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Research Article

Transfer Factors From Animal- And Plant-Based Sources: A Proteomics Comparison And Preclinical Effects On The Immune System.

Vaclav Vetvicka[1]* and David Vollmer[2].

1University of Louisville, Department of Pathology, 511 S. Floyd, Louisville, KY 40202, USA , Email: vaclav.vetvicka@louisville.edu.
2Research and Development Department, 4Life Research, Sandy, UT 84070, USA

Abstract

Transfer factors have long been used as natural immune-supporting supplements, traditionally derived from bovine colostrum and chicken egg yolks. However, limitations in sourcing, cost, and sustainability have driven interest in plant-based alternatives. This study investigates upcycled Brassica napus protein isolates as a potential plant-based transfer factor.

Proteomic analysis revealed high bio-similarity among Brassica species, particularly in defensin peptides, which are functionally analogous to immune-modulating peptides found in animal-based transfer factors. Nano-LC/MS/MS analysis confirmed the presence of defensin-like protein 1 (Brassica napus) and beta-defensin 5 (bovine colostrum), both associated with antimicrobial defense and immune activation.

Preclinical studies demonstrated significant immune-enhancing effects across multiple assays. Brassica napus, Sample A showed dose-dependent activity in phagocytosis, cytokine production (IL-2), NK cell-mediated tumor killing, and humoral immunity, though effects varied between samples. TriFactor, an established animal-based transfer factor, consistently showed the strongest response. Notably, high-dose Brassica napus, Sample A revealed greater activity than Sample B in several measures, underscoring their differences in low molecular-weight peptides and proteins.

These findings highlight the potential of Brassica napus as a scalable, plant-based alternative to traditional transfer factors. Further research is needed to refine this formulation and evaluate clinical efficacy.

Keywords : Transfer factor; plant-based; proteomics; immunity, phagocytosis, IL-2.

INTRODUCTION

Transfer factors are substances derived from human blood and transfer antigen-specific immunity.[1] Initially, they were believed to mediate immunity through cell injection, but was later demonstrated that partial immunity could be transferred even after immune cell lysis. These dialyzable leukocyte extracts have since been explored for their potential to enhance antigen-specific immune responses.[2,3] While their exact composition is still uncharacterized, evidence suggests they consist of low molecular-weight peptides[4] containing conserved sequences.[5]

Transfer factors have also been sourced from animals, primarily bovine colostrum and chicken egg yolk, with one study showing similar immune system activity between human blood-based and animal-based transfer factors.[6] These various sources facilitate the transfer of immunological memory from parent to offspring, offering protection against infections and chronic diseases.

More recently, transfer factor-like peptides have been found in plants. Low molecular-weight peptides and proteins isolated from brassica seeds show similarities to animal-based transfer factors in molecular size, structure, and immune activity.[7] This suggests a parallel mechanism in plants, where immunological memory may be passed through their seeds for intergenerational defense against biotic threats, despite the absence of an adaptive immune system.

Bovine colostrum and egg yolk-based transfer factors are commercially produced as dietary supplements and have been rigorously investigated for immune system health[8,10] and safety.[11] Earlier preclinical studies have examined their direct immunological effects and potential synergies with other bioactive molecules of botanical origin.[12,1]

***Corresponding Author:** **Vaclav Vetvicka**, University of Louisville, Department of Pathology, 511 S. Floyd, Louisville, KY 40202, USA,
Email: vaclav.vetvicka@louisville.edu.
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This study compares plant-based transfer factors from Brassica napus seeds to a combination of bovine colostrum and egg yolk-based transfer factors using proteomic experiments to evaluate molecular similarities in size and structure as well as preclinical experiments investigating immune system activity.

MATERIAL AND METHODS

Proteomics Experiments

Sample Preparation & 1D SDS-PAGE

For each sample, 50 mg powder from *Brassica napus* or bovine colostrum was solubilized then exchanged using spin column into 50 mM ammonium bicarbonate buffer. Protein concentration was measured using Bio-Rad Protein Assay Kit II #500-0002 according to manufacturer's protocol. For each sample, 40 µg protein was loaded in each lane in a 12% SDS-gel. The SDS-gel was run at 15°C until the dye front ran out of the gels. Afterwards, the gels were fixed and stained with Coomassie Blue. Gel images were scanned at once using Typhoon TRIO (GE Healthcare). Specific bands under the 10-kDa marker were selected, excised, and transferred into Eppendorf tubes containing 25 mM NH_4CO_3 for mass spectrometry in-gel sample preparation.

