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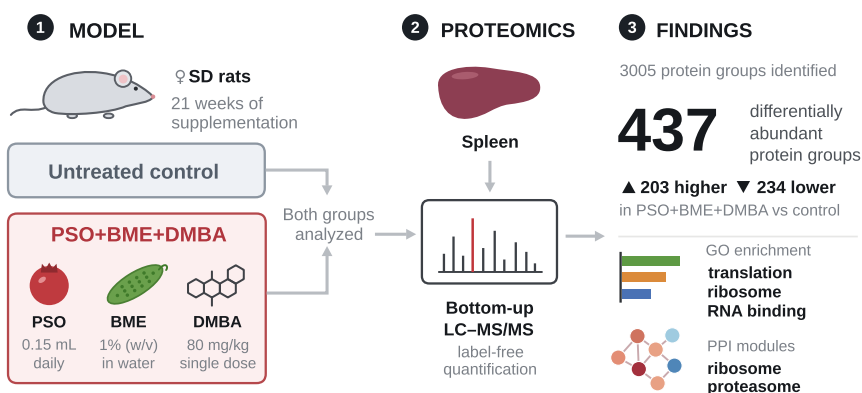
Bottom-Up Proteomic Profiling of Rat Spleen Under Combined DMBA Exposure and Dietary Supplementation

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This study applied bottom-up LC-MS/MS proteomics to compare spleen tissue from untreated female Sprague–Dawley rats with tissue from rats exposed to 7,12-dimethylbenz[a]anthracene (DMBA) while receiving combined pomegranate seed oil (PSO) and bitter melon extract (BME) supplementation. Previously published ICP-MS data obtained using the same experimental model were considered only as contextual information on

macro- and trace-element levels. Differential-abundance analysis identified 437 protein groups between the two endpoint groups, including 203 with higher and 234 with lower relative abundance in the PSO+BME+DMBA group. Functional enrichment highlighted translation-, RNA-binding-, and ribosome-related annotations, while protein–protein interaction analysis revealed interconnected modules within the differentially abundant protein-group set. The previously published elemental study indicated that carcinogenic status was the principal factor associated with splenic elemental composition and reported group-dependent differences in selected elements, including Fe and Ca. Because proteomics was performed only for the untreated control and PSO+BME+DMBA groups, the observed differences cannot be attributed separately to DMBA, PSO, BME, or their interactions. Direct element–protein relationships were not established. The study therefore provides an exploratory proteomic comparison interpreted in the context of previously reported elemental data.

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INTRODUCTION

Currently, one of the United Nations Sustainable Development Goals is to reduce deaths from non-communicable diseases. Cancer remains one of the leading non-communicable diseases with the highest mortality rates,¹ and its complex etiology necessitates a diverse array of treatments, often accompanied by undesirable side effects.² The pursuit of more effective cancer therapy and prevention has drawn significant attention to the potential benefits of dietary supplementation.^{3–7} Essential macronutrients and micronutrients, such as elements, fats, carbohydrates, vitamins, and water, are critical for the proper functioning of organisms. Essential elements participate in metabolic pathways as enzyme cofactors and contribute to protein structure and function.⁸ The elemental composition of organisms can undergo profound changes due to immune system disorders, physical conditions, dietary interactions, or the onset of diseases. Markers of diseases manifest as alterations in the elemental content of tissues and body fluids.⁹ Deficiencies in these elements can weaken immunity and induce carcinogenic effects,¹⁰ underscoring the importance of exploring new methods to support treatment.

Previous studies have used inductively coupled plasma mass spectrometry (ICP-MS) to investigate associations between dietary supplementation and tissue macro- and trace-element levels.^{11–13}

It is well-established that diet and supplementation significantly influence the absorption of bio-elements, offering a promising avenue for improving health outcomes.^{6,14,15} Dietary compounds may also influence molecular processes, including gene expression and protein relative abundance.

Two botanicals, *Momordica charantia* (bitter melon) and *Punica granatum* (pomegranate), have been used in various forms (extracts, oils, fruit, or powders) in traditional medicine due to their recognized health benefits. The biological activities reported for these plants include antioxidant, anticancer,^{16–26} hypolipidemic,²⁷ hypotensive,^{28,29} antidiabetic,^{30,31} and neuroprotective effects.²³

These plants are rich sources of various nutrients and bioactive compounds including triterpenoids, phenolic acids, triterpene glycosides, lectins, flavonoids, sterols, saponins, and proteins, known for health-promoting effects.³²

Several peptides and proteins involved in the anticancer mechanism were identified and characterized from bitter melon seeds or extracts: Momordica antiviral protein 30 kDa (MAP30),^{18,33,34} the peptide BG-4,³⁵ momorcharin (α -, β -, γ -, δ - and ϵ -),³⁶ 14 kDa ribonucleases (RNase MC2), or lectin (MCL).³⁷ Most of them belong to the ribosome-inactivating proteins (RIPs), which inhibit protein synthesis. Their molecular roles include the induction of reactive oxygen species generation, the activity of detoxification enzymes, and the reduction of pro-inflammatory cytokines.

