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Immune Activation by a Nutraceutical Blend: Rapid Increase in Immune-Modulating Cytokines, Followed by Induction of Anti-Inflammatory and Restorative Biomarkers

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Abstract: Immune cells express Pattern Recognition Receptors (PRRs) to recognize potentially pathogenic microbial forms. Nutraceutical compounds can induce immune cell activation through PRRs. The nutraceutical immune blend (IB), QuickStart™, contains botanical and yeast-derived ligands for PRRs, along with vitamin C and zinc. We evaluated immune-activating effects of the IB and its ingredients in vitro. Human peripheral blood mononuclear cells were treated with either the IB or single ingredients: elderberry extract, the proprietary *Saccharomyces cerevisiae* fermentate EpiCor™ (Sacc), the plant-based hemicellulose preparation Natramune (PDS-2865)™ (Hemi), vitamin C (VitC), or zinc gluconate (Zinc). The IB triggered sequential waves of immune activation. Initial cytokine induction by the IB at 2 h involved the immune-activating cytokines IL-6, IL-8, MIP-1α, and TNF-α, and the stem cell-mobilizing growth factor G-CSF, as did Sacc and Hemi. The 24 h immune-activation by the IB included increases in IL-1β, IL-17A, IP-10, GM-CSF, Basis FGF, PDGF-BB, and the anti-inflammatory cytokine IL-10. Increased CD69 expression by the IB was also seen for VitC and Sacc. Increased CD25 expression by the IB on monocytes was also seen for Sacc. The IB triggered rapid immune activating events of higher magnitude than the single ingredients, involving immune-activating cytokines and restorative growth factors. Clinical research is warranted to evaluate rapid immune-modulating events upon consumption.

Keywords: anti-inflammatory; CD25; CD69; cytokines; immune-modulation; restoration

Citation: McGarry, S.V.; Yu, L.; Cruickshank, D.; Iloba, I.; Jensen, G.S. Immune Activation by a Nutraceutical Blend: Rapid Increase in Immune-Modulating Cytokines, Followed by Induction of Anti-Inflammatory and Restorative Biomarkers. *Nutraceuticals* **2024**, *4*, 35–49. https://doi.org/10.3390/nutraceuticals4010003

Academic Editor: Mario Allegra

Received: 13 September 2023
Revised: 10 November 2023
Accepted: 1 December 2023
Published: 2 January 2024

1. Introduction

Health, as defined by the absence of illness, depends on the constant activity of our immune system. Immune cells travel throughout the body in a state of passive awareness but are rapidly engaged to a higher state of alertness upon recognition of potential threats. Our immune system responds to potentially harmful substances rapidly and communicates system-wide by means of cellular surveillance activity and secreted cytokines.

Immune cells express Pattern Recognition Receptors (PRRs) on the cell surface for the recognition of microbial lifeforms. Immune cell PRRs include Toll-Like Receptors (TLRs) and C-type Lectin Receptors (CLRs) (Table 1) [1–3]. These receptors are well characterized for the recognition of bacterial and fungal antigens, including beta-glucans. CLRs also recognize patterns associated with plant-based polysaccharides such as arabinogalactans, arabinoxylans, and alpha-mannans [4–7]. The engagement of PRRs on antigen-presenting immune cells such as macrophages and dendritic cells leads to an elevated state of alertness, where additional signals are required to mount a full state of immune defense activation. The co-stimulation of immune cells via multiple PRRs has synergistic effects [8].

Table 1. Pattern Recognition Receptors of relevance to nutraceutical products.

Receptor Class	Receptor Type	Ligands
Toll-Like Receptors	TLR-1	Lipoprotein [B]
	TLR-2	Peptidoglycans [B], Beta-glucans [F]
	TLR-4	Lipopolysaccharides [B(GN)]
	TLR-2/TLR-6	Beta-Glucans [F]
C-Type Lectin Receptors	Dectin-1	Beta-Glucans [F]
		Arabinoxylans [P]
	Dectin-2	Alpha-mannans [F,P]

[B]: bacterial origin; [B(GN)]: Gram-negative bacteria; [F]: fungal origin; [P]: plant-based origin.

From a nutraceutical perspective, there is abundant potential for designing blends with different, complementary mechanisms of action for a more robust support of innate immune alertness than any single ingredient alone. When consuming nutritional supplements such as probiotic bacteria and yeasts, PRRs on the surface of antigen-presenting dendritic cells are engaged in the gut mucosal lining. The activation of immune cells via PRRs mediates protective alertness of the community of immune cells residing in the gut mucosal immune tissue, and via cytokine secretion, also communicates to distant immune cells [9].