Mass Spectrometry Sample Preparation

The gel bands were washed a few times alternating between 25 mM NH_4CO_3 and 50:50 buffer (50% ACN, 50% 50mM NH_4CO_3). DTT was added to a final concentration of 10 mM and incubated at 60°C for 30 min, followed by cooling down to room temperature. Iodoacetamide was then added to a final concentration of 10mM and incubated in the dark for 30 min at room temperature. The proteins were then digested by Trypsin/Lys-C Mix, Mass Spec Grade (Promega, V5073), overnight at 37°C.

NanoLC-MS/MS

NanoLC was carried out using a Thermo Fisher Ultimate 3000. Mobile phase solvents A and B were 0.1% Formic acid (v/v) in water and 0.1% Formic acid (v/v) in Acetonitrile, respectively. Tryptic peptides were loaded into a µ-Precolumn Cartridge and then separated on a reverse-phase analytical HPLC column (Thermo Fisher Scientific, ES900). The following gradient was applied: 4 min from 4% B to 15% B, 18 min from 15% B to 30% B, 4 min at 99% B, and 4 min at 4% B. Mass spectrometry analysis was performed on an Orbitrap Exploris (Thermo Fisher Scientific) in the data dependent mode. MS full scan settings were as follows: 80000 resolution, 300-2000 m/z scan range, 100% AGC target, maximum injection time set to auto. MS/MS settings were as follows: 30,000 resolution, 200% AGC target, maximum injection time set to auto, 30% normalized HCD collision energy, 1.0e4 intensity threshold.

Database Search

Data analysis was performed on Proteome Discoverer 2.5 (Thermo Fisher Scientific) against a SwissProt database consisting of all protein sequences from the appropriate species (*Brassica napus, Bos taurus* (i. e., bovine colostrum)), as of July 2024. The search was performed using the Sequest HT algorithm with the following settings: Enzyme set to Trypsin, Min Peptide Length 5, Max Peptide Length 144, Precursor Mass Tolerance: 10 ppm, Fragment Mass Tolerance: 0.02 Da. The following dynamic modifications were used: Oxidation-M, N-terminal Acetylation, N-terminal Met-loss. The following static modifications were used: Carbamidomethyl-C. Label-free quantitation of protein abundances was performed using the Minora Feature Detector. In both processing and consensus steps, the results were filtered using a maximum 1% false discovery rate. The protein abundance values and ratios were normalized on total peptide amount.

Preclinical Experiments

Transfer Factor Samples

All samples were generously provided by 4Life Research, Sandy, UT, USA.

The combined animal-based transfer factor proteins, referred to as TriFactor, were isolated from defatted bovine colostrum and chicken egg yolk. The bovine colostrum transfer factor was purified to a high total protein content through a phase-separation process, followed by ultrafiltration, enriching low molecular-weight proteins and peptides. Deionized water was used to solubilize the low molecular-weight proteins and peptides prior to ultrafiltration. The chicken yolk transfer factor was purified through phase separation from the egg whites. Each material was then spray-dried into a powder and combined into a proprietary composition.

Two distinct versions of plant-based transfer factor proteins were isolated from mechanically defatted, non-GMO *Brassica napus* seeds. The resulting seedcakes were purified to a high total protein content through a solvent-free ultrafiltration process, which selectively enriches low molecular-weight proteins and peptides. A buffer solution was used to preferentially solubilize the low molecular-weight proteins and peptides prior to ultrafiltration. The two versions (*Brassica napus*, sample A and *Brassica napus*, sample B) were manufactured using different solubilization techniques: one with sodium chloride buffer adjusted with citric acid, and the other with ascorbic acid. These two versions were studied separately in preclinical experiments.

Using human-to-mouse allometric scaling[14] with 600 mg of TriFactor as the reference dose, the following treatment groups were studied:

Table 1.

Treatment Group	Mouse Doses (mg/kg)	HED (mg)
PBS (no treatment)	0	0
TriFactor (animal-based)	123 (HIGH)	600 (HIGH)
Brassica napus, sample A (plant-based)	21 (LOW) 62 (MED) 123 (HIGH)	100 (LOW) 300 (MED) 600 (HIGH)
Brassica napus, sample B (plant-based)	123 (HIGH)	600 (HIGH)

Materials

Wright stain and RPMI 1640 medium were bought from Sigma (St. Louis, USA), fetal calf serum (FCS) from Hyclone Laboratories (Logan, UT, USA).

