

Algal and cyanobacterial phycocyanins are promising alternatives to serum albumin in cellular agriculture

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ABSTRACT

Cultured meat may require fewer resources and is less polluting than animal meat, but it remains costly to produce. One of the critical challenges in cultivated meat science is to identify/develop fetal bovine serum (FBS) alternatives as growth supplements. Among numerous molecules present in FBS, albumin is the most abundant component, which is crucial for transporting fatty acids and the antioxidant activity of serum. Significant efforts are being invested in finding albumin alternatives due to its functional importance. This study explored the potential of phycocyanins from cyanobacteria (C-Phycocyanin) and red macroalgae (R-Phycocyanin) as albumin alternatives in media for cultivating Japanese quail myoblasts. We examined their fatty acid-binding potential using fluorescence spectroscopy, molecular docking, and dynamics. Both C-Phycocyanin and R-Phycocyanin bind stronger unsaturated FAs than saturated ones, while the α -linolenic acid exhibits the highest affinity, with multiple stable binding sites confirmed. Notably, the antioxidant activity of phycocyanins surpassed that of albumin. Cell viability assays and live-dead staining revealed enhanced cell growth in the presence of C-Phycocyanin and R-Phycocyanin. These results suggest that C-Phycocyanin and R-Phycocyanin effectively bind fatty acids and support cell proliferation, highlighting their potential as functional albumin substitutes, an essential prerequisite for the sustainable production of cultured meat.

1. Introduction

Projections suggest that the human population will reach 10 billion by 2050. The global demand for meat is projected to rise by 50% by the same year (van Dijk et al., 2021). Animal agriculture occupies 77% of all agricultural land on Earth, yet it supplies only 17% of humanity's food supply (Rauw et al., 2023). This inefficiency drives the need for agricultural expansion, the single most significant driver of land ecosystem damage. Indeed, animal farming is responsible for approximately 60% of agricultural emissions and between 11% and 19% of global greenhouse gas emissions (Xu et al., 2021). Accordingly, the development of products based on animal-free, alternative proteins is one of the main goals of today, which should reduce the environmental impacts of our

food system, feed more people with fewer resources, avoid contributing to the growing threat of antibiotic resistance, and free up land and ocean ecosystems for biodiversity restoration and recovery. Alternative protein-based products are produced from plants, animal cells (cultivated meat), or microbial fermentation (Liu et al., 2023). While plant-based and fermentation-derived products are currently available to consumers, other options, such as cultivated meat, are still in development, with only a limited number of options currently on the market (Caputo et al., 2024). Cultivated meat, cell-based or lab-grown meat, is produced by culturing animal cells in a lab setting without raising and slaughtering live animals. This approach can revolutionise how we produce meat, making it more sustainable, ethical, and environmentally friendly. Cultured meat has the potential to significantly reduce carbon

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emissions and land use associated with traditional animal agriculture (El Wali et al., 2024). Moreover, it may better recapitulate animal meat than plant-based and fermentation-derived meat products because cultivated meat products are closest to animal meat products in terms of biochemical composition, taste, appearance, and texture (van Dijk et al., 2023).

Conversely, cultured meat remains costly to produce, primarily due to the high cost of cell culture media (Soleymani et al., 2024). One of the significant challenges in the cultivated meat field is the development of serum-free media to ensure sustainability and scalability, as the serum used in traditional media is derived from fetal bovine serum (FBS) (Nikkhah et al., 2023). The use of FBS as a component of cell medium is expensive and raises ethical concerns, which contradict one of the primary benefits of cultivated meat: animal welfare (Soleymani et al., 2024). Therefore, there is an urgent need to replace FBS in cell media used for cultivated meat to achieve low-cost, eco-friendly, and cruelty-free cultivated meat products. FBS represents a high-protein-containing mixture that includes transport proteins (albumin, transferrin, etc.), growth factors, hormones, lipids, antioxidants, and other molecules essential for cell growth (Lee et al., 2022). While recombinant production of growth factors and hormones could satisfy the cultivated meat market in the future, the high demand for albumin for the cultured meat industry (tens of millions of kilograms per year) requires non-recombinant albumin alternatives (Schenzle et al., 2025; Swartz, 2023). Moreover, albumin contributes to nearly a third of the cost of growth medium, making its substitution an important prerequisite for cost reduction in the cultured meat industry (Pasitka et al., 2024).

Albumin is a single polypeptide globular protein composed of three homologous α -helical domains (I, II, and III), each containing two subdomains (A and B) (Bujacz, 2012). Its high solubility and abundance in serum (35–50 mg/ml) are responsible for maintaining the colloidal osmotic pressure of blood. Furthermore, albumin serves as a transport molecule of various nutrients, including long-chain fatty acids (FAs) and microelements such as copper and zinc (Merlot et al., 2014). Moreover, the presence of the free thiol group and its high serum concentration confer significant antioxidant activity on albumin (Roche et al., 2008). Therefore, to develop non-recombinant, low-cost, functional substitutes for albumin in meat cultivation, it is necessary to select proteins that can be produced sustainably in high amounts with the ability to bind FAs and microelements and provide substantial antioxidant capacity.

Phycobiliproteins (PBPs) represent photosynthetic, water-soluble proteins in cyanobacteria and red algae with potent antioxidant activity (Minić et al., 2024) and a propensity to bind various microelements such as copper, iron, and zinc (Minić et al., 2016; Simovic et al., 2022; Xing et al., 2024). These properties are ascribed to the presence of tetrapyrrole chromophores, covalently attached to the cysteine residues of PBPs (Minić et al., 2024). The presence of α -helix as the dominant secondary structure in PBPs and their globin fold gives rationale to hypothesise their ability to bind non-polar ligands, including FAs (Chintapalli et al., 2016). Phycocyanins are the most abundant PBPs, consisting of α and β subunits with a high propensity to oligomerise into trimers ($\alpha\beta$)₃ and hexamers ($\alpha\beta$)₆ (Padyana et al., 2001). They can be divided into R-Phycocyanin (R-PC) from red algae and C-Phycocyanin (C-PC) from cyanobacteria (Li et al., 2019). Considering the above-mentioned structural and functional properties of PBPs, as well as the high content of phycocyanins that can go above 10% of the dry biomass of cyanobacteria and/or algae (Minić et al., 2024), they have substantial potential to be positioned as non-recombinant albumin alternatives for the development of sustainable and cost-effective cell media for meat cultivation.

In this study, we aimed to investigate the potential of major cyanobacterial (C-PC) and algal (R-PC) PBPs as replacements for albumin in cell media for meat cultivation. For this purpose, we utilise two sources of phycocyanins, whose production is well established and has a long tradition in nutrition: the cyanobacterium *Arthrospira platensis* and the

red macroalgae *Porphyra haitanensis*. In the first part of the paper, we probed the two crucial characteristics of phycocyanins, the prerequisites for functional albumin replacement: FA binding potential and antioxidant activity. The fluorescence quenching approach was utilised to determine and compare the binding affinities of saturated and unsaturated FAs to phycocyanins. Molecular docking revealed the most probable location of FA binding sites on phycocyanins, while molecular dynamics demonstrated the stability of phycocyanins-FA complexes. The second part of the study probed the ability of phycocyanins to replace albumin in cell media to cultivate myoblasts from Japanese quail (QM7 cell line). For this purpose, we evaluate cell viability and growth using both one-passage and multi-passage cultivation approaches.

2. Materials and methods

2.1. Materials

Fetal bovine serum of South American origin (cat. no. 10270106) and tryptose phosphate broth (TPB, 18050039) were purchased from Gibco (Grand Island, NY, USA). Medium 199 (M4530), 100x L-glutamine (G7513), 100x penicillin-streptomycin (P07B1), 1 M HEPES buffer solution (H0887), dimethyl sulfoxide (DMSO), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, NR), 7-hydroxy-3H-phenoxazin-3-one-10-oxide (resazurin or Alamar Blue), trypan blue (TB), 0.25% trypsin-EDTA, recombinant human albumin (A9731) and all other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Merck (Darmstadt, Germany). Essential 8 (E8) medium was purchased from STEMCELL Technologies (Vancouver, Canada). Cells were maintained in T-75 and T-25 BioLite™ tissue culture-treated flasks with filter caps from Thermo Fisher Scientific, Inc., Waltham, MA, USA. Cell-based experiments were performed in clear, flat-bottom, 24- or 96-well BioLite™ Plastic sterile labware (Thermo Fisher Scientific, Inc.).

2.2. Purification of C-PC and R-PC

Purification of C-Phycocyanin (C-PC) was from the protein extract of commercial Spirulina powder (Hawaiian Spirulina Pacifica, Nutrex, USA) according to the previously described protocol (Chen, 1999). Extraction of C-PC was done using 20 mM sodium phosphate buffer, pH 7.0; the powder-buffer suspension was mixed for 3 h at room temperature. The resulting suspension was centrifuged at 10,000 x g for 30 min at 4 °C. Proteins in the supernatant were precipitated with ammonium sulfate (65% final concentration). The pellet was dissolved in 20 mM phosphate buffer, pH 7.0, and was dialysed against the same buffer. The dialysed solution was applied to the DEAE-Sepharose Fast Flow column, which had been previously equilibrated with 20 mM phosphate buffer, pH 7.0. Unbound proteins were flushed with an equilibration buffer. Bound proteins were eluted stepwise, increasing the ionic strength of NaCl from 50 to 300 mM. C-Phycocyanin-containing fractions ($A_{618}/A_{280} > 3.5$) were pulled and dialysed against 20 mM phosphate buffer (pH 7.0) in MilliQ water. Dialysed solution was filtered through a 0.2 μ m filter and further used for cell culture experiments and evaluation of antioxidant activity. For the characterisation of PBPs and fatty acid characterisation, traces of other proteins were removed by size exclusion chromatography using a Superdex Increase 200 10/300 GL column, which had been previously equilibrated with 20 mM MES buffer (pH 5.7) containing 150 mM NaCl. The flow rate was kept at 0.5 mL/min. Purified C-PC was stored in 40% (v/v) glycerol solution at –20 °C.

