezing point of pore water depends entirely on heterogeneous ice nucleation (HIN). The increase in osmotic potential at the ice-nucleus-liquid interface sharply limits the formation of crystals in the pores. This allows the hybrid network to restore its shape after thawing due to the poroelastic reabsorption mechanism [20].

Studies confirm the effectiveness of enthalpic polymer hydration and Gibbs-Thomson nanoconfinement mechanisms for protecting polymer systems from cryo-syneresis [21]. Despite these mechanistic insights, the problem of lipid exudation upon heating has not yet been solved, and there is a lack of comprehensive approaches that would combine thermodynamic mitigation of geochemical toxicity of phytoremediants with macromolecular engineering of proteins. These limitations stem from the isolated consideration of biomass decontamination and rheological stabilization processes, coupled with the fact that traditional dialysis remains a cost-prohibitive hurdle for industrial scale-up [22]. Promising approaches to overcome these barriers include inducing a phase transition of the system into an irreversible chemical gel [23], as well as the generation of dicarbonyl bonds and the *in situ* assembly of Pickering armor [24, 25], which reliably prevent lipid thermal coalescence. However, the synergy of these kinetic processes with the conformational transformation of decontaminated RuBisCO remains poorly understood.

Consequently, elucidating the mechanisms of thermodynamic detoxification of RuBisCO and its conformational engineering is of paramount importance for the stabilization of cryoresistant hybrid cheese models with zero lipid exudation.

## 3. The aim and objectives of the study

The aim of the study is to determine the mechanisms of thermodynamic detoxification and conformational engineering of RuBisCO from Lemna minor for the construction of a cryoresistant hybrid cheese model. This will enable the exploration of the limits of thermodynamic endurance of macromolecules and the validation of the devised protocol on the specified macroscopic model, substantiating the fundamental pathways of upcycling phytoremediants for non-food applications.

To achieve this aim, the following objectives were formulated:

– to establish the ecotoxicological profile of *L. minor*, to screen for background $Ni^{2+}$, and to evaluate the efficacy of the thermodynamic decontamination protocol (mucilage depolymerization, acid shock, and dialysis) in reducing the levels of contaminants ($Pb^{2+}$, $Cd^{2+}$, and $Cu^{2+}$) and recovering a decontaminated RuBisCO isolate;

– to determine the physicochemical and electrokinetic characteristics of the resulting Pickering emulsion and to optimize the kinetic parameters of its ionotropic gelation;

– to assess the hydration stability and resistance to cryo-syneresis of the constructed cheese models with different levels of volumetric replacement of the lipid phase by a Pickering emulsion gel (0% to 75% v/v) at −18°C;

– to characterize the macroscopic thermomechanics of the constructed cheese models (modified Schreiber test at 85°C) to confirm the macrostructural loss of thermoplasticity (transition to an irreversible gel state) and to assess the efficiency of lipid phase encapsulation.

## 4. Materials and methods

### 4. 1. Object and hypothesis

The object of the study is the construction of thermostable and cryoresistant hybrid cheese models using Pickering emulsion gels based on the RuBisCO isolate from *Lemna minor*.

The principal hypothesis states that the synergy of thermodynamic detoxification and protein conformational engineering will enable the formation of an *in situ* Pickering armor capable of blocking lipid thermocoalescence and cryo-syneresis. The adopted assumptions postulate that the efficacy of the demetallization protocol validated on an *in situ* stress model with a high level of contamination remains applicable to raw materials with lower background concentrations of heavy metals. The accepted simplifications involve neglecting the influence of background mineral micro-impurities on gelation and imputing instrumental values below the limit of detection (< LOD) as mathematical zeros for ANOVA.

### 4. 2. Materials and sampling

The biomass of the aquatic macrophyte *Lemna minor* was collected from the surface water layers in the riparian zones of the Syrovatka River (Sumy Oblast, Ukraine). The choice of location was dictated by the high military and industrial load on the aquatic environment (detonation and corrosion of munitions, destruction of infrastructure). The intensive accumulation of heavy metals in the local biomass, attributed to the high bioconcentration factor (BCF) of *L. minor* [26], allowed its use as a representative *in situ* stress model to validate the demetallization protocol. The native raw material was mechanically separated from macroscopic impurities and subjected to fivefold washing with deionized water (resistivity ≥ 18.2 MΩ·cm) for the complete removal of surface mineral sediments and epiphytic microflora. Subsequently, the biomass was dried in a forced-air convection oven at 40°C to constant mass to prevent thermal denaturation of endogenous proteins.