Animals

Female, 8-week-old BALB/c mice were bought from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO2 asphyxiation.

Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier.[15] Briefly: peripheral blood cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The test tubes were incubated at 37° C for 60 min., with intermittent shaking. Smears were stained with Wright stain which allowed us to see the microparticles inside the cells. The cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

IL-2 secretion

Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) obtained from mice fed with samples were added into wells of a 24-well tissue culture plate. Cells were incubated for 48 hrs. in a humidified incubator (37° C, 5% CO_2/95% air). Addition of 1 µg of Concanavalin A (Sigma) was used as a positive control. At the end point of incubation, supernatants were collected, filtered through 0.45-µm filters and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

Antibody formation

The technique was described earlier.[16] Briefly: formation of antibodies was evaluated using ovalbumin (Sigma) as an antigen. Mice were injected twice, two weeks apart, with 100µg of albumin and the serum was collected 7 days after the last injection. Experimental groups were getting daily ip. injection of glucan. Levels of specific antibodies against ovalbumin were detected by ELISA. A combination of ovalbumin and Freund's adjuvant (Sigma) was used as a positive control.

NK cell assay

Splenic cells were isolated from the spleen of mice by standard methods. A cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. Cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6/ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with 50 µl of target cell line YAC-1. After spinning the plates at 250 x g for 5 min, the plates were incubated for 4 h at 37°C.

The cytotoxic activity of cells was determined using CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 10 µl of lysis solution was added into appropriate control wells 45 min before the end of incubation. The plates were spun at 250 x g for 5 min, followed by transferring 50 µl of the supernatant into flat-bottomed, 96-well microplates. After 50 µl of reconstituted substrate was added into each well, plates were covered and incubated for 30 minutes at room temperature in the dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC, USA) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity) = 100 X [(OD_{492}experimental − OD_{492} spontaneous)/(OD_{492}maximum−OD_{492}spontaneous)] as described in the manufacturer's instructions, where spontaneous release was that from target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

Statistical analysis

Paired t-test statistical significance was evaluated (GraphPad Prism 5.04; GraphPad Software, USA). An average and standard deviation was evaluated after determining standard value composition (D'Agostino, Pearson).

RESULTS

Proteomics Experiments

A comparative analysis of three brassica species—*Raphanus sativus, Brassica juncea*, and *Brassica napus*—was conducted using the UniProt database to assess protein biosimilarities. The results revealed a significant overlap among the species, as illustrated in **Figure 1**, with many of the identified proteins and peptides being smaller than 25 kDa.

SDS-PAGE analysis of the tested materials revealed a clear similarity between the *Brassica napus* and bovine colostrum samples, as evidenced by the presence of identical molecular-weight bands, particularly around 25 kDa and below, as shown in **Figure 2**. Nano-LC/MS/MS analysis of the <10 kDa

excised bands from each sample further confirmed this similarity, with peaks observed at approximately 7.2, 9.5, 13.1, 15.5, and 18.9 minutes, as depicted in **Figure 3**.

Figure 1. Venn diagram of identified proteins from Raphanus sativa, Brassica juncea, and Brassica napus obtained from the Uniprot database.