R-phycocyanin (R-PC) was purified from Nori flakes (*Porphyra haitanensis*) as described previously (Velićković et al., 2023). Nori flakes were frozen with liquid nitrogen and then pulverised using a mortar and pestle. The powdered sample was then suspended in 20 mM phosphate buffer, pH 7.0 (1 g of powdered sample per 20 mL of buffer). Extraction of phycobiliproteins (PBPs) was performed for 3 h at room temperature with constant stirring. The resulting slurry was centrifuged at 10,000 x g

for 30 min at 4 °C. Proteins in the supernatant were precipitated with ammonium sulphate (65% final concentration). The pellet was dissolved in 2.5 mM phosphate buffer, pH 7.0, and was dialysed against the same buffer. The dialysed solution was applied to the CHT ceramic hydroxyapatite (HA) column, which had been previously equilibrated with the same buffer. Non-bound proteins (mainly R-PE) were washed away with the equilibration buffer. At the same time, fractions enriched with R-PC were obtained using a stepwise elution protocol, which employed increasing concentrations of sodium phosphate salt (pH 7.0) from 2.5 to 100 mM. R-Phycocyanin fractions were dialysed against 20 mM phosphate buffer (pH 7.0) in MilliQ water. Dialysed solution was filtered through a 0.2 µm filter and further used for cell culture experiments and evaluation of antioxidant activity. For the characterisation of PBPs and fatty acid complexes, traces of other proteins were removed by anion-exchange chromatography using DEAE-Sepharose Fast Flow resin, which had been previously equilibrated with 20 mM phosphate buffer, pH 7.0. Unbound proteins were flushed with an equilibration buffer. Bound proteins were eluted stepwise, increasing the ionic strength of NaCl from 50 to 200 mM. The R-PC fractions were pulled, desalted, and concentrated. Purified R-PC was stored in 40% (v/v) glycerol solution at −20 °C.

2.3. Fluorescence spectroscopy

The binding potential of five FAs, including palmitic, stearic, oleic, linoleic, and α-linolenic acids, to phycocyanins was probed by fluorescence quenching of tetrapyrrole chromophores of R-PC and C-PC.

The binding was characterised using a FluoroMax spectrofluorimeter (HORIBA Scientific, Japan) at 37 °C in phosphate-buffered saline (pH 7.4). C-Phycocyanin (18 nM, final concentration) or R-PC (90 nM) was titrated by the stock solution of FAs (in ethanol) within the concentration range from 0.5 to 30 µM. Since ethanol *per se* induces quenching of C-PC and R-PC fluorescence, the titration of both proteins was also performed with pure ethanol, and the quenching contribution of ethanol was deduced from the quenching of the ethanol solution of FAs. Phycobiliproteins, alone and in the presence of ligands, were excited at 590 nm (specific excitation of phycocyanobilin, PCB), and emission spectra were recorded from 625 to 670 nm, with both excitation and emission slits set at 5 nm. The percentage of chromophore quenching is equivalent to the ratio of bound and total protein, and it was calculated by the following equation:

$$\text{quenching (\%)} = \frac{F_0 - F}{F_0} * 100 = \frac{[\text{Bound}]}{[\text{Total}]} * 100 \quad (1)$$

where F_0 and F represent fluorescence of C-PC (at 645 nm) and R-PC (at 635 nm) without and with the addition of FAs, respectively. The results are expressed as the percentage of quenched chromophore as a function of FA concentration. The dissociation constants (K_d) were determined by fitting the quenching curves with the Hill equation adopted from Weiss (1997):

$$\text{quenching (\%)} = \frac{\frac{[\text{FA}]^n}{K_d}}{1 + \frac{[\text{FA}]^n}{K_d}} \quad (2)$$

where K_d , n , and $[\text{FA}]$ represent the dissociation constant, Hill coefficient, and fatty acid concentration, respectively. Curve fitting was performed using GraphPad Prism 8.0 software. Considering the binding of linoleic and α-linolenic acids results in quenching curves with multiple transitions and defined plateaus, each transition was fitted separately as an independent binding event.

2.4. Computational methods

Crystal structures of C-PC (PDB ID: 1HA7) and R-PC (PDB ID: 1F99) were retrieved from the PDB bank. For the C-PC protein, two hexamer

units were found, and only the first was used in the calculation. For the R-PC protein, the hexamer unit was constructed using the author-prescribed symmetry operations with the Bio3D program (Grant et al., 2006). A cavity search was conducted with the KVFinder program (Guerra et al., 2023).

The protonation state of histidine and other titratable residues was determined on the αβ heterodimer and transferred to other chains, using the H++ program (Gordon et al., 2005). A docking experiment was conducted on hexamer and trimer protein structures. Modified residue in C-PC protein α-chain, N-methyl asparagine (residue name MEN), was transferred to asparagine by deleting the additional methyl group and renaming the residue to Asn. Polar hydrogens and Gasteiger partial charges were added, and protein structures were prepared for docking using the AutoDockTools (version 1.5.7) program (Morris et al., 2009). The geometries of all five fatty acid anions are optimised in extended conformations using the B3LYP/6–311G** method and basis set in Gaussian09 software (Frisch et al., 2013). All double bonds are put in *cis* conformation. Finally, ligands were prepared for docking by adding partial charges and setting all single bonds to rotatable in the AutoDockTools program. Docking was done with the AutoDock Vina program (Trott and Olson, 2010), using the ‘large number of small grid boxes’ method. In short, a grid box of size 30 × 30 × 30 Å (large enough to fit the ligand) was translated by 8 Å in all three dimensions until the entire surface and volume of the protein were covered. For C-PC 2250, boxes were constructed. Then, a docking run was done in each box, with the exhaustiveness value set to 50. This ‘large number of small grid boxes’ method was proven successful when dealing with large proteins (Đurđević Delmas et al., 2025; Šunderić et al., 2024).

Before molecular dynamics simulations, force-field parameters for new residues (PCB and PEB chromophores) and ligands (α-linolenate and stearate) were derived using a standard procedure as prescribed by the Amber developers. While the molecular dynamics study used deprotonated fatty acids (α-linolenate and stearate), for the sake of simplicity in the further text, we will use the terms α-linolenic and stearic acid. For bonds, valence angles, torsion and improper torsion angles, and Lennard-Jones parameters, standard generalized Amber force-field version 2 (gaff2) parameters were used (Wang et al., 2004). Partial atomic charges were determined by first calculating the Mertz-Kollman electrostatic potential at the MP2/def2-TZVP level of theory and then deriving partial charges with the RESP procedure (Bayly et al., 1993).

Molecular dynamics simulations were done on two model systems for each protein. Each model system contains one α- and one β-chain, connected via an α–β interface (Model system 1) or a β–α interface (Model system 2). To each model system, eight ligands were added. Initial ligand positions were determined by docking: the first three ligands were docked structures with the highest binding energies, and the next five were randomly chosen from the other 15 structures with the highest docking score. Each simulation was performed for 400 ns and conducted in triplicate, resulting in a total simulation time of 1.2 ms for each model system. Preparation for molecular dynamics was done with the tleap program from the AmberTools (version 23) suite of programs (Case et al., 2023). The standard Amber ff19sb (Tian et al., 2020) force field was used for “standard” protein residues, and the OPC model (Izadi et al., 2014) for water and ions. The system was neutralised with Na⁺ ions and solvated in a Truncated Octahedron box of water, with at least 10 Å between the box edges and the protein residues. Additional Na⁺ and Cl[−] ions were added to simulate the 0.15 M salt concentration. Simulations were performed using the Amber program (version 24) (Case et al., 2024) under periodic boundary conditions, and the Particle Mesh Ewald summation method was employed for calculating long-range electrostatics. The system was initially minimised for 2000 steps using the Newton-Raphson algorithm, then slowly heated from 100 K to 310 K over 1 ns with a time-step of 1 fs, while keeping the volume constant. The initial restraint on protein and ligand atoms was 100 kcal/mol Å². Next, the system was relaxed for a total of 6 ns with a 1

fs time step in the NPT ensemble by slowly lifting restraints (to 10.0, 1.0, and 0.1 kcal/mol Å²). Finally, 5 ns of equilibration in the NPT ensemble with a 1 fs time step and no restraints was conducted. For a 400 ns production run, the NPT ensemble was chosen with a Langevin thermostat and a Berendsen barostat and a 2 fs time step. The SHAKE algorithm was employed to impose constraints on hydrogen atoms.

Molecular dynamics trajectory analysis and collective variable calculations were done with the cpptraj program (Roe and Cheatham, 2013). Two collective variables were chosen for describing the topology of the ligand binding site: d – distance between the ligand centre of mass and the chain α centre of mass, and Θ – spherical angle between vectors connecting the ligand and chain α centres of mass and the z axis. The Gromacs sham utility (Abraham et al., 2015) was used for free energy landscape (FEL) calculations. FEL images are shown only for ligands that remain in the same binding pocket in all three simulations. Due to insufficient sampling time for non-biased molecular dynamics (1.2 μ s), calculated free energy values are not accurate representations of actual absolute ligand binding free energies and therefore are not displayed in corresponding figures. FEL images are used only for the analysis of ligand binding site topologies described by molecular dynamics simulations. Reported ligand binding free energies are calculated with the Molecular Mechanics/Poisson-Boltzmann Surface Area (MMPBSA) method using the MMPBSA.py program (Miller et al., 2012). Calculated ligand binding free energies were translated into the dissociation constant using the equation:

$$\Delta G = -RT \ln \left(\frac{1}{K_d} \right) \quad (3)$$

The PLIP (protein ligand interaction profiler) program (Salentin et al., 2015) was used for calculating protein-ligand interactions from the MD trajectory. Images are created using the Pymol, VMD, and Discovery Studio Visualizer programs.