Commercially pasteurized, lactose-free water buffalo milk (7.7% w/w fat, 4.1% w/w protein) served as the continuous phase of the cheese model. Enzymatic coagulation of the polymer dispersion was induced by an aqueous extract of *Withania coagulans* phytoproteases. Food-grade sodium alginate (SA), calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$), tetrasodium pyrophosphate (TSPP), and refined sunflower oil (model lipid phase) were utilized to form Pickering emulsion gels. The multienzyme preparation Viscozyme® L (Novozymes) was applied for enzymatic depolymerization of macrophyte

mucilage. Semipermeable regenerated cellulose dialysis membranes (molecular weight cut-off [MWCO] of 3.5 kDa) were employed for metal extraction. For sample preparation, concentrated $HNO_3$ (65% w/w), $H_2O_2$ (30% w/w), 5.0 mol/L KOH, ionic strength adjustment buffer (ISAB) reagents ($KNO_3$ and ascorbic acid), and dimethylglyoxime (DMG) were used. All chemical reagents were of analytical grade (purity ≥ 99.0%).

**4. 3. Sample mineralization and analytical control**

Biomass sample preparation (equivalent to 100 g of wet mass) was carried out according to the extended pseudo-total acid leaching protocol. Dried biomass samples were placed in borosilicate reactors previously decontaminated with a 10% (v/v) nitric acid solution. A volume of 9.0 mL of concentrated $HNO_3$ and 3.0 mL of 30% (v/v) $H_2O_2$ solution (volume ratio 3:1) were slowly added to the sample. The mixture was kept at room temperature for 30 min to suppress the initial intense exothermic oxidation reaction. Further mineralization was carried out in a closed thermoblock using a two-stage temperature gradient: initial heating at 95°C for 60 min with subsequent temperature increase to 105°C. The process was maintained under a reflux mode until the organic matrix was completely destroyed and the release of brown nitrogen oxide vapors ceased. The resulting transparent digestate was evaporated to a residual volume of 1.5–2.0 mL and cooled in an ice bath. After neutralization with 5.0 mol/L KOH solution to pH 4.0 ± 0.1, the sample was quantitatively transferred to a volumetric flask and adjusted to 50 mL with ISAB buffer (pH 5.5). As a background electrolyte, 1.0 mol/L $KNO_3$ was used. To avoid interference from background $Fe^{3+}$ ions without complexing the target heavy metals, reductive masking was used by adding 0.1 mol/L ascorbic acid (which quantitatively converts iron into the non-interfering form $Fe^{2+}$).

A complex biological matrix has a high ionic strength, which creates matrix effects. To minimize them, the quantitative determination of free ions ($Pb^{2+}$, $Cd^{2+}$, $Cu^{2+}$) by the potentiometric method was carried out exclusively according to the algorithm of standard additions. For this purpose, microvolumes of standard solutions of target metals were sequentially added to a fixed volume of the test sample. The initial concentration of metal ions ($C_x$) was calculated using equation (1)

$$C_x = \frac{C_s \times V_s}{V_x \times \left(10^{\frac{\Delta E}{S}} - 1\right)}, \quad (1)$$

where $C_x$ is the unknown initial concentration of metal ions in the sample, mg/L;

$C_s$ is the concentration of the introduced standard solution, mg/L;

$V_s$ is the volume of the added standard, mL;

$V_x$ is the initial volume of the test sample, mL;

$\Delta E$ is the change in electrode potential after the introduction of the standard, mV;

$S$ is the empirically determined slope of the electrode function (Nernst slope), mV/decade.

The limit of detection (LOD) of ion-selective electrodes was conservatively set at 0.10 mg/kg. The efficiency of thermodynamic detoxification ($E_{detox}$, %) of the isolate was calculated using formula (2)

$$E_{detox} = \frac{C_i - C_f}{C_i} \times 100, \quad (2)$$

where $C_i$ is the initial concentration of the target heavy metal in the native biomass, mg/kg; $C_f$ is the final residual concentration of the metal in the purified RuBisCO isolate, mg/kg (for instrumental values below the limit of detection (LOD), the mathematical value of $C_f$ = LOD was imputed). The presence and extraction of $Ni^{2+}$ ions were additionally checked by a qualitative colorimetric method using Chugaev's reagent (dimethylglyoxime, DMG) in a weakly alkaline medium.