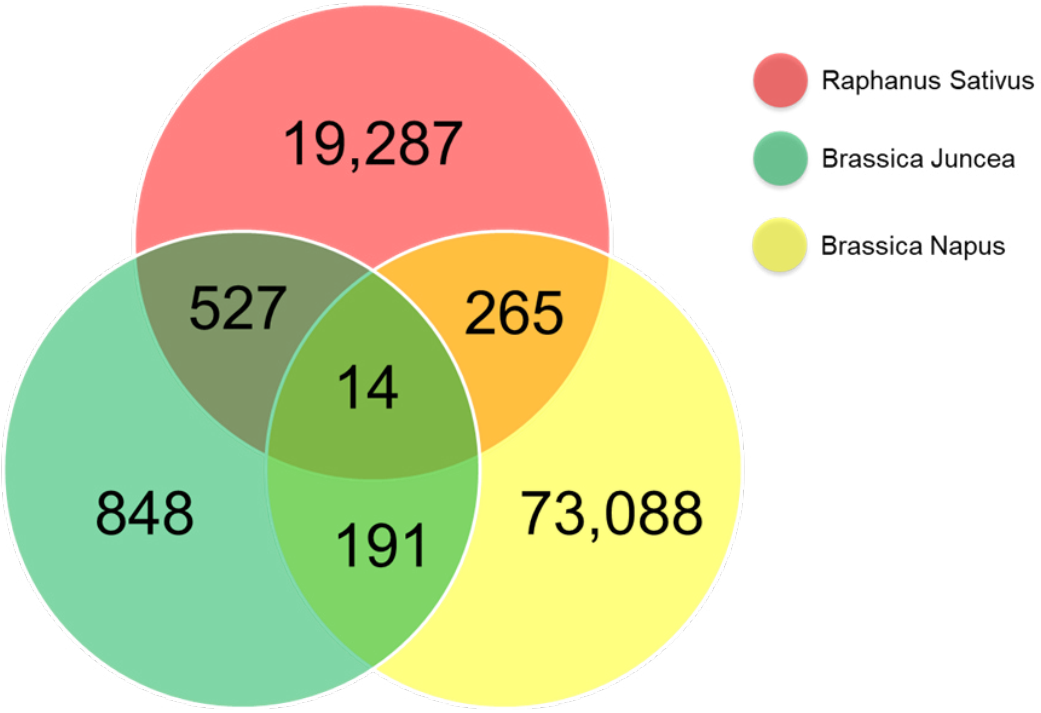


Figure 2. 1D Gel of 40 mg of (1) Brassica napus and (2) bovine colostrum. M is molecular weight markers.

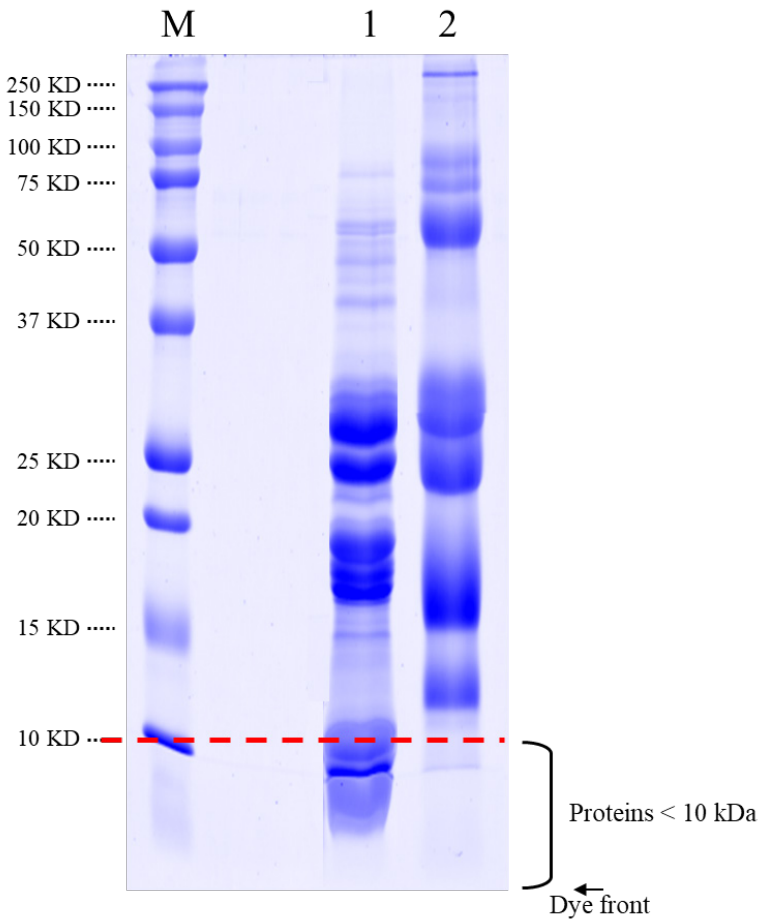
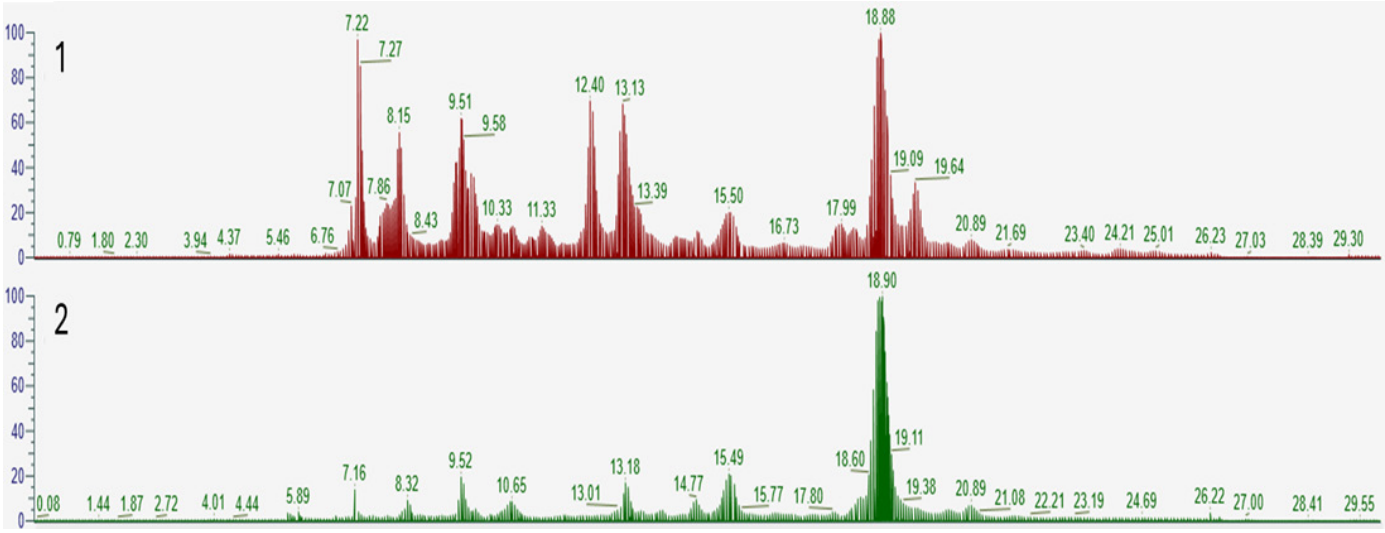