2.5. Removal of albumin from FBS

Albumin was chromatographically removed from FBS to test the potential of C-PC and R-PC as a substitute for albumin. The Cibacron Blue (Capto Blue, Cytiva, United States of America) resin was used, which selectively binds albumin, while other FBS components pass through as a *flow-through* (unbound or de-albuminised fraction). The column was equilibrated with 10 mM citrate buffer, pH 5.5, with 150 mM NaCl, at standard pressure and a constant flow rate of 200 μ L/min in a 20 mL column. After equilibration of the column, 5 mL of FBS was applied (the pH of the FBS had been previously adjusted to 5.5). Once the entire sample volume was applied to the matrix, the flow was stopped, and the column was incubated for 10 min. After incubation, the flow was continued, and fractions were collected at the same flow rate. The presence of proteins was proven by a spot test with the Bradford reagent. After no further positive reaction to the proteins, albumin from the column was eluted with 20 mM phosphate buffer with 2 M NaCl. All albuminous and unbound (*flow-through*) fractions that showed a positive spot test were separated and combined. The purity of unbound fractions was tested by protein resolving via sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 4% concentrating gel and 12% separating gel, CBB staining; Fig. S1).

2.6. Cultivation of QM7 cell line

QM7 cells were maintained in Medium 199 formulated with Earle's balanced salts supplemented with 10% (v/v) FBS, 10% (v/v) TPB, and 1% penicillin-streptomycin (v/v) at 37 °C in a humidified atmosphere with 5% CO₂. QM7 cells were used between passages 15 and 25 (Sibinčić et al., 2024).

2.7. Neutral red assay

The neutral red uptake (NRU) assay was performed according to the protocol described by Repetto et al. (2008). QM7 cells were seeded at 10,000 cells per well in media containing 10% FBS and left overnight to allow cell attachment. The next day, the old media was exchanged for 200 μ L of media containing either dealbuminized FBS (DA FBS) at a protein concentration of 0.1 mg/mL, DA FBS with C-PC, R-PC, or recombinant albumin at a concentration of 0.2 mg/mL or 0.5% FBS (FBS-positive control). After 72 h of incubation, the media were discarded, and 100 μ L of 5 μ g/mL neutral red (NR) in medium without serum was added to each well. The cells were then incubated with NR at 37 °C for an additional 4 h. After incubation, the cells were spun at 500 $\times$ g for 10 min. The cells were gently washed with 150 μ L of phosphate-buffered saline (PBS) and spun again. After discarding the PBS, the NR dye was extracted by adding 150 μ L of 50% (v/v) ethanol with 1% (v/v) glacial acetic acid to each well. The microplate was incubated on a plate shaker (200 RPM) for at least 10 min or until the NR had been extracted from the cells and formed a homogeneous solution. The solution with the extracted dye was transferred into fluorescence-compatible plates (Sarstedt, Nümbrecht, Germany), where the NR fluorescence was measured using a BioTek multimode plate reader (Agilent Technologies Inc, Santa Clara, CA, USA) with the excitation/emission filter configured at 530/25 nm for excitation and 590/35 nm for emission. The viability of the treated cells was determined by normalising the fluorescence of the samples to that of the untreated cells (DA FBS).

The same assay was performed under serum-free conditions in the chemically defined E8 medium. Briefly, QM7 cells were seeded at 10,000 cells per well in media containing 10% FBS and left overnight to allow cell attachment. The next day, the old media was exchanged for 200 μ L of E8 media in the absence and the presence of C-PC, R-PC, or recombinant albumin at a concentration of 0.2 mg/mL. After 48 h of incubation, the assay was performed as described above. The viability of the treated cells was determined by normalising the fluorescence of the samples to that of the untreated cells (containing only E8 media).

2.8. Resazurin (alamar blue) assay

The resazurin assay was performed according to the method described by Kumar et al. (2018). The assay was conducted on QM7 cells at the same seeding density, FBS, and protein concentration as described for the Neutral red assay. After 72 h, cells were spun for 10 min at 500 $\times$ g, and the old medium was replaced with 100 μ L of 0.15 mg/mL resazurin in a medium without FBS. The plate was incubated for approximately 3 h at 37 °C in a cell culture incubator (until the solution changed from blue to pink). Seventy μ L of solution from each well was transferred to a fluorescence-compatible 96-well plate, and the fluorescence was recorded and evaluated as described for the Neutral red assay.

The same assay was performed under serum-free conditions in the chemically defined E8 medium in the absence and the presence of C-PC, R-PC, or recombinant albumin (0.2 mg/mL each) after 48 h of incubation.

2.9. Trypan blue exclusion assay

QM7 cells were seeded in 24-well plates at a density of 30,000 cells per well in 500 μ L of 10% supplemented media. The next day, the media was replaced with fresh media supplemented with either DA FBS (at a protein concentration of 0.1 mg/mL) or DA FBS with C-PC, R-PC, or recombinant albumin at a concentration of 0.2 mg/mL or 0.5% FBS. The cells were incubated for 72 h, with morphology monitored daily (at 24, 48, and 72 h). On the third day, the TB exclusion assay was performed. The cells were spun at 500 $\times$ g for 10 min, culture media were discarded, and the cells were detached with 100 μ L of 0.25% trypsin-EDTA. The enzyme was inactivated with 400 μ L of Medium 199 containing 10%

FBS, and the cells were spun at 500 x g for 10 min. Finally, the cells were resuspended in 100 μ L of Medium 199 in the absence of FBS and mixed in a 1:1 (v/v) ratio with 0.4% (w/v) TB solution in PBS. The cells were counted in all fields of the Fast Read 102 microscope slides (Heinz Herenz Medizinallbedarf GmbH, Hamburg, Germany) under a light microscope.

The effects of phycocyanins on the QM7 cells' proliferation were also probed in the chemically defined E8 medium. QM7 cells were seeded in 24-well plates at a density of 40,000 cells per well in 500 μ L of 10% supplemented media. The next day, the media was replaced with E8 media in the absence and the presence of C-PC, R-PC, or recombinant albumin at a concentration of 0.2 mg/mL. The cells were incubated for 48 h, with morphology monitored daily (at 24 and 48 h). After 48 h, the TB exclusion assay was performed as described above.

2.10. Multi-passage cultivation of cells in low-serum media supplemented with C-PC or R-PC

At the beginning of the experiment, the QM7 cells were seeded in 24-well plates at 40,000 cells per well in 500 μ L media containing 10% FBS and left overnight to allow cell attachment. The next day, the media was replaced with 500 μ L of fresh media containing 1% FBS, C-PC, R-PC, and recombinant albumin at a concentration of 0.2 mg/mL. Control cells in 1% FBS were also included. Considering the total protein concentration in 100% FBS is around 40 mg/mL, the protein concentration of 0.2 mg/mL corresponds to 0.5% FBS. In this regard, we introduced an additional control containing 1.5% FBS without phycocyanins or recombinant albumin. Moreover, the third control, which comprised QM7 cells cultivation in media containing 10% FBS, was also included. Cell counting was conducted for the three consecutive passages, and the period between each passage was kept at 48 h. The passaging and counting of the cells were done with TB, as described in the previous section. After each passage, fresh, low-serum media (1% FBS) were added, both in the absence and presence of phycocyanins or recombinant albumin, and the cells were reseeded in 24-well plates at the same density. The number of treated cells was normalised to that of the control (1% FBS).

2.11. Reducing power assay

A reducing power assay was performed as previously described (Minic et al., 2016). In short, 100 μ L (0.2 mg/mL of C-PC, R-PC, or recombinant albumin) of the sample was pipetted into a mixture of 100 μ L of 200 mM phosphate buffer, pH 6.6, and 100 μ L of a 1% solution of potassium ferricyanide dissolved in MilliQ water. The same procedure was repeated with 100 μ L of 20 μ M Trolox instead of the sample. Following the addition of Trolox or the sample, the mixture was incubated at 50 °C for 20 min. Then, 62.5 μ L of 20% trichloroacetic acid solution is added. After that, 200 μ L of this described mixture is mixed with 200 μ L of MilliQ water and 24 μ L of a 0.1% FeCl₃ solution dissolved in MilliQ water. 200 μ L of the final mixture was pipetted into a 96-well plate, and after 10 min, the absorbance at 700 nm was measured.

2.12. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

ABTS radical scavenging assay was performed as previously described with minor modifications (Re et al., 1999). ABTS was dissolved in water to a concentration of 7 mM. ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS stock solution with 2.5 mM potassium persulfate (final concentration) and allowing the stock solution to stand in the dark at room temperature overnight. The working solution was made by diluting the stock solution (20 mM phosphate buffer, pH 7.4) to achieve an absorbance of 0.9 at 734 nm. The working solution (750 μ L) was mixed with 250 μ L of the sample (C-PC, R-PC, or recombinant albumin at a concentration of 0.2 mg/mL) and allowed to react for 30 min. The obtained mixture was pipetted into a 96-well plate,

and the absorbance at 734 nm was measured.

2.13. Statistical analysis

Student *t*-test was used to compare the albumin-replacement potential of C-PC and R-PC in terms of their antioxidant potential and the viability of QM7 cells with the corresponding controls. Each cell-based experiment was performed in three independent experiments, and each experiment was performed at least in triplicate. The GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA) was used to create the figures.