In the case of pomegranate, the extract has been reported to induce apoptosis and to downregulate the expression of genes involved in DNA double-strand break repair, including MRE11, RAD50, RAD51, NBS1, BRCA1, BRCA2, and BRCC3,³⁸ to decrease cyclin B1 and D1,³⁹ to up-regulate the cyclin-dependent kinase inhibitor p21,⁴⁰ and to inhibit constitutive NF- κ B activity in prostate cancer models.⁴¹

Despite the well-documented effects of consumption, particularly in light of their antagonistic actions, a comprehensive understanding of these effects remains limited.^{42–44} There are not many papers on studies of gene expression induced by the presence of the mentioned plants in the diet due to the complicated nature of the experiment conducted on animals. Both the liver and spleen are pivotal organs, holding key roles in various diseases affecting other organs. Notably, spleen immune cells present an enticing target for gene therapy, especially in the context of immune system diseases, including cancer.⁴⁵

While existing research has unveiled the anti-carcinogenic properties of both pomegranate and bitter melon,^{23,26,46} comprehensive investigations into the elemental composition of tissues and their proteomic profiles remain limited.¹³ To address this knowledge gap, the present study examined the splenic proteomic profile using bottom-up LC-MS/MS proteomics.

This study aimed to characterize differences in splenic protein relative abundance between untreated control rats and rats exposed to DMBA while receiving combined PSO and BME supplementation. Bottom-up LC-MS/MS proteomics was used to identify and quantify differentially abundant protein groups between these two endpoint conditions. Previously published ICP-MS data obtained using the same experimental model¹³ were used solely as contextual information on macro- and trace-element levels. Because the proteomic analysis included only these two endpoint groups, the study design did not allow the independent effects of DMBA exposure, PSO supplementation, BME supplementation, or their interactions to be distinguished, nor did it permit direct element–protein correlations. The analysis therefore examined whether the two endpoint conditions differed in protein relative-abundance patterns and in the functional annotations represented among the differentially abundant protein groups.

MATERIALS AND METHODS

Animals, dietary supplementation, tissue collection

The experiment was conducted using female 30-day-old Sprague-Dawley rats ($n = 96$) after obtaining ethical approval (Approval No. 56/2013 and 54/2015) from the 2nd Local Commission of Animal Experiment Ethics situated at Warsaw University of Life Sciences (WULS, Poland).¹³ All relevant welfare guidelines and protocols for animal handling were meticulously adhered to throughout the entire experimental period, and these practices are further described elsewhere.^{47,48} Following a one-week adaptation period, the animals were divided into eight groups, each consisting of 12 individuals. These groups were distinguished by the specific dietary supplementation they received (see Table I for details¹³). In groups supplemented with pomegranate seed oil (PSO), in addition to the standard diet and water, the animals received a daily intragastric dose of 0.15 mL of PSO (PSO and PSO+DMBA). For the groups receiving a bitter melon-based diet (BME and BME+DMBA), standard drinking water was replaced with an aqueous extract from bitter melon (BME). In groups receiving both BME and PSO supplements (PSO+BME and PSO+BME+DMBA), the animals were provided with an aqueous BME *ad libitum* and 0.15 mL of PSO daily *via* an intragastric probe. The control groups (Con and Con+DMBA) were not subjected to any dietary modifications and continued to receive standard feed and unrestricted access to water.

Dietary supplementation was maintained over a period of 21 weeks. On day 50 of the study, all animals assigned to the DMBA-treated groups received a single intragastric administration of 7,12-dimethylbenz[a]anthracene (DMBA) at a dose of 80 mg per kilogram of body weight, thereby initiating tumorigenesis. The carcinogen was prepared in rapeseed oil for the Con+DMBA and BME+DMBA groups, whereas pomegranate seed oil served as the solvent in the PSO+DMBA and PSO+BME+DMBA groups.

At week 22 of the study, the animals were euthanized by decapitation in accordance with the procedures approved by the 2nd Local Commission for Animal Experiment Ethics at the Warsaw University of Life Sciences (Approval Nos. 56/2013 and 54/2015). The spleens were subsequently excised, divided into two portions, and stored at -70 °C until analysis.