**4. 4. Enzymatic extraction and thermodynamic detoxification of ribulose-1,5-bisphosphate carboxylase/oxygenase**

Extraction of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from native biomass was carried out in an aqueous solution at a solid-to-liquid ratio of 1:15 (w/v) and pH 8.0 with the addition of 10 mmol/L ascorbic acid. Endogenous apiogalacturonans of the macrophyte cause a high pseudoplastic viscosity of the extract. To eliminate it, the pH was reduced to 4.5 and the multienzyme complex Viscozyme L (1.5% (v/w) of dry weight) was added. The temperature of 35°C was chosen to enable sufficient depolymerization of polysaccharides [27] while preventing thermal denaturation of native RuBisCO. After incubation, the suspension was subjected to coarse centrifugation (8000 × g, 15 min, 4°C) to remove insoluble fiber.

For efficient dissociation of coordination bonds between metals and native RuBisCO, the pH of the clear supernatant was sharply reduced to 3.0 by dropwise titration of 1.0 mol/L HCl (acid shock). The exposure time (20 min) was chosen empirically to ensure desorption of metals by proton competition [28] without inducing irreversible protein denaturation. At the pH 3.0 stage, the solution was dialyzed against acidified deionized water (pH 3.0) at 4°C with continuous stirring. This prevented re-association of heavy metals with protein groups during neutralization. The volume ratio of retentate to dialysate was 1:50. The process was accompanied by complete replacement of the dialysate every 8 h for 48 h until complete leaching of free toxic metal ions along the concentration gradient. After dialysis, the pH of the retentate was titrated with 1.0 mol/L NaOH to 4.5, which corresponds to the isoelectric point (pI) of isolated RuBisCO from native biomass [4]. The decontaminated protein precipitate was separated by centrifugation (MLW T 23, 4000 × g, 20 min at 4°C). To prevent thermal denaturation of native RuBisCO, the recovered protein isolate was not subjected to total drying but was stored in the form of a wet precipitate. In subsequent aqueous dispersions, it was necessary to ensure an accurate protein concentration (2.5% (w/v)). For this purpose, the mass fraction of dry matter was determined in a separate microaliquot of the wet precipitate by the rapid thermogravimetric method (Chizhov device, TVU-2.1-0.01 balance).

**4. 5. Conformational engineering and formation of Pickering emulsion gels**

An equivalent weight of the wet isolate was redispersed in deionized water to a concentration of 2.5% (w/v). The system was then subjected to a pH-shift (11.0 → 7.0), which initiated the intermolecular hydrophobic assembly of macromolecules into protein nanoparticles [29]. Refined sunflower oil was added to the protein dispersion (aqueous phase-to-oil ratio of 8:2). The mixture was dispersed in a standard laboratory

high-speed rotary homogenizer. The process was carried out at a speed of ≥ 10,000 rpm for 10 min. To prevent thermal denaturation of RuBisCO due to hydrodynamic heating (which can reach 60–80°C), the homogenization process was carried out in an aluminum crystallizer on an ice bath.

This hydrodynamic regime generates an intense shear force to overcome the Laplace pressure. According to hydrodynamic scaling models, these conditions enable the formation of droplets with a theoretical size of 1.5–2.0 μm. To confirm the calculations, the mean diameter ($D_{32}$), polydispersity index (PDI), and zeta potential (ζ) were determined instrumentally. Measurements were performed by dynamic and electrophoretic light scattering (DLS/ELS) methods. The analysis was performed on a standard laboratory laser particle analyzer at 25°C. To prevent multiple scattering effects, the samples were pre-diluted with deionized water (1:100 (v/v)). The absence of macroscopic coalescence was additionally confirmed visually by optical microscopy (MICROmed XS-2610 microscope).

The emulsion was then mixed with a solution of food-grade sodium alginate (protein/alginate ratio 5:1). To the aqueous phase, 0.2% (w/w) TSPP was pre-added as a competitive chelator (kinetic trap). Crosslinking of the alginate matrix was initiated by hydrodynamic injection of a $CaSO_4 \cdot 2H_2O$ suspension (pre-dispersed in glycerol) into the intensive mixing zone. The use of TSPP ensured a homogeneous distribution of $Ca^{2+}$ ions until gelation, forming a statistically isotropic, heterogeneous fibrillar network of an ionotropic gel. The operational technological window (pot-life) before the onset of ionotropic gelation was estimated by the macroscopic tube inversion method. For this purpose, the samples were thermostated using a UT-15 ultrathermostat at 20°C, recording the time until the system completely lost its fluidity (no surface deformation when tilted by 180°).