3. Results

3.1. In vitro binding of fatty acids to C-phycocyanin and R-phycocyanin

Phycobiliproteins exhibit strong fluorescence in the visible range, which is a valuable property for probing the binding of various ligands. The addition of saturated FAs induces modest quenching of the phycocyanin chromophores (Fig. 1A and B), with the quenching efficiency at the highest concentration ranging from 10 to 15%. The presence of saturated FAs due to low quenching efficiency gives noisy binding (quenching) curves, with stearic acid exhibiting stronger binding than palmitic acid (Figs. 1A, B, S2, and S3).

On the other hand, unsaturated FAs exhibit more pronounced quenching, resulting in binding curves with two or more transitions (Fig. 1C, D, S4 and S5), featuring local plateaus, which suggests the presence of several non-equivalent binding sites on C-PC and R-PC. Quenching of both phycocyanins by oleic acids results in a steep decrease in fluorescence intensity (Fig. 1C and D), reaching saturation at a ligand concentration of ~10 μ M. Meanwhile, polyunsaturated FAs (linoleic and α -linolenic) induce the discrete quenching at lower ligand concentrations (up to 10%), followed by substantial quenching of C-PC and R-PC at higher concentrations (up to 70%). Therefore, it seems that saturation in FA structures significantly influences the mode of FAs binding to phycocyanins and, consequently, the shape of quenching (binding) curves.

Based on the shape of quenching curves (presence of one or more plateaus) and Eqs. (1) and (2), we determined the binding affinities (dissociation constants) for one or more non-equivalent binding sites of FAs to phycocyanins. The highest binding affinities are determined for α -linolenic acid to both proteins. Interestingly, the quenching of C-PC and R-PC by α -linolenic acid also demonstrated the highest number of non-equivalent binding sites. This result showed the most significant potential of C-PC and R-PC to accommodate α -linolenic acids within their different binding sites. In general, all FAs, except palmitic acid, demonstrate relatively high affinity, with K_d below 10 μ M (Table 1). Furthermore, there is no substantial difference in the binding affinities of FAs between C-PC and R-PC, which could be ascribed to their high structural similarity.

3.2. Detection of fatty acid binding sites on phycocyanins by molecular docking and dynamics

Phycocyanins are large hexameric proteins composed of two doughnut-shaped trimeric units stacked on top of each other. Each of the trimeric units is composed of three $\alpha\beta$ heterodimers arranged to form a closed circular structure. One tetrapyrrole chromophore is covalently bound to the Cys residue of the α -chain via a thioether bond, while the β -chain binds two chromophores in the same manner. While C-PC solely binds one type of chromophore (phycocyanobilin), two different types of tetrapyrroles (phycocyanobilin and phycoerythrobilin) are attached to R-PC. The α -chain of R-PC binds only the phycocyanobilin chromophore, while the β -subunit binds both chromophores.

We first conducted a molecular docking study on the hexameric structure of the C-PC and R-PC. The highest energy binding site for all

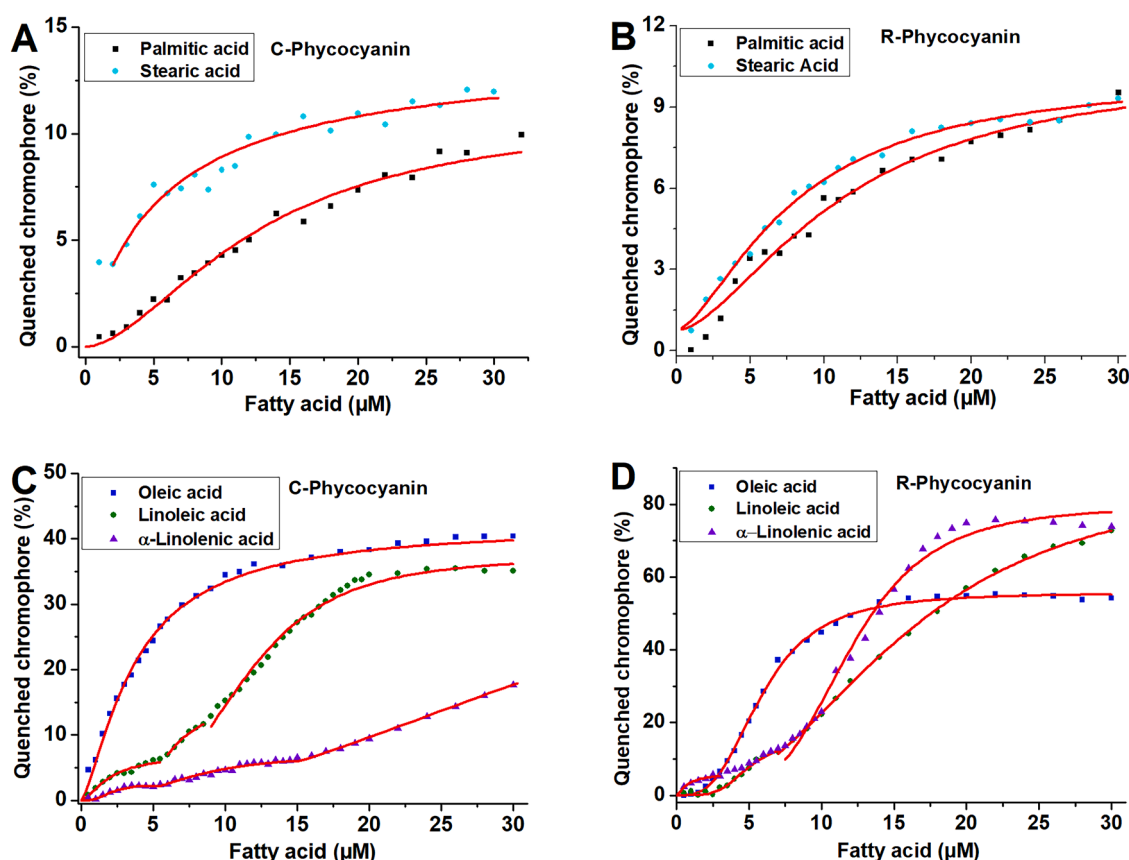


Fig. 1. Binding of fatty acids to C-phycoerythrin (C-PC) and R-phycoerythrin (R-PC) obtained by fluorescence quenching of phycoerythrin chromophore (excitation at 590 nm, emission at 635 and 645 nm for R-PC and C-PC, respectively). **Figures A and B** demonstrate the binding of saturated FA to C-PC and R-PC, respectively. **Figures C and D** represent the binding of unsaturated FA to C-PC and R-PC, respectively. The fitting curves (full red lines) are obtained using the Hill equation.

Table 1

Dissociation constants (K_d) of fatty acids binding to C-Phycocyanin and R-Phycocyanin obtained by fluorescence quenching of phycoerythrin chromophore at 37 °C and pH 7.4.

	C-Phycocyanin			R-Phycocyanin		
	K_d (μM)					
Palmitic acid	12.6			11.3		
Stearic acid	5.6			8.0		
Oleic acid	3.7			5.9		
	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}
Linoleic acid	2.0	6.1	11.3	4.9	15.8	None
α-Linolenic acid	1.9	7.4	42.7	0.7	14.9	None

investigated FAs on both proteins was located in the large cavity of the hexameric unit (Tables S1 and S2), situated between two α -chains of different trimeric units, far away from the tetrapyrrole chromophore binding sites, with a distance of around 1 nm. There are three such cavities per hexameric unit (Figs. 2A and S6). The docking experiment produced hits for all investigated FAs in all three cavities. The best-found docking pose and 2D interaction diagrams for every investigated FA in the inter-trimeric cavity are shown in Figs. 2B, S7 and S8. In this cavity, two different orientations of FAs are found. The carboxylic head of the FAs forms a hydrogen bond with Ser127 of the α -chain, but due to the symmetry of the cavity, both orientations have similar binding energy. Due to the presence of double bonds, unsaturated FAs have different orientations (Figs. 2B, S7, and S8) and the highest docking energy compared to saturated ones (Table 2). All FAs' binding scores are slightly higher for C-PC than for R-PC. No other high-energy binding sites were found in the hexamer structure of both phycoerythrin.

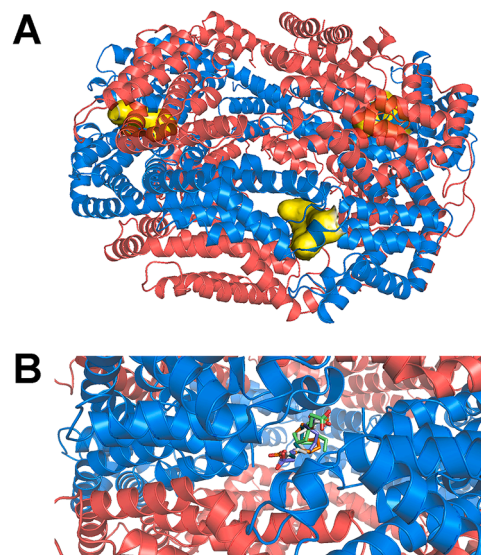


Fig. 2. (A) Three large cavities (yellow surfaces) were found between two trimeric units in the C-Phycocyanin hexamer; (B) All five investigated fatty acids (sticks) docked in the large cavity of the hexameric unit located between two α -chains of different trimeric units. Colour code: α -chain – blue; β -chain – red; α -linolenic – grey; linoleic – purple; oleic – orange; stearic – green; palmitic – blue.

To probe the ability of FAs to bind to C-PC and R-PC in the vicinity of tetrapyrrole chromophores, we further perform docking analysis on the

Table 2

Binding energies (kcal/mol), calculated by docking study, in hexameric and trimeric structures of C-PC and R-PC.