Commercially available cold-pressed pomegranate seed oil (ECOSPA, Great Britain) was stored in a dark glass bottle at 8 °C and administered daily to animals after reaching ambient temperature. A fresh infusion of bitter melon tea (Hung Phat Corp, Vietnam) was prepared daily to obtain 1% extract (w/v) with 80 °C water. After cooling to ambient temperature, the extract was administered to animals *ad libitum*.

Table I. Applied group diets with supplementation of extract of bitter melon (BME) and/or pomegranate seed oil (PSO) or without any supplementation (Con)^{13*}

Group	Supplementation	Carcinogenic Factor
Con	not applied	not applied
PSO	0.15 mL PSO per day	not applied
BME	1% BME in water <i>ad libitum</i>	not applied

(continued on next page)

Table I. Applied group diets with supplementation of extract of bitter melon (BME) and/or pomegranate seed oil (PSO) or without any supplementation (Con)^{13*} (continued)

Group	Supplementation	Carcinogenic Factor
PSO+BME	0.15 mL PSO per day and 1% BME in water <i>ad libitum</i>	not applied
Con+DMBA	not applied	DMBA in rapeseed oil
PSO+DMBA	0.15 mL PSO per day	DMBA in PSO
BME+DMBA	1% BME in water <i>ad libitum</i>	DMBA in rapeseed oil
PSO+BME+DMBA	0.15 mL PSO per day and 1% BME in water <i>ad libitum</i>	DMBA in PSO

*Ref. No. 13: Białek, M.; Lepionka, T.; Wojtak, W.; Ruszczyńska, A.; Bulska, E.; Czauderna, M.; Białek, A. Splenic Elemental Composition of Breast Cancer-Suffering Rats Supplemented with Pomegranate Seed Oil and Bitter Melon Extract. *Molecules* **2024**, 29 (9), 1942. <https://doi.org/10.3390/molecules29091942>. [Article published by MDPI under an open access license. No special permission is required to reuse all or part of the article.]

Proteomic analysis was performed for the untreated control and PSO+BME+DMBA groups as an exploratory comparison of two endpoint conditions. Spleen samples from all 12 animals of each of these groups ($n = 12$ per group) were analyzed individually; samples were not pooled, and each biological sample was analyzed in a single LC-MS/MS injection. This comparison captures the overall difference between the two composite experimental conditions but does not permit separation of the individual effects of DMBA exposure, PSO supplementation, BME supplementation, or their interactions. Consequently, all proteomic interpretations were restricted to differences between these two groups.

Previously published elemental dataset and its contextual use

The elemental composition of spleen samples from all eight experimental groups was previously determined by inductively coupled plasma mass spectrometry (ICP-MS) and reported by Białek et al.¹³ No new ICP-MS measurements were performed for the present study. The previously published elemental results were used solely as contextual analytical information for the interpretation of the targeted proteomic comparison and were not directly integrated with the proteomic dataset at the individual-sample level.

Because the elemental and proteomic datasets differed in experimental-group coverage and were not analyzed as a matched sample-level dataset, direct correlations between elemental levels and protein relative abundance were not evaluated. Consequently, potential relationships between elemental homeostasis and proteomic pathways are considered contextual biological hypotheses rather than experimentally demonstrated associations.

Bottom-up proteomic workflow and LC-MS/MS analysis

Sample preparation and LC-MS/MS measurements were performed according to previously established procedures,⁴⁹ whereas the quality-assurance strategy was based on the approach described by Gawor and Bulska.⁵⁰

Approximately 50 mg of frozen spleen tissue was homogenized for 15 min at room temperature in 1 mL of lysis buffer containing 1% SDS and cOmplete™ EDTA-free protease inhibitor in 100 mmol L⁻¹ ammonium bicarbonate using an Ultra-Turrax mechanical homogenizer. The homogenates were centrifuged for 30 min at 20,000 × g, and the protein concentration in the resulting supernatants was determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Aliquots corresponding to 100 µg of total protein were precipitated overnight with 800 µL of acetone at -20 °C. After centrifugation at 20,000 × g and 4 °C, the supernatants were discarded, and the dried protein pellets were resuspended in 50 µL of 0.1% RapiGest in 50 mmol L⁻¹ ammonium bicarbonate.

Proteins were reduced with 75 μL of 5 mmol L^{-1} 1,4-dithiothreitol for 45 min at 56 °C and alkylated with 75 μL of 30 mmol L^{-1} acrylamide for 30 min at room temperature in the dark. The alkylation reaction was quenched by adding an equimolar amount of 1,4-dithiothreitol, followed by incubation for 15 min at room temperature. The samples were digested with sequencing-grade trypsin for 18 h at 37 °C using a protein-to-enzyme mass ratio of 100:1. Digestion was stopped by adding 150 μL of 5% formic acid, and the peptides were purified using Pierce™ Peptide Desalting Spin Columns. The dried peptide mixtures were reconstituted in 100 μL of 5% acetonitrile containing 0.1% formic acid before LC-MS/MS analysis.