**4. 6. Construction of a thermoresponsive cheese model**

Lactose-free buffalo milk was thermostated at 35°C and adjusted to pH 5.6 with food-grade lactic acid. After that, *W. coagulans* extract was added – a validated phytoprotease complex effective under mild conditions [30]. After 45–50 min, the formed dense *para*-κ-casein curd was cut into cubes with a side of ca. 10 mm and separated from the whey. The experimental design provided for four variants of the cheese model. The basic control DFC contained native animal fat. In other samples, volumetric replacement of lipids with emulsion gel was performed at levels of 25%, 50%, and 75% (coding REG25, REG50, REG75). For thermal inactivation of proteases, the curd was ground (fraction ≤ 5 mm) and subjected to hydrothermal shock in a water bath. The inactivation parameters (90°C, 150 s) were established empirically, taking into account the high thermal stability of *W. coagulans* aspartic proteases [30]. Immediately after the thermal shock, the hot mass (> 70°C) was subjected to thermomechanical consolidation in cylindrical molds under pressure to form a monolithic block of coagulated microparticles. The formed samples were kept at 4°C for 24 h.

**4. 7. Evaluation of hydration stability (water-holding capacity and resistance to cryo-syneresis)**

The water-holding capacity (WHC) was determined by the gravimetric method with controlled low-speed centrifugal expression to preserve the microporous structure of the model. Samples of the cheese model weighing approximately 5.0 g were placed in two-chamber centrifuge tubes with mesh filters (pores 100 μm). This design allowed the separated liquid to be drained into the lower reservoir without reverse capillary suction. Centrifugation was performed at 500 × g for exactly 5 min at 20°C. The WHC index was calculated using formula (3)

$$WHC = \frac{W_1 - W_{ex}}{W_1} \times 100, \quad (3)$$

where $W_1$ is the initial mass of the cheese model sample before centrifugation, g;

$W_{ex}$ is the mass of the expressed liquid (supernatant) separated during centrifugation, g.

The stability to syneresis (cryo-test) was assessed by freezing the samples in airtight packaging (–18°C, 24 h). They were then thawed at room temperature (22 ± 1°C) on weighed filter paper for 4 h. The moisture loss or degree of cryo-syneresis (CS, %) was calculated gravimetrically using equation (4)

$$CS = \frac{M_0 - M_t}{M_0} \times 100, \quad (4)$$

where $M_0$ is the mass of the test disk before freezing, g;

$M_t$ is the mass of the sample after thawing and thorough removal of surface exudate, g.

**4. 8. Thermoresponsive mechanics and glycation kinetics**

The macroscopic melting was assessed using a standardized closed-system thermomechanical spreading test. Cylindrical disks with a standardized initial diameter ($D_0$) of 17.6 ± 0.1 mm and a height of 5.0 ± 0.1 mm were cut from a monolithic block of cheese model using a calibrated cork borer. The samples were heated on a heat-conducting aluminum plate in hermetically sealed glass Petri dishes at 85°C for 15 min. Inducing a closed vapor-liquid equilibrium enabled the preservation of moisture as an internal plasticizer. After cooling to room temperature, the final spreading diameter ($D_f$) was measured with a digital caliper in six radial directions to eliminate the effects of asymmetric melting. The melt expansion coefficient (*EF*, %) was calculated using formula (5)

$$EF = \frac{D_f - D_0}{D_f} \times 100, \quad (5)$$

where $D_f$ is the average final diameter of the cheese model disk after melting, mm;

$D_0$ is the initial diameter of the cheese model disk, mm.

Free lipid exudation was estimated by the diameter of the lipid spot on filter paper under similar heating conditions. Qualitative assessment of macroscopic signs of melanoidin formation (an indicator of the late stages of the Maillard reaction) as a probable consequence of crosslinking of free galactose and ε-amino groups of RuBisCO lysine residues was carried out by directional visual inspection of the surface of the melted cheese model.