Binding site	Fatty acid				
	α -Linolenic	Linoleic	Oleic	Stearic	Palmitic
C-Phycocyanin					
Hexamer structure					
Cavity between two trimers	−7.3	−7.2	−6.8	−6.6	−6.3
Trimer structure					
β chain helices H β 1 and H β 2	−5.6	−5.3	−5.2	−4.9	−4.9
β – α interface	−5.5	−5.2	−5.1	−4.7	−4.5
α – β interface	−4.9	−4.7	−4.7	−4.6	−4.6
α chain helices H α 1, H α 2, H α 10	−5.2	−4.8	−4.8	−4.6	−4.5
R-Phycocyanin					
Hexamer structure					
Cavity between two trimers	−7.1	−6.8	−6.6	−6.4	−6.2
Trimer structure					
β – α interface (BS1)	−5.4	−5.2	−5.2	−4.7	−4.8
β – α interface (BS2)	−4.9	−5.0	−4.7	−4.3	−4.3
α -chain helices H α 1, H α 2, H α 10	−4.9	−4.8	−4.7	−4.6	−4.6

trimeric structure. Since the trimeric structure is highly compact, cavity search only found small cavities, with the largest one located on the surface of the β – α interface (Tables S1 and S2; Figs. S9 and S10). A detailed analysis of the docking results revealed four distinct binding sites for the C-PC (Fig. S11) and three distinct binding sites for the R-PC (Fig. S13), respectively. These binding sites are located at the protein surface with relatively low binding energies between −5.6 and −4.3 kcal/mol (Table 2). Unsaturated FAs have higher docking scores than saturated ones, while all FAs are bound more firmly to C-PC than R-PC (Table 2). In general, all FA binding sites found in the trimer structure are located at the interface between α and β subunits or interacting with the secondary structure elements involved in binding between subunits (Figs. S11–S13). Furthermore, all binding sites are close to the surface of the proteins, and some are located near the tetrapyrrole chromophores (~0.5 nm distance).

Since the docking study on the trimeric structure yielded only lower energy binding sites on protein surfaces near chain interfaces, molecular dynamics simulations were employed on different model systems to verify whether ligands would remain stable under dynamic conditions. For this purpose, we probed the binding of α -linolenic and stearic acids, the two ligands with the highest affinities among unsaturated and saturated FAs, respectively. Considering the large size of the trimeric unit (($\alpha\beta$)₃), the molecular dynamics was performed on $\alpha\beta$ monomers. In this regard, two model systems were constructed: model system 1 for binding sites at the α – β interface ($\alpha_1\beta_1$) and model system 2 for binding sites at the β – α interface ($\beta_3\alpha_1$). Model System 2 contains a substantially smaller subunit interface compared to Model System 1. Initial positions of the ligands were determined by molecular docking; the 8 best, nonoverlapping docking poses were selected for α -linolenic and stearic acids. The main criteria for evaluating the binding site were ligand binding energy (calculated using the MMPBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) method), the number of interactions between the ligand and protein, and the ligand's tendency to dissociate into the solution.

Molecular dynamics simulations revealed that the highest affinity binding site in Model System 1 is located in the β -chain, between the H β 1 and H β 2 helices (Figs. S14–S17, S25–S29, and S36–S40). Molecular docking calculations also identify this binding site as the highest energy binding site for the C-PC trimer. Molecular dynamics simulations confirm docking results that α -linolenic acid will have a higher binding energy in this binding site than stearic acid. This binding site in R-PC has significantly lower binding energies (Table 3). Hence, the highest

Table 3

Ligand binding energies obtained from molecular dynamics simulations and expressed in kcal/mol. The values in parentheses represent the K_d values calculated from the ligand binding energies (Eq. (3)).

Model System 1				
Protein	C-Phycocyanin		R-Phycocyanin	
Fatty acid	α -linolenic	Stearic	α -linolenic	Stearic
Between H β 1 and H β 2 helices	−5.12 (243 μ M)	−4.61 (557 μ M)	−3.23 (5.3 mM)	−1.90 (45.6 mM)
Between H α 4 and H α 6 helices	None	−2.21 (27.6 mM)	−1.17 (149.3 mM)	−3.50 (3.4 mM)
Between H α 1 and H α 2 helices	None	None	None	−2.69 (12.6 mM)
Model system 2				
Protein	C-Phycocyanin		R-Phycocyanin	
Fatty acid	α -linolenic	Stearic	α -linolenic	Stearic
β – α interface and helix H α 6	−7.24 (8 μ M)	−6.18 (43 μ M)	−7.51 (5 μ M)	−6.79 (16 μ M)
β – α interface and helix H β 6	−3.77 (2.2 mM)	−4.53 (635 μ M)	−5.68 (98 μ M)	None
Between H β 3 and H β 6 helices	−2.17 (29.4 mM)	−4.08 (1.3 mM)	None	None
Between H β 4 and H β 9 helices	None	None	−1.34 (113.3 mM)	None

binding energy in model system 1 is observed for C-PC: α -linolenic acid (5.12 kcal/mol); however, this binding energy yields a dissociation constant of ~250 μ M, a value that we could not detect using fluorescence quenching studies (Table 1).

The binding site with the highest binding energy for all investigated proteins and FAs is located at the β – α interface (Model system 2; Fig. 3A). This binding site is characterised by hydrophilic head interacting via salt bridges with arginine-rich region at the interface between α - and β -chain (Fig. 3A and B). Also, this region is rich in Tyrosine residues, that can form hydrogen bonds with ligand head and/or hydrophobic interactions with ligand tail, depending on the ligand orientation (Figs. 3B, S18–S21, S30–S33, S41–S47). The proposed binding site of C-PC and R-PC also contains the Cys82 residue in the β -chain, covalently bound to a phycocyanobilin chromophore. The free energy landscape image (Fig. 3C) shows one extensive minimum with different ligand orientations in the binding site, characterised by significant variation in the value of spherical angle theta. Most of the time, the hydrophobic tail of the FA is oriented towards α -chain helix H α 6, which stabilises the ligand binding with hydrophobic interactions. The hydrophilic head remains locked in the arginine-rich region of the β – α interface (Figs. 3A, S20, S32, S43, and S46). Based on MMPBSA interaction energy calculations, this binding site has a higher affinity for binding α -linolenic acid than stearic acid by 0.7 to 1.0 kcal/mol (Table 3). In comparison, both FAs are strongly bound to R-PC than C-PC by 0.3 to 0.6 kcal/mol (Table 3). When free energies calculated by MMPBSA are translated to the dissociation constant, the obtained values range from 5 to 43 μ M, which reasonably agrees with the dissociation constant obtained by the fluorescence quenching study (Table 1). The binding of FAs to other sites in model 2 yields less stable complexes, prone to dissociation, with substantially lower binding energies (Table 3 and Figs. S23, S24, S34, and S35).

Other binding sites found by molecular dynamics have low binding energy and high ligand mobility. Typically, the ligand's hydrophilic head is oriented towards the solution, interacting with water molecules and sodium ions and promoting the dissociation of the entire ligand into the solution. A detailed description of the molecular dynamics results is provided in the Supplementary material.

3.3. Antioxidant activities of C-phycocyanin and R-phycocyanin

Besides FAs binding, albumin's important physiological role is its significant antioxidant activity. We compared the antioxidant activity of

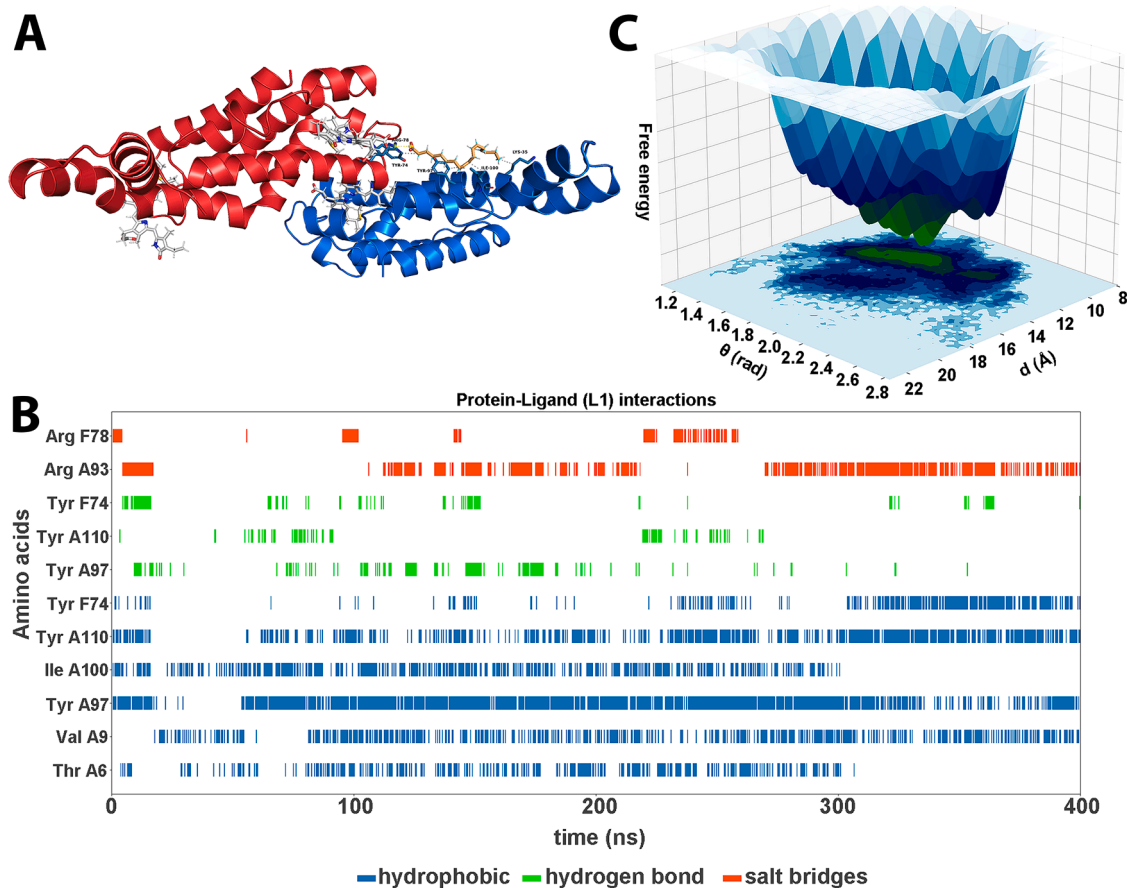


Fig. 3. (A) 3D interaction diagram, the most prominent protein-ligand interactions (B) and free energy landscape profile (C) for α -linolenic acid (L1) binding to C-Phycocyanin Model system 2. Colour code: α -chain – blue; β -chain – red; α -linolenic – orange. Tetrapyrrole chromophores are shown in grey.