One microgram of the peptide mixture was analyzed using an UltiMate 3000 nano-UHPLC system (Thermo Fisher Scientific, USA) coupled online to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, USA). Peptides were separated on an in-house-packed fused-silica C18 analytical column measuring 75 $\mu\text{m} \times 500 \text{ mm}$ and containing 1.9 μm ReproSil Gold 120 C18 particles (Dr. Maisch, Germany). Mobile phase A consisted of 0.1% formic acid in water, whereas mobile phase B consisted of 0.1% formic acid in 80% acetonitrile and 20% water. Chromatographic separation was performed over a 90-min gradient at a flow rate of 300 nL min^{-1} . Peptides were ionized in positive-ion nanoelectrospray mode using a capillary voltage of 1.9 kV. Mass-spectrometric data were acquired in data-dependent acquisition mode with a dynamic-exclusion time of 60 s. Survey spectra were recorded over an m/z range of 350–1600 in the Orbitrap mass analyzer at a resolving power of 60,000, with an automatic gain control target of 1×10^5 and a maximum ion-injection time of 30 ms. Multiply charged precursor ions with intensities above 1×10^6 were selected for higher-energy collisional dissociation using a normalized collision energy of 30. Tandem mass spectra were acquired in the Orbitrap mass analyzer at a resolving power of 15,000, with an automatic gain control target of 5×10^4 and a maximum ion-injection time of 60 ms.

LC-MS/MS equipment performance was monitored throughout the analytical sequence by assessing peak width, peak shape, chromatographic resolution, retention-time consistency, and the mass accuracy of detected peptide ions. A pooled quality-control sample was prepared by combining aliquots from all biological samples included in the proteomic experiment. The pooled QC sample was analyzed at the beginning and at the end of the analytical sequence, as well as between consecutive experimental groups, to monitor chromatographic and mass-spectrometric stability throughout the measurements.

Protein identification, label-free quantification, and statistical analysis

Raw LC-MS/MS data were processed using MaxQuant software, version 1.6.1.0 (Max Planck Institute of Biochemistry, Martinsried, Germany), with the integrated Andromeda search engine. Tandem mass spectra were searched against the *Rattus norvegicus* reference proteome obtained from the UniProt database. Trypsin was specified as the proteolytic enzyme, and up to two missed cleavages were allowed during database searching. Cysteine propionamidation was defined as a fixed modification, whereas methionine oxidation and protein N-terminal acetylation were included as variable modifications. The precursor- and fragment-ion mass tolerances were set to 5 and 10 ppm, respectively. Only peptides containing at least seven amino acid residues were considered. Peptide-spectrum match and protein-level false discovery rates were controlled at 1% using a target–decoy strategy. Common contaminants were included during database searching. Protein groups were quantified using the MaxQuant label-free quantification algorithm based on unique and razor peptides, with a minimum ratio count of two. The resulting protein-group dataset was imported into Perseus software, version 1.6.14.0 (Max Planck Institute of Biochemistry, Martinsried, Germany). Reverse database hits, proteins identified only by site, and potential contaminants were removed before statistical analysis. LFQ intensity values were $\log_2$ -transformed. Proteins were retained for quantitative analysis when at least eight valid LFQ values were available in at least one experimental group. Missing values were imputed from a normal distribution representing signals below the detection limit.

Differences in protein relative abundance between the untreated control and PSO+BME+DMBA groups were evaluated using a two-sample Student's *t*-test. Multiple-testing correction was performed using a

permutation-based false discovery rate of 1%, with an s_0 parameter of 1. Proteins meeting the applied statistical criteria were considered differentially abundant. Mean LFQ intensities and standard errors were calculated for each experimental group to describe protein-abundance differences and variability among biological samples.

RESULTS AND DISCUSSION

Contextual elemental findings reported previously

A previous study using the same experimental model reported group-dependent differences in splenic macro- and trace-element levels across the eight experimental groups.¹³ Selected elements, including Fe and Ca, differed among the experimental conditions, while multivariate analysis indicated that carcinogenic status was the principal factor associated with splenic elemental composition. Because these elemental results were published previously and were not matched with the present proteomic measurements at the individual-sample level, they are included here solely as contextual analytical information. No direct associations between elemental levels and protein relative abundance were evaluated in the present study.