**4. 9. Statistical analysis**

All experiments were performed in three biological and analytical replicates ($n$ = 3). Statistical processing of data sets was carried out in base R (version 4.6.0). The data were

preliminarily checked for normality of distribution using the Shapiro-Wilk test and for homogeneity of variances (homoscedasticity) using the Levene test. If these assumptions were confirmed, one-way analysis of variance (one-way ANOVA) was used with subsequent determination of the significance of differences between groups using the Tukey's HSD a *posteriori* test at $p < 0.05$. The results are represented as the arithmetic mean ± standard deviation (Mean ± SD). All differences described in the manuscript between the compared experimental groups are statistically significant ($p < 0.05$).

## 5. Results of studies on the mechanisms of thermodynamic detoxification of proteins and properties of hybrid cheese models

### 5. 1. Ecotoxicological profile of biomass and detoxification of RuBisCO isolate

Analysis of native *L. minor* biomass revealed an initial lead ($Pb^{2+}$) content of 6.9 ± 2.3 mg/kg and a cadmium ($Cd^{2+}$) content of 0.60 ± 0.35 mg/kg. These indicators substantially exceed the maximum permissible levels (MPLs) for standard agricultural raw materials, regulated by Commission Regulation (EU) 2023/915 (≤ 0.20 mg/kg for $Pb^{2+}$ and ≤ 0.10 mg/kg for $Cd^{2+}$). Copper ($Cu^{2+}/Cu^{+}$) was present at a concentration of 20.3 ± 9.8 mg/kg, which is within the strict ecotoxicological limits according to FAO/WHO recommendations (≤ 40.0 mg/kg). Qualitative screening using Chugaev's reagent (dimethylglyoxime (DMG)) gave a negative result for the presence of nickel ions ($Ni^{2+}$) in the raw material (color change at the background level). Table 1 shows the dynamics of detoxification and the ecotoxicological profile.

Table 1

Ecotoxicological profile of *Lemna minor* biomass and validation of the depth of decontamination of the RuBisCO isolate

| Heavy metal | Content in native biomass, mg/kg | Content in decontaminated RuBisCO isolate, mg/kg | Detoxification efficiency, % | Regulatory ecotoxicological limit (MPL), mg/kg | Decontamination status |
|---|---|---|---|---|---|
| Lead ($Pb^{2+}$) | 6.9 ± 2.3 | < LOD | ≥ 98.5 | ≤ 0.20 (EU Reg. 2023/915) | Target markers < LOD |
| Cadmium ($Cd^{2+}$) | 0.60 ± 0.35 | < LOD | ≥ 83.3 | ≤ 0.10 (EU Reg. 2023/915) | Target markers < LOD |
| Copper ($Cu^{2+}/Cu^{+}$) | 20.3 ± 9.8 | 3.1 ± 1.9 | ≥ 84.6 | ≤ 40.0 (FAO/WHO) | Thermodynamic extraction limit |
| Nickel ($Ni^{2+}$) | Traces (at background level) | Not detected | – | – | No contamination |

After applying the detoxification protocol (acid shock pH 3.0 and dialysis), the content of $Pb^{2+}$ and $Cd^{2+}$ in the RuBisCO isolate decreased to values below the detection limit of the instruments (< the limit of detection (LOD), ≤ 0.10 mg/kg), which fully meets the criteria for deep decontamination. The calculated detoxification efficiency was ≥ 98.5% for lead and ≥ 83.3% for cadmium. The copper content was significantly reduced ($p < 0.05$) by 84.6% to 3.1 ± 1.9 mg/kg. These empirical results confirm the high efficiency of acid shock and dialysis for overcoming the ecotoxicological barrier and recovering a deeply decontaminated isolate.

### 5. 2. Physicochemical and electrokinetic characteristics of the Pickering emulsion

After treatment of the extract with Viscozyme L and pH-shift (11.0 to 7.0), a kinetically stable Pickering lipid-water emulsion stabilized by structurally reassembled protein nanoparticles was formed. Table 2 shows the physicochemical and electrokinetic characteristics of the resulting system.

Table 2

Physicochemical and electrokinetic parameters of Pickering emulsion and hydrogel

| Indicator | Value |
|---|---|
| Zeta potential of emulsion at pH 7.0 (mV) | −35.77 ± 0.57 |
| Average diameter of lipid droplets, $D_{32}$ (μm) | 1.8 ± 0.3 |
| Polydispersity index (PDI) | 0.22 ± 0.03 |
| Time to onset of gelation (pot-life), min | 26.0 ± 2.4 |

The average diameter of lipid droplets ($D_{32}$) was 1.8 ± 0.3 μm with a polydispersity index (PDI) of 0.22 ± 0.03. The zeta potential of the system at pH 7.0 was recorded at −35.77 ± 0.57 mV. The addition of 0.2% (w/w) TSPP to the system provided an operational technological window before the onset of gelation of 26.0 ± 2.4 min at 20°C. The recorded zeta potential and technological window parameters confirm the sufficient colloidal and operational stability of the emulsion for its further volumetric integration into the cheese model.