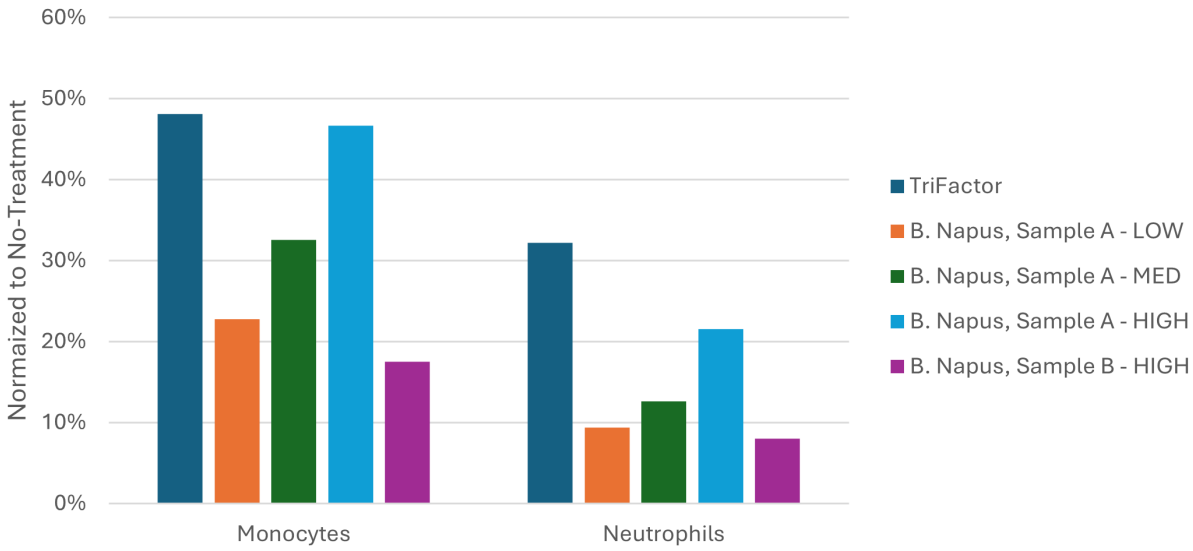
Figure 3. Nano-LC/MS/MS chromatographic plots of less than 10 kDa bands for (1) Brassica napus and (2) bovine colostrum.