Proteomic data quality and protein identification

Bottom-up LC-MS/MS analysis identified 2448 protein groups and 2676 unique peptide sequences in the PSO+BME+DMBA group, whereas 2094 protein groups and 2284 unique peptide sequences were identified in the untreated control group. The overlap between the identified protein-group sets is presented in the Venn diagram in Figure 1. Across both experimental groups, a total of 3005 protein groups were identified, of which 1537 were detected in both groups. These shared protein groups represented 62.8% of the protein groups identified in the PSO+BME+DMBA group and 73.4% of those identified in the control group. Under the applied identification criteria, 911 protein groups were detected only in the PSO+BME+DMBA group and 557 only in the control group. Differential-abundance analysis identified 437 protein groups with statistically significant differences in relative abundance between the untreated control and PSO+BME+DMBA groups, based on an FDR of 1%, an s_0 value of 1, and an adjusted p -value threshold of 0.01. Identification, statistical parameters, and individual $\log_2$ LFQ intensity values for the differentially abundant protein groups are provided in Supplementary Table S1.

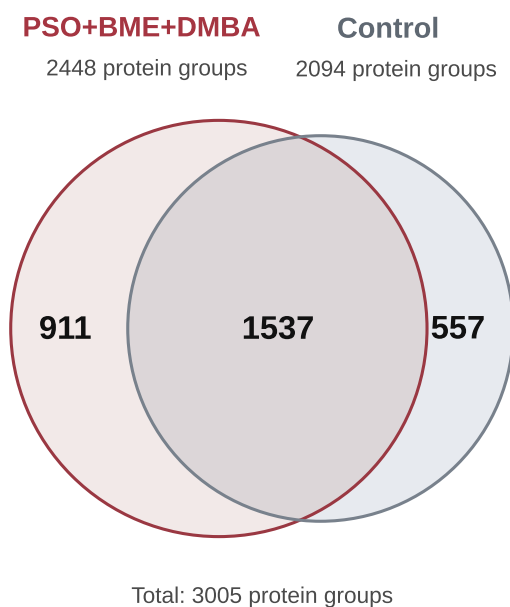
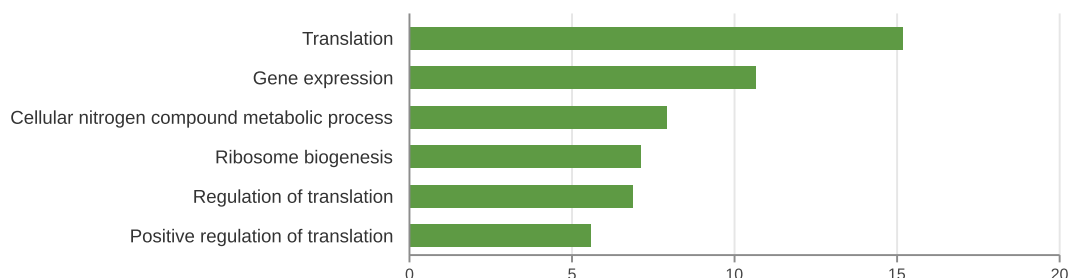


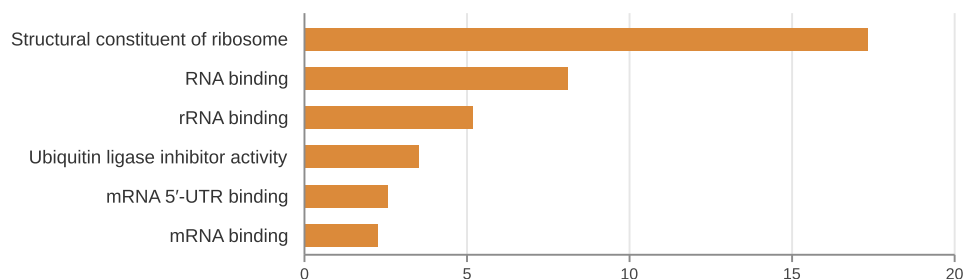
Figure 1. Venn diagram showing the overlap between protein groups identified in the untreated control and PSO+BME+DMBA groups.

Gene Ontology (GO) enrichment analysis of the differentially abundant protein groups was performed using STRING (<http://string-db.org/> accessed on 12 September 2022). The criteria in the GO analysis were set to be identical to the previous work.⁴⁹ GO classifies protein functions into specific groups, biological processes (i), molecular functions (ii), and cellular components (iii). For each category, the six top-ranked terms were selected (Figure 2). For (i) these are: translation, gene expression, cellular nitrogen compound metabolic process, ribosome biogenesis, regulation of translation, and positive regulation of translation. Looking in terms of (ii): structural constituent of ribosome, RNA binding, rRNA binding, ubiquitin ligase inhibitor activity, mRNA 5'-UTR binding, and mRNA binding. And for (iii) factors: cytosolic ribosome, ribosomal subunit, cytosolic small ribosomal subunit, cytosolic large ribosomal subunit, intracellular non-membrane-bounded organelle, and synapse.