### 5. 3. Hydration stability and resistance to cryo-syneresis

Replacing the native lipid phase with Alginate-RuBisCO REG substantially increased the structural water-holding capacity (WHC) under the action of low-speed centrifugal moisture expression (500 × *g*). Table 3 shows the dynamics of hydration stability and resistance to syneresis.

Table 3

Hydration stability of cheese models depending on the level of volumetric replacement of the lipid phase with Pickering gel

| Cheese model variant | Water-holding capacity (WHC, at 500 × g), % | Moisture loss after cryo-test (Syneresis), % |
|---|---|---|
| DFC-0% | 49.0 ± 3.2$^{c}$ | 14.0 ± 2.1$^{a}$ |
| REG-25% | 59.5 ± 3.5$^{c}$ | 11.1 ± 1.4$^{a}$ |
| REG-50% | 74.6 ± 5.6$^{b}$ | 5.2 ± 0.8$^{b}$ |
| REG-75% | 87.0 ± 4.2$^{a}$ | 1.5 ± 0.3$^{c}$ |

*Note: Values bearing different superscript letters (a–c) within the same column differ significantly (Tukey's HSD test, $p < 0.05$).*

The WHC increased from 49.0 ± 3.2% in the Control (DFC-0%) sample to 87.0 ± 4.2% in the REG-75% sample. Statistical analysis revealed no significant difference between the DFC-0% and REG-25% samples ($p > 0.05$); however, a significant increase in WHC was recorded between the REG-25% and REG-50% samples ($p < 0.05$). Moisture loss after the freeze/thaw cycle (cryo-syneresis) demonstrated the opposite trend: moisture loss significantly decreased ($p < 0.05$) from 14.0 ± 2.1% (DFC) to 1.5 ± 0.3% (REG-75%).

Such macroscopic behavior directly indicates that the integration of Pickering gel allows for intensive moisture immobilization and the minimization of the cryo-syneresis defect.

### 5. 4. Thermoresponsive parameters and lipid encapsulation

In the modified Schreiber test (85°C, 15 min in a sealed chamber), the macroscopic melting and coalescence of lipids were investigated under conditions of vapor-liquid equilibrium. Table 4 shows the thermoresponsive parameters of the cheese models.

Table 4

Thermoresponsive and physical parameters of cheese models after heating simulation

| Cheese model variant | Spreading diameter (Schreiber test), mm | Free lipid spot diameter, mm |
|---|---|---|
| DFC-0% | $38.4 \pm 1.7^a$ | $47.5 \pm 4.3^a$ |
| REG-25% | $32.6 \pm 1.3^b$ | $37.6 \pm 1.6^b$ |
| REG-50% | $22.3 \pm 0.4^c$ | $23.4 \pm 1.1^c$ |
| REG-75% | $17.8 \pm 0.2^d$ | $17.7 \pm 0.2^c$ |

*Note: values bearing different superscript letters (a–d) within the same column differ significantly (Tukey's HSD test, $p < 0.05$).*

The control sample demonstrated a typical viscoelastic melt, macroscopic spreading (diameter 38.4 ± 1.7 mm), and large-scale thermocoalescence of the dispersed phase accompanied by intense free lipid exudation (spot 47.5 ± 4.3 mm). With increasing REG fraction, the polymer system underwent irreversible chemical gelation: for the REG-75% sample, the spreading diameter was 17.8 ± 0.2 mm. The free lipid spot for REG-75% was 17.7 ± 0.2 mm. No significant difference between lipid exudation in the REG-50% and REG-75% samples was detected ($p > 0.05$). Visual examination confirmed the formation of melanoidins on the surface of the heated samples, and during further storage at 4°C, no signs of macroscopic degradation or softening of the polymer framework were detected. The equivalence between the spreading and free lipid spot diameters for the REG-75% sample indicates zero lipid exudation and the successful formation of an irreversible thermostable gel.