Preclinical Experiments

Phagocytosis was assessed using synthetic HEMA particles, which minimize nonspecific adhesion to cell membranes. Peripheral blood monocytes and neutrophils were analyzed, with results shown in **Figure 4**. TriFactor and *Brassica napus* Sample A -MED, HIGH treatment groups significantly enhanced neutrophil activity, while all treatment groups stimulated monocyte phagocytosis. A dose-dependent response was observed in *Brassica napus* Sample A, with higher doses exhibiting greater activity than Sample B.

Figure 4. Phagocytosis of microparticles by peripheral blood neutrophils and monocytes with animal- and plant-based transfer factors. Experiments were done in triplicate, normalized to the no-treatment group and results reported as mean ± SD. All treatment groups were significantly different ($P \leq 0.05$) than no-treatment group, except for neutrophils for B. Napus, Sample A – LOW and B. Napus, Sample B – HIGH.



IL-2 secretion by splenocytes was measured to assess cytokine production. All treatment groups significantly increased IL-2 levels, with TriFactor having the strongest effect. *Brassica napus* Sample A showed a dose-dependent response, though not statistically significant, as shown in **Figure 5**. At higher doses, Sample A exhibited similar activity to Sample B.

NK cell activity was evaluated to assess the impact on cellular immunity. All treatment groups significantly increased YAC-1 cell killing, with TriFactor showing the strongest effect (**Figure 6**). A dose-dependent response was observed in *Brassica napus* Sample A, with the high-dose group exhibiting greater activity than Sample B.

Humoral immunity was assessed by measuring antibody production in mice immunized with ovalbumin, with samples administered alongside two intraperitoneal antigen injections. Ovalbumin with Freund's adjuvant served as a positive control.

As shown in **Figure 7**, all treatment groups significantly enhanced antibody production, with TriFactor having the strongest effect. *Brassica napus* Sample A exhibited dose-dependent responses, though Sample B elicited a higher overall response.

Figure 5. IL-2 secretion in splenocytes. Addition of Concanavalin A was used as a positive control Experiments were done in triplicate, normalized to the no-treatment group and results reported as mean ± SD. All treatment groups were significantly different ($P \leq 0.05$) than no-treatment group.

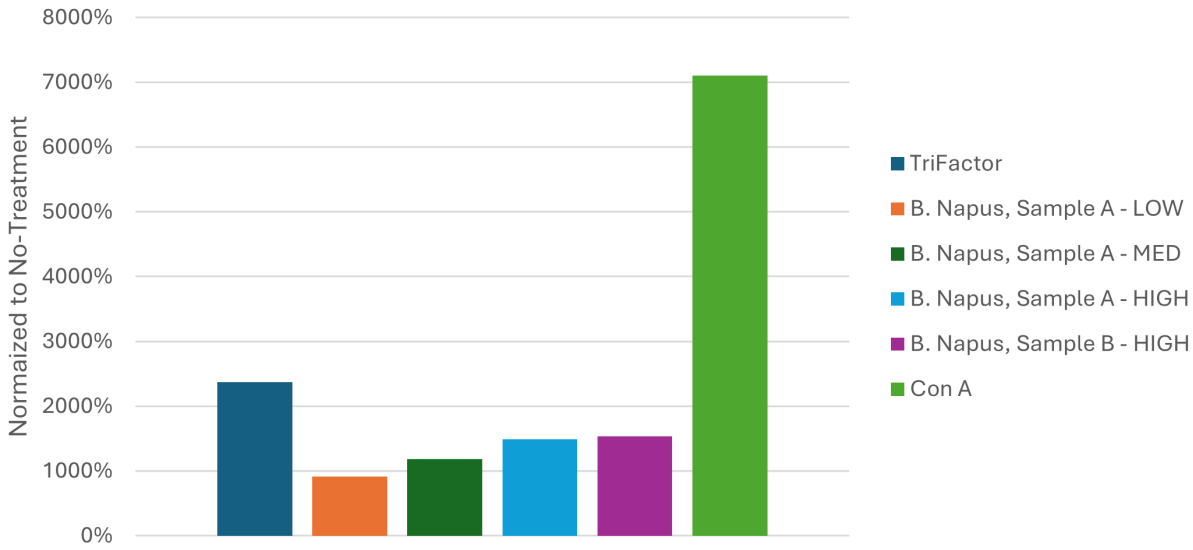


Figure 6. NK cell activity against YAC-1 tumor cells with animal- and plant-based transfer factors using a target to effector ratio of 50:1. Experiments were done in triplicate, normalized to the no-treatment group and results reported as mean ± SD. All treatment groups were significantly different ($P \leq 0.05$) than no-treatment group.