A Biological process



B Molecular function



C Cellular component

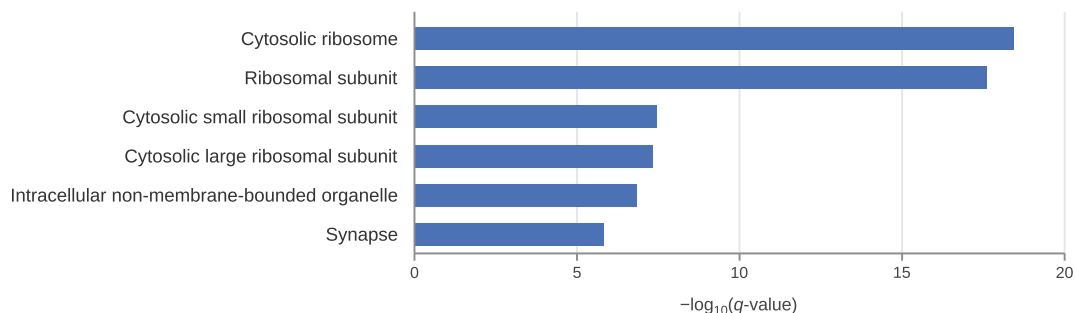


Figure 2. Gene Ontology enrichment analysis of differentially abundant protein groups: (A) biological process, (B) molecular function, and (C) cellular component. The x-axis represents $-\log_{10}(q\text{-value})$, where q-values were obtained after correction for multiple testing using the Benjamini–Hochberg procedure. Higher values indicate greater statistical significance of enrichment.

Protein-protein interaction analysis was performed using the STRING database. A minimum required interaction score of 0.900 was applied, corresponding to the highest-confidence interaction level. The maximum number of additional interactors was set to zero, and disconnected nodes were omitted from the displayed network. The resulting PPI network is presented in Figure 3.