C-PC and R-PC with albumin at the concentrations used for cell-based assays (0.2 mg/mL).

In the reducing power assay, antioxidants reduce the Fe^{3+} -ferricyanide complex to Fe^{2+} ions, and the increased absorbance of the reaction mixture is directly proportional to the reducing power capacity. C-Phycocyanin and R-PC exhibit substantially higher reducing power (up to 4.5 times) than albumin (Fig. S48A). Among all protein amino acid residues, only cysteine (Cys) exhibits a substantial reducing power capacity (Zheng et al., 2016b). C-Phycocyanin, R-PC, and albumin contain 3, 2, and 1 free cysteine residues, respectively. In terms of this, the substantially stronger reducing power of phycocyanins than albumin could not only be ascribed to the higher number of Cys residues but also due to the presence of tetrapyrrole chromophores, the major contributor of reducing power in phycocyanin (Minic et al., 2016). In the ABTS scavenging assay, C-PC and R-PC also exhibit significantly stronger activities (up to 2.5 times) than albumin (Fig. S48B). The higher relative antioxidant activity of albumin in the ABTS assay compared to the reducing power test could be ascribed to the fact that, besides the Cys residue, Tyr and Trp residues significantly scavenge ABTS cation radical (Zheng et al., 2016a).

3.4. Validation of albumin replacement potential of C-phycocyanin and R-phycocyanin in QM7 cell culture

The potential of C-PC and R-PC to replace albumin in the QM7 cell culturing medium was validated using several complementary assays. The NRU assay quantifies the ability of viable cells to uptake the survival NR dye within their lysosomes. Supplementation of culture medium with de-albuminised serum containing 0.1 mg/mL total protein, DA FBS, with C-PC and R-PC substantially increased QM7 cell

viability in NRU assays when compared to the sole DA FBS (Fig. 4A). The addition of albumin also increased cell viability, but not as efficiently as C-PC or R-PC (Fig. 4A). QM7 cell viability was also assessed by resazurin assay, where the metabolically active living cells reduce the nonfluorescent dye resazurin to the strongly fluorescent dye resorufin. Both resazurin and NRU are complementary functional assays, as they measure different parameters (cellular redox potential vs. lysosomal integrity) and, when combined, can provide a more realistic outcome (Sibinčić et al., 2024). Similar to the NRU assay, the resazurin assay revealed that both C-PC and R-PC supplemented cells showed higher cell viability compared to DA FBS (Fig. 4B). However, the viability of the cells in the presence of 0.5% FBS was significantly higher than that of C-PC and albumin. Out of all three proteins, R-PC excelled in restoring DA FBS potential to support QM7 cell growth and proliferation, as monitored in both the NRU and Resazurin assays (Fig. 4B). While the previous two assays provide information on cell health and metabolic activity, the TB exclusion staining provides a direct count of live cells. This assay confirmed a significant increase in cell proliferation upon the addition of both phycocyanins compared to the control (DA FBS; Fig. 4C). Moreover, the number of live QM7 cells in the presence of C-PC and R-PC approaches the cell count in the presence of 0.5% complete FBS (Fig. 4C), supporting the potential of phycocyanins as albumin-like supplements. We also monitored the morphology of the QM7 cells before and after incubation (72 h) with different media compositions. The obtained micrographs supported the albumin-like biological activity of C-PC and R-PC under the experimental conditions employed; the QM7 cells confluency in the presence of both phycocyanins was higher compared to DA FBS, while it was comparable with 0.5% FBS and albumin (Fig. 4D).

While the concomitant presence of DA FBS in a concentration of 0.1

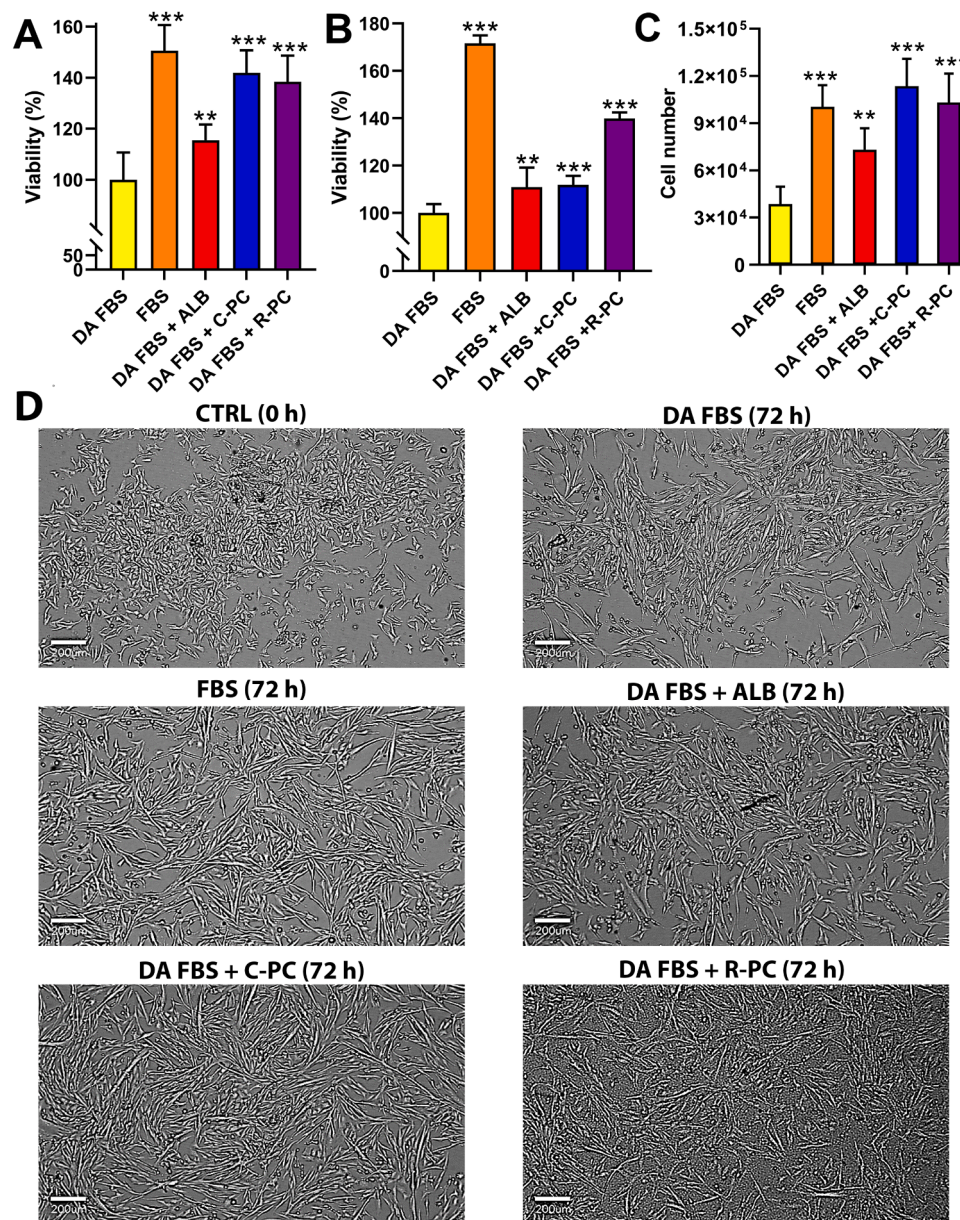


Fig. 4. The effects of C-Phycocyanin (C-PC) and R-Phycocyanin (R-PC) on the QM7 cell viability and proliferation – Viability assays: neutral red (A) and resazurin (B); (C) trypan blue exclusion assay. DA FBS (0.1 mg/mL protein content) and FBS (0.5%) denote de-albuminised and complete fetal bovine serum, respectively. Concentrations of albumin (ALB), C-PC, and R-PC were kept at 0.2 mg/mL. Statistically significant differences between the control (DA FBS) and other groups are labelled with ** $p \leq 0.01$ and *** $p \leq 0.001$; (D) Morphology of QM7 cells before (0 h; CTRL) and after (72 h) incubation with different media compositions.

mg/mL of total proteins and phycocyanins or albumin induces cell proliferation in the previous assays, propagation of QM7 cells via multiple passages could not be achieved. The removal of albumin from FBS by the Cibacron Blue matrix could also be accompanied by the binding of other components from serum, which are required for the maintenance of QM7 cells (attachment, growth, and proliferation via multiple passages). Considering this, we probed the effects of C-PC, R-PC, and recombinant albumin on QM7 cells in the presence of 1% FBS. For this purpose, we used the following controls: 1% FBS and 1.5% FBS. We also include the third control with 10% FBS in the cell culture medium. The difference in protein concentration between these two serum samples corresponds to the concentration of C-PC, R-PC, and albumin used in this experiment (0.2 mg/mL). QM7 cells tolerated 1% FBS conditions and achieved good morphology and high confluency after three passages (Fig. S49). The addition of C-PC and R-PC in all three passages substantially enhances the cell proliferation when compared to 1% FBS,

based on the TB exclusion cell counting (Fig. 5). The QM7 cell number in the presence of 1% FBS with C-PC or R-PC approaches the cell numbers achieved in the presence of 1.5% FBS in the first passage and even slightly exceeds the extent of cell proliferation in the third passage (Fig. 5). On the other hand, the supplementation with recombinant albumin boosts the QM7 cell growth in only the first two passages (Fig. 5). Furthermore, both C-PC and R-PC induce slightly higher proliferation of QM7 cells than recombinant albumin in the first and second passages. In comparison, a substantially higher proliferation was observed in the third passage (Fig. 5). In all samples, the cell proliferation decreases as the passage number increases, while the medium containing 10% FBS demonstrated the highest resistance to cell number decline (Fig. 5).