## 6. Discussion of results related to the study on the mechanisms of thermodynamic detoxification of RuBisCO and the properties of cryoresistant hybrid cheese models

The observed high level of heavy metals confirms the effectiveness of *L. minor* as a biological filter (phytoremediant). However, the critical excess of the limits of European legislation (in particular, Regulation EU 2023/915, based on the ALARA principle) makes such raw materials unsuitable for direct food use. We observed the reduction of $Pb^{2+}$ and $Cd^{2+}$ to levels < LOD (Table 1), which is attributed to the high efficiency of the applied acid shock. At pH 3.0, competitive protonation occurs: a high concentration of protons competes with metal ions for oxygen and nitrogen ligands, chemically displacing them from the coordination centers of the protein. Macroscopic unfolding of polypeptides serves to consolidate this outcome, destroying the formed chelate complexes. This mechanism is consistent with molecular dynamics data [8], which demonstrate a critical dependence of the stability of metal-protein complexes on the conformational state.

The achieved level of detoxification substantially exceeds the efficiency of traditional methods of aqueous-salt extraction of plant biomass, which are usually unable to break these coordination bonds [6]. An additional critical step in decontamination was the use of the multienzyme complex Viscozyme L, which destroyed the apiogalacturonan mucilage and eliminated the phenomenon of polyelectrolyte complexation [9], thereby preventing localized secondary binding of metals during dialysis. Conversely, the incomplete removal of copper (Table 1) is fully attributed to the theory of hard and soft acids and bases (HSAB) and the mechanism of the "redox trap". Ascorbic acid reduces copper to the Cu(I) ion, which forms thermodynamically stable covalent thiol complexes that are resistant to proton substitution. This mechanism has been directly confirmed in recent studies [10]. However, the residual copper content meets strict ecotoxicological criteria. It is well below the regulatory limit (≤ 40.0 mg/kg) established as a reference marker for safe agricultural raw materials (Joint FAO/WHO Program).

Conformational engineering of RuBisCO (pH-shift from 11.0 to 7.0) induced intermolecular hydrophobic assembly of unfolded protein macromolecules into functional nanoparticles. Similar dynamics of conformational reorganization and increase in surface activity have previously been successfully validated for other plant proteins after alkaline shift [13]. The high negative zeta potential of the emulsion (Table 2) indicates exposure of internal anionic domains, which provided electrostatic repulsion. In combination with the particulate steric hindrance of the rigid nanoparticles, this created a strong mechanical barrier against the coalescence of lipid droplets. This behavior is a classic mechanism of Pickering emulsion stabilization by plant proteins [14]. The accurately predicted gelation time (Table 2) is governed by the action of TSPP molecules as a competitive chelator and kinetic trap [18]. Unlike the uncontrolled instantaneous coagulation of native proteins, such kinetic control provides the necessary technological window for the homogeneous formation of cheese models.

A statistically significant increase in hydration indices (Table 3) between the REG-25% (v/v) and REG-50% (v/v) samples confirms the crossing of the percolation threshold. The pronounced increase in water-holding capacity is attributed to the Donnan osmotic swelling of the strongly charged anionic polyelectrolyte network of alginate, which is fully consistent with theoretical models [17]. Consequently, the atypical decrease in cryo-syneresis (Table 3) is driven by a dual mechanism. First, intensive hydration converts water into tightly bound water (enthalpy effect) [19]. Second, free capillary water undergoes the Gibbs-Thomson nanoconfinement effect, which effectively suppresses heterogeneous ice nucleation in nanopores [20]. The achieved stability contrasts sharply with the behavior of traditional protein and polysaccharide hydrogels, which typically undergo irreversible microstructural destruction upon thawing [16, 21].

The observed suppression of macroscopic spreading of the cheese model at 85°C (Table 4) indicates the phase transition of the system from a viscoelastic melt to an irreversible thermostable gel [22]. The absence of residual proteolytic activity confirms that the chosen thermal profile successfully inactivated the stable proteases of *W. coagulans* [30]. The observed reduction of free lipid exudation to almost zero (Table 4) is macroscopic confirmation of *in situ* interfacial cross-linking of the Pickering armor [23]: we hypothesize that the RuBisCO nanoparticles are covalently crosslinked directly at the interface, blocking lipid coalescence. In addition, the rapid

interaction of free D-galactose with ε-amino groups of lysine residues (Maillard reaction) [24], which probably formed intermediate dicarbonyl crosslinks, could have played a critical role. Visual observation of the darkening of the polymer system serves as a macroscopic indicator of their formation, although the precise chemical mechanism will require further instrumental validation. This behavior differs profoundly from commercial vegetable cheese analogs, which often exhibit intense lipid exudation upon heating [15].