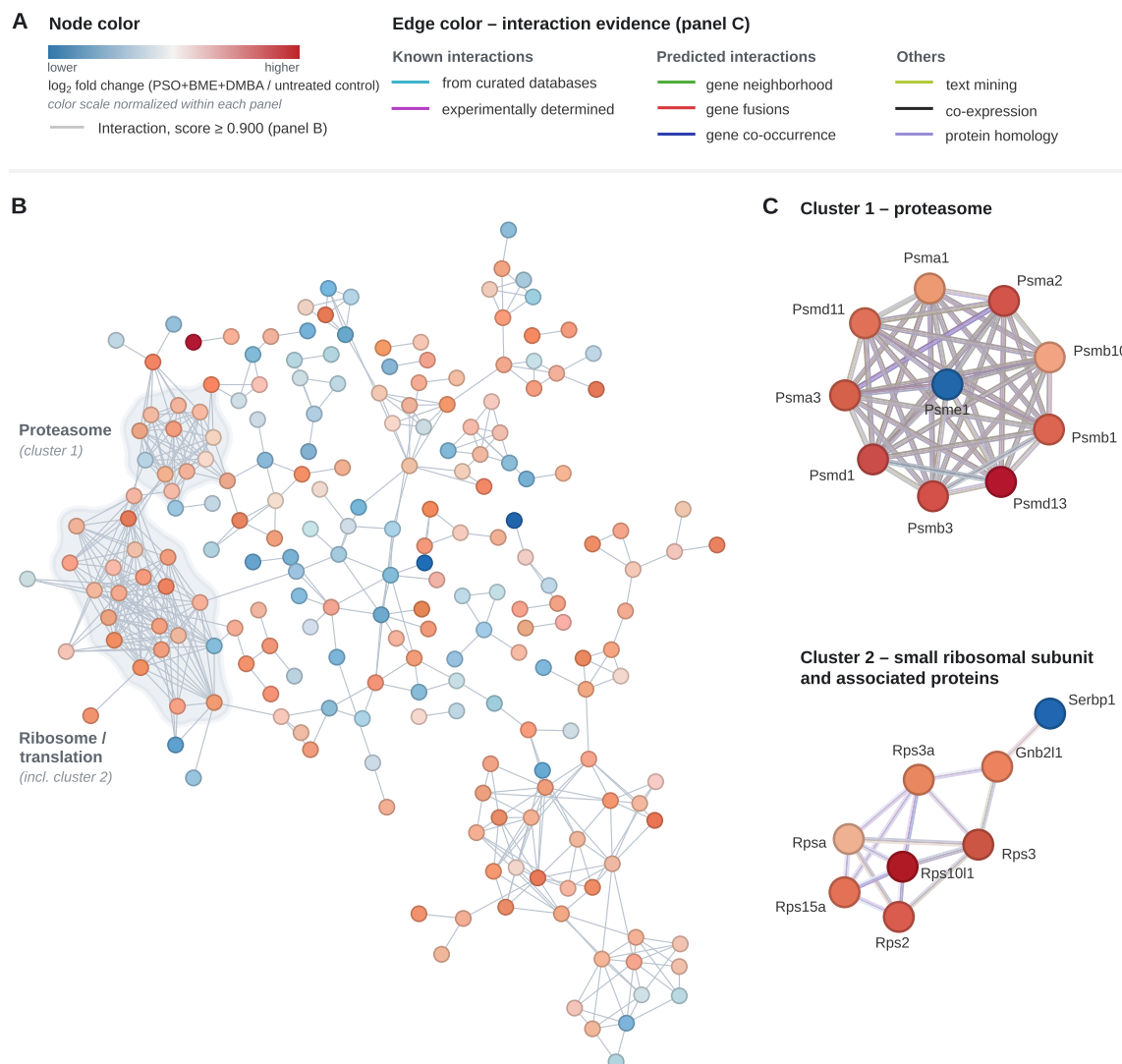


Figure 3. STRING-based protein–protein interaction analysis of protein groups with significantly different relative abundance between the PSO+BME+DMBA and untreated control groups: (A) legend describing the sources of interaction evidence; (B) interaction network of the connected differentially abundant protein groups; and (C) selected interaction subnetworks. Edge colors indicate the sources of interaction evidence used by STRING. Disconnected nodes are not shown. Node colors indicate the log₂ fold change in relative abundance between the PSO+BME+DMBA and untreated control groups (red, higher; blue, lower relative abundance in the PSO+BME+DMBA group).

The network comprised 396 nodes (differentially abundant protein groups mapped to STRING) and 460 edges; nodes without interactions at the applied confidence threshold are not shown in Figure 3B. The mean node degree was 2.32, the average local clustering coefficient was 0.402, and the expected number of edges was 283. The PPI enrichment *p*-value was below 1.0×10^{-16} , indicating that the network contained significantly more interactions than expected for a random protein set of comparable size.

Inspection of the protein sets associated with the interaction network revealed a prominent group of ribosomal and translation-associated proteins. Representative proteins included RPL10A, RPLP0, RPL19, RPS7, RPS3A, RPS2, EIF3J, RPS3, RPS15A, RPSA, ETF1, EEF1G, RPS18, EIF3D, RPL5, RPL11, RPLP2, RPS14, and MCTS1. These proteins represent components or regulators of the small and large ribosomal subunits and of translation initiation, elongation, and termination.

A further subnetwork comprised proteasome-associated proteins, including PSMA1, PSMA2, PSMA3, PSMB1, PSMB3, PSMB10, PSMD1, PSMD11, PSMD13, and PSME1 (Figure 3C, Cluster 1). The presence of these proteins among the differentially abundant protein groups suggests differences in the relative abundance of components involved in proteasomal protein degradation. However, proteasome activity was not measured, and its functional alteration cannot be inferred from the interaction network alone.

Another protein set comprised proteins associated with extracellular-matrix organization, cell adhesion, cytoskeletal regulation, vascular functions, and innate immune signaling. Representative members included NID1, LUM, THY1, PTK2, ITGB1, PODXL, CD14, VWF, ICAM1, EZR, and LAMB2. Additional proteins in this set, including RAP1A, RAP1B, ARHGEF2, ARHGEF7, FLNC, and SPTAN1, were associated predominantly with cytoskeletal organization, cell migration, and adhesion-related signaling.

A smaller subnetwork (Figure 3C, Cluster 2) comprised the small ribosomal subunit proteins RPSA, RPS2, RPS3, RPS3A, RPS10L1, and RPS15A, together with the ribosome-associated proteins GNB2L1 (RACK1) and SERBP1. Because several of these proteins were also present in the larger ribosome-associated set, the protein lists were interpreted as overlapping functional subnetworks rather than as fully independent biological pathways.

The ribosome- and translation-associated subnetworks were consistent with the prominence of translation- and ribosome-related annotations in the functional analysis (Figure 2). However, the PPI network indicates predicted or previously reported functional connectivity and does not itself demonstrate changes in pathway activity. In Figure 3C, edge colors represent the different sources of interaction evidence used by STRING, as defined in the legend in Figure 3A.