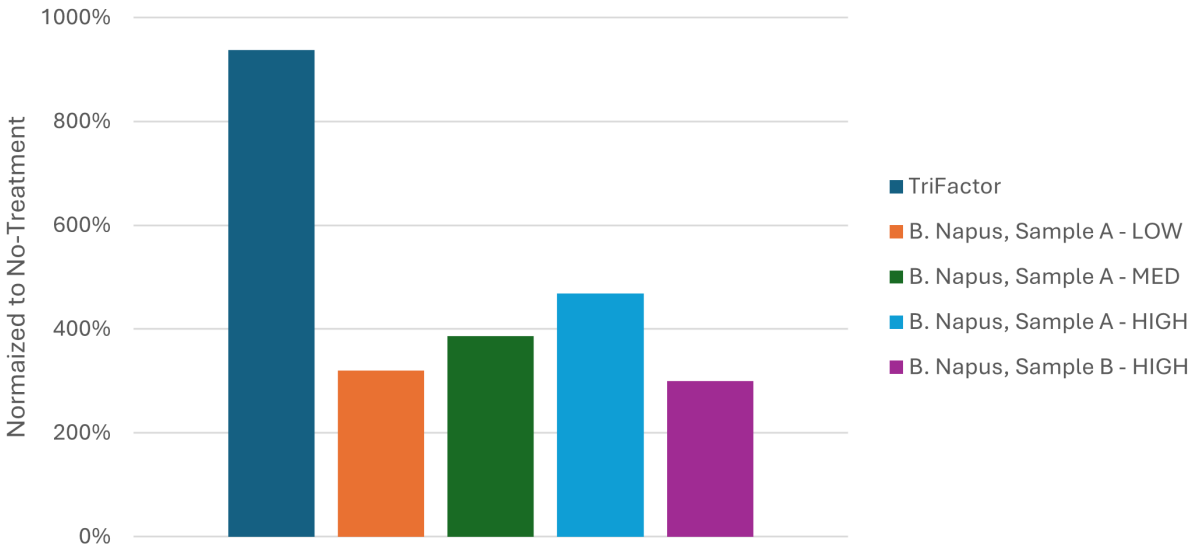
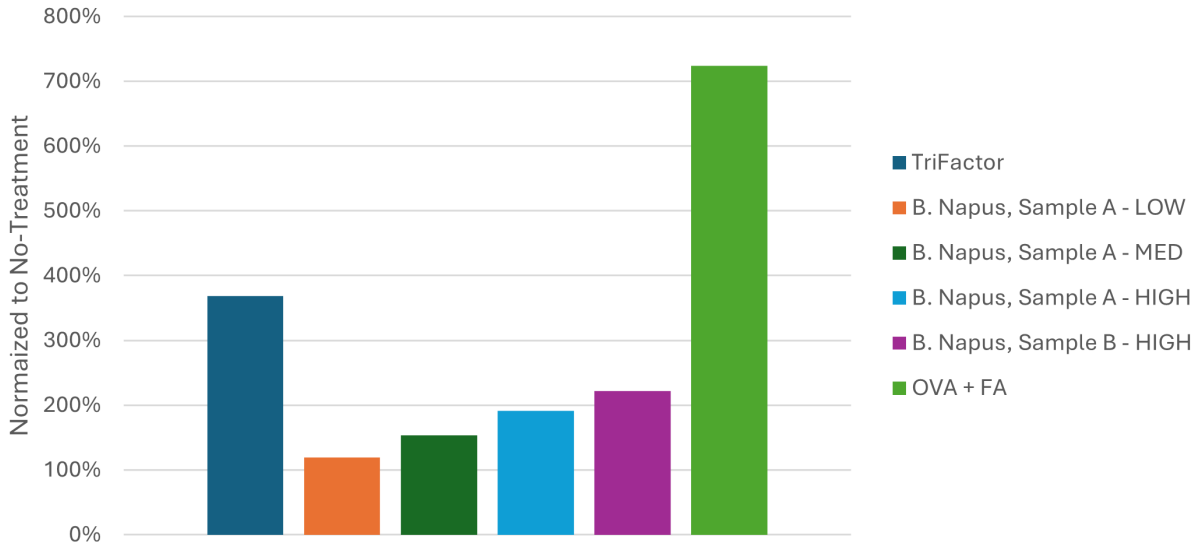