In addition, we performed experiments under fully serum-free conditions using Essential 8 (E8), a chemically defined medium. In these experiments, both phycocyanins and albumin significantly enhanced the viability and growth of QM7 cells in a single-passage setup (Fig. S50),

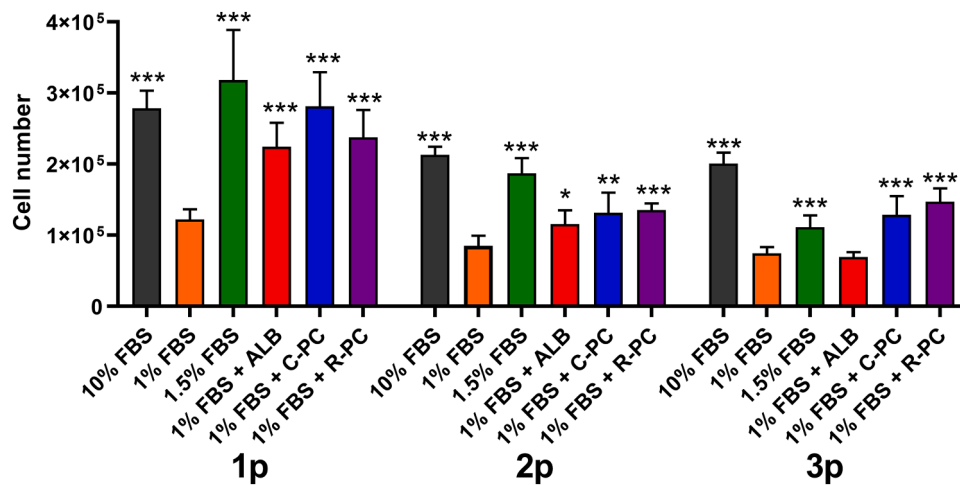


Fig. 5. The effects of phycocyanins on QM7 cell proliferation across 3 passages (1p, 2p, or 3p) were monitored by trypan blue exclusion staining. The concentrations of albumin (ALB), C-Phycocyanin (C-PC), and R-Phycocyanin (R-PC) were kept at 0.2 mg/mL. Statistically significant differences between the control (1% fetal bovine serum, FBS) and other groups are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

indicating a short-term supportive effect under serum-free conditions. However, in multi-passage experiments under serum-free conditions using E8 medium, we did not obtain reproducible results for either albumin or phycocyanins (data not shown). Therefore, we cannot conclusively claim sustained enhancement of cell proliferation across multiple passages. One possible explanation is that, in the single-passage experiment, cells are transitioned from 10% FBS to E8 medium, which likely induces a stress response; under such conditions, both albumin and phycocyanins may exert protective effects. In contrast, during multi-passage cultivation, once the cells adapt to the E8 medium, the protective effect of albumin and phycocyanins becomes less pronounced. Rather, our findings suggest that phycocyanins may provide albumin-like supportive functions during the initial phase of serum-free culture. Further optimisation of the culture medium and systematic multi-passage studies will be required to determine whether they can support long-term serum-free cultivation.

4. Discussion

This study demonstrated the potential of major PBPs, C-PC and R-PC, to replace albumin, the most abundant protein component in cell media, for sustainable meat cultivation. Both phycocyanins bind FAs with medium to high affinity, indicating the presence of at least several binding sites, as revealed by fluorescence spectroscopy, molecular docking, and dynamics studies. The antioxidant activity of C-PC and R-PC is substantially greater than that of albumin. Cell-based assays demonstrated the ability of C-PC and R-PC to promote the growth of the quail muscle cell line (QM7) with a higher potential comparable to recombinant albumin.

One of the critical functions of serum albumin is the ability to bind and transport various FAs and other nutrients required for normal cell growth. C-PC and R-PC bind saturated (palmitic and stearic) and unsaturated (oleic, linoleic, and α -linolenic) FAs with dissociation constants from the high to the low micromolar range as demonstrated by fluorescence spectroscopy. Moreover, similar to serum albumin, both phycocyanins exhibit the potential to interact with FAs at different binding sites with different affinities. Moderate to high-affinity binding was detected for each FA. Additionally, fluorescence spectroscopy revealed that polyunsaturated FAs can bind to secondary binding sites on phycocyanins with medium to low affinity. Although the fluorescence quenching study did not prove the existence of several non-equivalent binding sites for saturated FAs and oleic acid, this possibility cannot be excluded. In this context, fluorescence spectroscopy cannot distinguish between non-equivalent binding sites if their affinities do not

differ significantly. Furthermore, low-affinity binding sites (K_d in the millimolar range) could not be detected because the poor solubility of FAs disables the incubation of phycocyanins with high concentrations of FAs. Indeed, molecular docking and dynamics demonstrated the propensity of either saturated or unsaturated FAs to interact with C-PC and R-PC at several binding sites.

High-affinity binding (low micromolar or sub-micromolar K_d values) of FAs influenced the low extent of quenching of the fluorescence of PCB chromophores, suggesting FAs bind to phycocyanins at a distant site compared to the PCB binding pocket. Indeed, the molecular docking approach revealed the ability of phycocyanins to bind FAs with the highest binding scores at the trimmer interfaces of hexamers, far away from the chromophore binding site (around 1 nm). Upon saturation of high-affinity binding sites, a further increase in FA concentration induces a substantial decrease in phycocyanin fluorescence, indicating binding close to the PCB chromophore with lower affinity. Both molecular docking and dynamics revealed the ability of FAs to bind with a low energy score in proximity to the PCB chromophore (around 0.5 nm distance) at the interface between α and β subunits. Moreover, molecular dynamics simulations demonstrated that the binding of FAs near PCB chromophores yields stable phycocyanin: FAs complexes, resistant to dissociation over a 400 ns timescale, which could explain the substantial quenching of PCB fluorescence at higher FAs concentrations. Interestingly, molecular dynamics (through the application of the free energy simulation method) delivered the binding constant for the FAs in proximity to the PCB chromophore, which is in the same order of magnitude as the lower affinity constants (K_d in the range 5–43 μ M) obtained by fluorescence quenching. Therefore, the binding constants obtained by molecular dynamics correspond to the secondary and tertiary binding sites identified by the fluorescence quenching approach.

Both molecular simulation and *in vitro* studies demonstrated similar affinities and binding sites between C-PC and R-PC. This could be ascribed to the high similarity in their 3D structures (Jiang et al., 2001; Padyana et al., 2001). However, some differences in the number of binding sites between C-PC and R-PC were observed by both approaches, with more binding sites identified for C-PC. The lower number of identified sites for polyunsaturated FAs could also be ascribed to the much higher extent of fluorescence quenching in R-PC than C-PC, which could mask the identification of low-quenching transitions in fluorescence quenching curves, corresponding to the non-equivalent binding sites of FAs on proteins.

In vitro studies revealed that the affinities of FAs to bovine serum albumin were one order of magnitude higher than those of phycocyanins (Spector et al., 1969). These results are expected, as the physiological

role of albumins is to bind lipophilic compounds. Identifying albumin substitutes in nature with simultaneous high production yields and albumin-like FA binding affinities is challenging. However, while FAs binding constants to C-PC and R-PC are not as high as for albumin, both phycocyanins exhibit higher affinities to essential, polyunsaturated FAs than saturated FAs. On the other hand, albumin binds saturated FAs with similar affinity or even more strongly than unsaturated ones (Spector, 1975; Spector et al., 1969). Therefore, the higher preferences of C-PC and R-PC for linoleic and α -linolenic acids could give them a comparative advantage in the more efficient delivery of essential FAs, strengthening the nutritional profile of cultured meat products. Considering the affinity differences between these two classes of proteins, careful dosage and selection of appropriate fatty acids are required to make cell media without albumin to achieve optimal growth conditions for QM7 or any other cells used for meat cultivation. The exact molecular mechanisms of phycocyanin-bound fatty acid uptake by these cells remain to be explored in the future. This would enable detailed tailoring of optimal growth media compositions used in meat cultivation. Interestingly, the presence of C-PC and R-PC more efficiently increases the viability and growth of QM7 cells than albumin, which could be ascribed to the excellent antioxidant properties of phycocyanins, which could be beneficial for cell growth, especially when cells are under stress, such as in media with low content of proteins and growth factors used in this study. Indeed, we demonstrated that the antioxidant activities of C-PC and R-PC are up to 4.5 times higher than those of the recombinant albumin used in this study. Furthermore, previous studies demonstrated that C-PC has one order of magnitude higher antioxidant capacity compared to BSA (Benedetti et al., 2010; Minic et al., 2016; Plantier et al., 2016). Furthermore, it has been previously demonstrated that C-PC stimulates cyclin-dependent kinase, thereby enhancing cell viability and proliferation, as shown in dermal fibroblasts (Liu et al., 2019; Madhyastha et al., 2008). Therefore, not only does the ability of phycocyanins to bind FAs determine their potency to replace albumin in cell cultivation, but other albumin-related as well as albumin-independent properties are also crucial for cell growth stimulation by C-PC and R-PC. In general, phycocyanins show potential to replace albumin both due to their ability to bind FAs, but also, they exhibit higher antioxidant capacities than albumin. Both of these effects make phycocyanins promising candidates for the replacement of albumin in cell culture media. Further experiments aiming specifically at one or the other property of phycocyanins should provide evidence which of these two effects is more significant for cellular cultivation.