The principal new finding of the present study is the difference in splenic protein relative-abundance patterns between the untreated control and PSO+BME+DMBA endpoint groups. Differential-abundance analysis identified 437 protein groups between these two groups, including 203 protein groups with higher and 234 with lower relative abundance in the PSO+BME+DMBA group. However, the comparison involved two composite endpoint conditions and therefore cannot distinguish whether the observed protein-abundance differences resulted from DMBA exposure, PSO supplementation, BME supplementation, or interactions among these factors. Accordingly, the results should not be interpreted as evidence of supplementation-specific or carcinogenesis-specific protein regulation.

Functional enrichment analysis indicated that translation-, RNA-binding-, and ribosome-related annotations were prominent among the differentially abundant proteins. These findings suggest coordinated differences in the relative abundance of proteins associated with translational machinery between the untreated control and PSO+BME+DMBA endpoint groups. However, enrichment of these annotations does not directly demonstrate changes in global protein-synthesis rates, ribosome activity, or translational regulation. Confirmation of such functional effects would require targeted biochemical and molecular assays.

The interpretation of the GO enrichment results is limited by the completeness and quality of the available *Rattus norvegicus* annotations in the STRING database. Because the analysis relied on species-level annotations rather than a separate strain-specific annotation dataset for Sprague–Dawley rats, proteins with incomplete or absent functional annotations may be underrepresented in the enriched categories. Therefore, the GO results should be interpreted as a hypothesis-generating functional summary of the differentially abundant protein groups rather than as direct evidence of altered biological activity.

Previous studies have reported that ribosome-inactivating proteins and other biologically active components of *Momordica charantia* may inhibit protein synthesis and affect apoptosis-related pathways in experimental cancer models.^{51,52} These literature findings provide a possible biological context for the prominence of ribosome-related annotations in the present proteomic dataset. However, the presence,

concentration, and biological activity of bitter melon-derived ribosome-inactivating proteins were not measured in the spleen samples. Therefore, the observed differences in protein relative abundance cannot be attributed directly to ribosome-inactivating protein activity or specifically to BME supplementation.

The previously published ICP-MS study using the same experimental model reported group-dependent differences in Fe, Se, and other elements.¹³ Because Fe and Se participate in redox-related biological processes, these observations raise the hypothesis that elemental homeostasis and oxidative balance may be relevant to the biological context of the proteomic findings. Nevertheless, direct correlations between elemental levels and individual proteins were not evaluated. Moreover, reactive oxygen species, antioxidant capacity, lipid peroxidation, glutathione metabolism, and ferroptotic cell death were not measured. Consequently, oxidative stress and ferroptosis should be considered possible directions for future investigation rather than mechanisms demonstrated by the present study.

Similarly, previously reported effects of pomegranate-derived compounds on DNA repair, cell-cycle regulation, apoptosis, and inflammatory signaling were observed mainly in cellular or tumor-focused experimental models.^{38–41,53–58} These findings may provide broader literature context but cannot be directly extrapolated to the splenic proteomic differences observed in the present study. Because proteomics was restricted to two composite endpoint groups, the detected differences cannot be attributed separately to PSO, BME, DMBA exposure, or their interactions. Future studies should include the intermediate experimental groups and targeted measurements of translation activity, oxidative-stress markers, lipid peroxidation, antioxidant systems, and ferroptosis-related pathways. Such measurements would be required to determine whether the hypotheses raised by the present exploratory findings have functional significance.

CONCLUSIONS

Bottom-up LC-MS/MS proteomics identified 437 protein groups with significantly different relative abundance between the untreated control and PSO+BME+DMBA endpoint groups. Among these protein groups, 203 showed higher and 234 showed lower relative abundance in the PSO+BME+DMBA group. Functional enrichment analysis indicated that translation-, RNA-binding-, and ribosome-related annotations were prominent within the differential-protein set, while protein–protein interaction analysis revealed interconnected protein modules.

Previously published ICP-MS results obtained using the same experimental model were considered solely as contextual information on splenic elemental composition. Because proteomic analysis was restricted to two composite endpoint groups and the elemental and proteomic datasets were not directly integrated at the individual-sample level, the present findings do not distinguish the individual effects of DMBA exposure, PSO supplementation, BME supplementation, or their interactions. They also do not establish direct relationships between elemental levels and protein relative abundance.

The present study therefore provides an exploratory characterization of differences in the splenic proteome between the two endpoint conditions. Further studies should include all intermediate experimental groups, matched elemental and proteomic measurements from the same biological samples, and targeted functional validation of the identified protein-related pathways.

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Conflicts of interest

The authors declare there are no financial conflicts of interest. All potential sources of bias, including affiliations and funding sources, have been appropriately disclosed in the manuscript.

Use of artificial intelligence tools

The authors declare that no artificial tools were used in the preparation of this article.

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SUPPLEMENTARY MATERIAL

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