Figure 7. Formation of antibodies against ovalbumin with animal- and plant-based transfer factors. Animals were injected twice (two weeks apart) with antigen and the serum was collected 7 days after the last injection. Freund's adjuvant was used as positive control. Experiments were done in triplicate, normalized to the no-treatment group and results reported as mean ± SD. All treatment groups were significantly different ($P \leq 0.05$) than no-treatment group.



DISCUSSION

Transfer factors have been used as natural supplements for decades, though originally studied for their antigen-specific immune support.[17] Over time, transfer factor production shifted from blood-based to bovine colostrum and chicken egg yolks, enabling their commercialization as dietary supplements with broader immune health claims.

Despite the many benefits of animal-based transfer factors, sourcing limitations, cost, and environmental concerns have driven interest towards plant-based alternatives. Plants own sophisticated defense mechanisms, often using low molecular-weight peptides for immune-modulating properties.[18,19] Recent research confirmed transfer factor-like activity in certain low molecular-weight plant-based proteins.[7] However, due to the lack of scalable protein isolates from *Raphanus sativa* and *Brassica juncea*, this study investigated upcycled *Brassica napus* as an alternative.

Proteomics analysis revealed high similarity among these brassica species, particularly in defensins—peptides with >90% bio-similarity.[20] Their presence may explain the immune activity observed here and suggests a functional[21] and conservatory[22] link to animal-based transfer factors. This connection was further supported by nano-LC/MS/MS analysis, which identified defensin-like protein 1 (P69240 · DEF1_BRANA) in *Brassica napus* and beta-defensin 5 (P46163 · DEFB5_BOVIN) in bovine colostrum, both involved in antimicrobial defense. Notably, plant defensins also exhibit potential anti-cancer properties presumably by activating adaptive immunity.[23]

Preclinical studies here also showed anti-tumor effects in all treatment groups using an NK cell assay against YAC-1, a murine lymphoblast tumor cell line.[24] As previously observed, animal-based transfer factors had the strongest effects.12,13 Additional immune responses, including enhanced phagocytosis, cytokine production, and antibody formation, suggest both plant- and animal-based transfer factors function as natural immunomodulators.

Dose-dependent responses were observed for *Brassica napus*, Sample A across all immune system activity measures, supporting allometric scaling for human applications. For animal-based transfer factors (e.g., TriFactor), a 600-mg dose has shown rapid immune activation in human clinical studies[10] which extrapolates to the high-dose treatment groups in this study.

Interestingly, the *Brassica napus*, sample A high-dose group generally surpassed sample B high-dose group in the various immune system activity measures, with a more potent response. This suggests there may be higher concentrations of isolated transfer factors from a slightly different manufacturing process between the two samples, where the Sample A process favors a more efficient isolation of the low molecular-weight peptides and proteins compared to Sample B process. Similar trends were observed in unpublished data comparing these two samples in *ex vivo* natural killer cytotoxicity experiments.[25]

CONCLUSION

This study highlights *Brassica napus* as a promising plant-based alternative to animal-based transfer factors. Proteomic analysis revealed that *Brassica napus* shares key immune-modulating peptides, such as defensins, with bovine colostrum. Preclinical tests confirmed that both plant- and

animal-based transfer factors enhance immune functions like phagocytosis and cytokine production. Notably, *Brassica napus*, sample A showed stronger immune responses than sample B, suggesting that the low molecular-weight peptides play a key role in immune system activity.

These findings also support the potential of *Brassica napus* as a sustainable, cost-effective source of natural immunomodulators, offering an environmentally friendly alternative to animal-based supplements. Further research is needed to refine its use for human application, potentially looking at varying combinations of animal- and plant-based transfer factors for broader immune system health.

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