To our knowledge, this is the first study demonstrating that purified phycocyanins can provide albumin-like supportive functions in animal cell culture media. While previous studies were based mainly on cell growth stimulation by C-PC-rich cyanobacterial extracts at low FBS conditions (Ghosh et al., 2023; Park et al., 2021; Sibinčić et al., 2024), this study demonstrates that purified C-PC and R-PC support QM7 cell growth under albumin-depleted conditions (de-albuminised serum in a concentration of 0.1 mg/mL of total proteins). However, FBS has fetuin, a protein that performs similar functions *in vitro* (Schenzle et al., 2025). Fetuin is particularly high in FBS, and its concentration could go above 20 mg/mL (Cartellieri et al., 2002). Meanwhile, based on our analysis, we estimate that the fetuin content in our system is limited. According to the manufacturer's specification for the Gibco FBS used in this study, the concentration of the α -globulin fraction, to which fetuin belongs, is around 13 mg/mL. Therefore, the actual fetuin content in our FBS must be lower than this value. Furthermore, based on SDS-PAGE analysis of the de-albuminised FBS (DA-FBS) samples (Fig. S1) and corresponding densitometric evaluation, we conservatively estimate that fetuin does not exceed ~12% of the total protein content in the DA-FBS sample. Accordingly, in our DA-FBS samples (concentration of 0.1 mg/mL of total proteins), the fetuin concentration would not exceed approximately 12 μ g/mL, whereas the concentration of phycocyanins or recombinant albumin used in our experiments was 200 μ g/mL, i.e., substantially higher. This difference in concentration suggests that the

contribution of residual fetuin is expected to be substantially lower than that of the added phycocyanins or recombinant albumin. Nevertheless, a contribution of residual fetuin cannot be completely excluded and should be considered when interpreting the observed biological effects. In terms of this, phycocyanins have some similar function to albumin and can be helpful in albumin-depleted/fetuin-containing samples. However, further studies are required to demonstrate phycocyanins activity in fully fetuin-depleted media.

Furthermore, we performed additional experiments in serum-free, chemically defined E8 medium. In these experiments, both phycocyanins and albumin substantially enhanced the viability and the growth of QM7 cells in a single-passage setup. Although, these experiments represent proof of concept in initial adaptation to serum-free conditions, single-passage experiments cannot exclude the contribution of residual serum-derived proteins carried over from previous culture conditions or transient cellular responses associated with serum withdrawal. Furthermore, multi-passage experiments performed in E8 medium did not yield reproducible results. Therefore, further optimisation of the culture medium together with systematic multi-passage studies will be required to demonstrate sustained long-term serum-free proliferation.

It is essential to emphasise that the purification of phycocyanins is a crucial prerequisite for stimulating the growth of QM7 cell lines at a protein concentration of 200 μ g/mL. Otherwise, applying a similar concentration range of the crude extract could not substantially increase cell growth due to the simultaneous presence of cytotoxic components in the algal extracts (Sibinčić et al., 2024), which counteract the proliferative activities of phycocyanins. Research data on identifying specific BSA alternatives is scarce, significantly limiting the development of meat cultivation. One of the rare studies focusing on albumin replacement validated the ability of partially purified rapeseed protein isolates to replace recombinant albumin in Beefy-9 serum-free media for bovine satellite cells, demonstrating enhanced cell growth (Stout et al., 2023).

This study further showed the ability of C-PC and R-PC to enhance QM7 cell proliferation across 3 passages under reduced serum conditions (1%). In accordance with viability assays, both C-PC and R-PC induced higher proliferation of QM7 cells than albumin, especially in the last passage. Low-serum conditions were used for mechanistic evaluation and were not intended to represent production-scale proliferation conditions. Standard 10% FBS media contain excess undefined proteins, lipoproteins, and antioxidants, which can obscure the effects of individual proteins. By reducing serum content, we created a sensitized system that permits evaluation of albumin-associated functions. However, cultivation of cells under reduced serum conditions leads to increased oxidative stress, which may impair cell proliferation rates (Chevallier et al., 2020). The substantially more potent antioxidant activity of C-PC and R-PC gives them an advantage over albumin and likely makes QM7 cells more resistant to oxidative stress under reduced serum conditions during prolonged cultivation.

Developing alternatives to albumin supplementation in cellular agriculture requires selecting candidates with desired albumin-like functional properties and the ability to produce them in substantial quantities (millions of tons). C-Phycocyanin is exceptionally abundant in cyanobacteria, contributing above 10% of the dry mass of *Spirulina* (Minić et al., 2024), while the content of R-PC in red macroalgae *Porphyra* is one order of magnitude lower (Cao et al., 2016). On the other hand, the annual production of *Porphyra* is two orders of magnitude higher than that of *Spirulina* (FAO, 2021). Considering the comparable albumin-like supportive activities observed for two phycocyanins as replacements for albumin, the substantially larger annual production of *Porphyra* makes R-PC may represent a more sustainable candidate for reducing albumin supplementation than C-PC. Hence, an increase in the production of cyanobacteria in the future is a prerequisite for establishing C-PC as a viable alternative to albumin. Applying the concept of the circular economy by utilising animal cell spent media for cyanobacterial and algal cultivation could substantially decrease the costs of their production and consequently increase the production yield (Yang

et al., 2023), enabling cost-effective meat cultivation. Another crucial condition required to establish both phycocyanins as albumin alternatives is the cost-effective purification of large-scale cyanobacterial and algal extracts to remove potential cytotoxic components that could significantly inhibit the growth-stimulating activities of these proteins. Further studies should be conducted to show the minimal purity level of phycocyanins required for efficient cell growth support, in order to make cultivation as cost-efficient as possible. Regarding phycocyanin purification technology at the moment, cost-efficient methods that show promise in this regard are membrane chromatography (Lauceri et al., 2019) and aqueous biphasic systems (Kovaleski et al., 2022), both of which have good scalability potential, and the final products are of high purity. Translation of phycocyanins from laboratory to industrial scale as potential albumin substitutes also requires stabilisation and controlled release over a long period to reduce the cost burden of cell media for meat cultivation. The stability of phycocyanins, especially their chromophores, is limited during prolonged incubations in cell culture media, as observed by our results (data not shown). One strategy to mitigate this is to add small amounts of fresh proteins more periodically or to have a delivery system inside the bioreactor that prolongs the release of phycocyanins in cell media. Therefore, encapsulation of phycocyanins into hydrogel matrices is a promising approach, as previously demonstrated by the incorporation of C-PC into Chitosan/Cellulose-Based Porous Nanofilms used for the mass proliferation of myoblasts in cultured meat under reduced serum conditions (Park et al., 2021). This finding is significant in prolonging the shelf life of phycocyanins, considering their limited stability, especially in the case of R-PC. Indeed, our previous study revealed that the encapsulation of R-PC into alginate hydrogel substantially increases its stability (Veličković et al., 2023). Further studies should focus on incorporating phycocyanins into other polysaccharide gels with better cell-adhesive properties. That way, cell support and prolonged delivery of phycocyanins can be achieved.

5. Conclusions

This study demonstrates the potential of two phycocyanins, from red algae (R-PC) and cyanobacteria (C-PC), to provide albumin-like supportive functions in cell culture media for meat cultivation. Both phycocyanins bind FAs with moderate to relatively high affinity, with micromolar dissociation constants for the primary binding sites. Essential polyunsaturated FAs have the highest affinities and several non-equivalent binding sites to C-PC and R-PC. Adding C-PC and R-PC to DA-FBS significantly enhanced QM7 viability and proliferation and, under these experimental conditions, outperformed recombinant albumin. In a chemically defined serum-free E8 medium, both phycocyanins also improved QM7 viability and proliferation in a single-passage experiment, indicating a short-term supportive effect. The growth-stimulating activities of C-PC and R-PC on QM7 cells were also demonstrated in multi-passage cultivation under low-serum conditions. Although additional optimisation of the culture medium and systematic multi-passage validation under fully serum-free conditions are required, the present findings identify phycocyanins as promising candidate proteins for providing albumin-like supportive functions in defined culture media.

The identification of cyanobacterial and algal phycocyanins, together with other phycobiliproteins, as proteins with albumin-like supportive properties represents a promising step towards reducing reliance on serum albumin in cultivated meat media. Such proteins may contribute to the development of more sustainable and cost-effective media formulations for cultivated meat production. Further advances in phycobiliprotein production, purification, stability and scalability will be essential for evaluating their practical implementation in future albumin-reduced or serum-free culture media, particularly within circular bioeconomy concepts.

Ethical statement - studies in humans and animals

The authors declare that the manuscript entitled “Algal and cyanobacterial phycocyanins are promising alternatives to serum albumin in cellular agriculture” does not contain studies in humans and animals.

CRediT authorship contribution statement

Aleksandar Ivanov: Writing – review & editing, Visualization, Investigation, Formal analysis, Conceptualization. **Miloš Milčić:** Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Nikola Gligorićević:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Luka Veličković:** Investigation. **Zorana Jovanović:** Investigation. **Nikolina Sibinčić:** Investigation. **Ljubodrag Aleksić:** Investigation. **Miloš Šunderić:** Investigation. **Marija Stojadinović:** Writing – review & editing, Investigation. **Tatjana Srdić Rajić:** Writing – review & editing, Investigation. **Marija Takić:** Writing – review & editing. **Milan Nikolić:** Writing – review & editing, Supervision. **Maja Krstić Ristivojević:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Simeon Minić:** Writing – review & editing, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis, 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2026.101168](https://doi.org/10.1016/j.fufo.2026.101168).

Data availability

Data will be made available on request.

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