Inherent technological and analytical limitations of the study include the constraints of the static isothermal Schreiber model, which does not fully reproduce the temperature gradients of open thermodynamic processes. Additional barriers are open acid mineralization (providing only pseudo-total leaching) and the use of classical dialysis, which in the future will require a transition to industrial ultrafiltration systems with water recirculation [25]. Concurrently, a fundamental toxicological barrier is that conformational engineering (pH-shift from 11.0 to 7.0) induces a transit state of the "molten globule", creating the risk of steric confinement of lipophilic xenobiotics. Since the ion-selective electrodes used are insensitive to electroneutral organics, confirmation of comprehensive toxicological safety without non-targeted molecular screening (LC-HRMS) is analytically unattainable.

Given these findings and the requirements in European legislation (Regulation (EC) No 178/2002), direct food integration of such biomass is strictly prohibited, and the constructed hybrid model is considered exclusively as a macroscopic rheological test system. This finally establishes the status of the isolate for technical upcycling in non-food applications.

Further studies might be aimed at overcoming the limitations of the copper "redox trap" and using the REG system as a bioink for 3D printing of technical polymer composites with subsequent complex tribological profiling.

## 7. Conclusions

1. Competitive protonation under acid shock conditions in synergy with enzymatic depolymerization of mucilage effectively destroys polyelectrolyte metal complexes. Given the instrumentally confirmed absence of background $Ni^{2+}$, subsequent dialysis provides deep demetallation of RuBisCO (< 0.10 mg/kg for $Pb^{2+}$ and $Cd^{2+}$), while the residual copper concentration (3.1 mg/kg) is consistent with the thermodynamic limitations of thiol redox traps (HSAB).

2. Determination of the physicochemical and electrokinetic characteristics of the obtained Pickering emulsion confirms the formation of a kinetically stable dispersed system resistant to macroscopic coalescence (zeta potential −35.77 mV, average diameter of lipid droplets 1.8 μm). Concurrently, optimization of kinetic parameters by adding a competitive chelator TSPP ensures tightly controlled kinetics of ionotropic gelation with the required operational technological window of about 26 min.

3. Volumetric integration of these emulsion gels (up to 75% v/v) alters the hydration dynamics of the cheese model. Donnan osmotic swelling intensively immobilizes water (WHC up to 87.0% w/w), while the dense poroelastic architectonics of the polymer framework sterically limits the mobility of free capillary moisture. Their synergy minimizes macroscopic cryo-syneresis at −18°C (reduction to 1.5% w/w).

4. Upon heating (85°C), the synergistic interaction of the ionotropic alginate network and the interfacially crosslinked Pickering armor *in situ*, likely supported by the formation of new disulfide bonds by RuBisCO, transforms the system into an irreversible thermostable gel. The process is accompanied by thermally induced macromolecular consolidation (with visual signs of late-stage Maillard reactions), effectively blocking thermal coalescence of lipids (zero exudation). The ability of the protein to stabilize such a thermodynamically complex system after chemical decontamination justifies the use of isolates derived from phytoremediants in the synthesis of non-food biocomposites and confirms the feasibility of extrapolating the protocol to standard food raw materials.

## Conflicts of interest

The authors declare that they have no conflicts of interest in relation to the current study, including financial, personal, authorship, or any other, that could affect the study and the results reported in this paper.

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## Data availability

All data are available in the main text of the manuscript.

## Use of artificial intelligence

In accordance with the editorial policy, the authors provide detailed information about the use of AI:

– Gemini (Gemini 3.1 Pro model from Google);

– the tool was used in Sections 1, 2, 3, 4, 5, and 6 of this manuscript;

– the tool was used exclusively for technical editing: checking grammar, spelling, and punctuation, which is fully consistent with the permitted tasks according to the journal policy. No stylistic changes, adaptation of scientific style, sentence shortening or content generation using AI were performed.

The authors performed a full manual check of each proposed technical edit to ensure that the corrections did not affect the content or accuracy of scientific terminology.

The use of the tool to correct grammatical errors in no way affected the data obtained, their interpretation, results, or conclusions of the study, which are entirely from the authors.

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**Author contributions**

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