A nutraceutical immune blend (IB), Quick-Start™, was formulated based on three ingredients that contain compounds which engage PRRs to seek a synergistic effect at the cellular level, where several types of immune cell surface receptors would be engaged on multiple types of immune cells. Those ingredients include a yeast-based fermentate, a hemicellulose-rich plant extract, and elderberry. The IB also contains vitamin C and zinc for additional support of various intracellular functions during an active immune response.

The nutritional yeast-based fermentate, EpiCor™, with immune-activating and -modulating properties, is a proprietary fermentate from *Saccharomyces cerevisiae* (here, abbreviated as Sacc), a yeast organism used in baking and brewing. Yeast cell walls contain fungal beta-glucans able to engage TLR-2 and TLR-2/6 receptors on immune cells. This postbiotic whole food fermentate has been shown to activate immune cells including NK cells and alter the expression of chemokine receptors on immune cells [10], suggesting increased chemotactic awareness and responsiveness to homing signals. The consumption of Sacc results in rapid changes in immune surveillance, specifically associated with increased trafficking and homing of NK cells and T cells in vivo, and increased alertness as seen by elevated expression of the activation markers CD25 and CD69 on NK cells in blood 1–2 h after consuming a single dose of Sacc [11]. Long-term consumption of Sacc was clinically proven to reduce the severity and duration of symptoms of cold and flu [12] as well as allergies [13]. In addition to the immune-activating properties, the Sacc fermentate also contains polyphenolic antioxidants likely contributing to the anti-inflammatory properties of the fermentate seen both in vitro and in a human cross-over trial of acute histamine-induced inflammation [10,14].

Plant-based polysaccharides have additional immune-activating properties in vitro and in vivo [15]. A hemicellulose-rich nutraceutical formulation, Natramune PDS-2865, (Hemi), contains predominantly arabinoxylan, a well-known immune-modulating plant-based polysaccharide [16], as well as immune-modulating arabinogalactans and mannans. This consumable formula has been shown to increase killer cell response rate and cytotoxicity [17], stimulate macrophage phagocytosis in vitro [18], and at the same time, exhibit anti-inflammatory properties via inhibition of T cell adhesion to fibronectin [19]. Consuming the Hemi for 8 weeks was associated with a mild but significant increase in the levels of circulating lymphocytes in human study participants [18].

Elderberry (*Sambucus nigra*) is a shrub in the *Adoxaceae* family of musk herbs used in traditional medicine throughout Europe, Northern Africa, Western Asia, and North America to aid the immune systems' ability to fight infections [20]. Elderberry contains numerous bioactive and immune-modulating compounds [21], including lectins with direct anti-viral properties [22]. Elderberry is also rich in polyphenolic compounds including

anthocyanins, which contribute to the color of the fruit and have potent antioxidant and anti-inflammatory properties [23–27]. Flavonoids also contribute immune-modulating effects, binding directly to viral agents and preventing their recognition and entry into cells [28]. Polysaccharides in elderberry were shown clinically to reduce upper respiratory symptoms when supplemented in patients with influenza A and B [29–32].

Vitamin C, or ascorbic acid, is an essential micronutrient that contributes to immune defense, acting as a co-factor in several regulatory and biosynthetic enzymes [33–36]. Primates, including humans, lack the capacity for endogenous vitamin C biosynthesis, so it must be obtained through diet, primarily through fruits, vegetables, and organ meat [37,38]. Vitamin C acts as an antioxidant and reduces oxidative damage in biological systems [39,40]. Vitamin C is an epigenetic regulator, promoting DNA demethylation, thereby activating genes and preventing hypermethylation, a source of genetic instability [41–44].

Zinc is an essential micronutrient that catalyzes enzymatic activity, mediates signal transduction, and directly regulates gene expression [45,46]. Zinc is a component of zinc fingers, which are small protein domains that constitute site-specific targeting domains, wherein zinc ions coordinate the 3D structure and therefore the function of the protein [47]. Zinc finger proteins serve as transcriptional regulators in wide arrays of cellular functions [48]. Zinc plays important roles in immunity where it is required for the development of peripheral blood mononuclear cells: macrophages, monocytes, and natural killer T cells [49,50]. Of particular interest to immune activation by PRRs, zinc ions participate as regulators of TLR-mediated signaling pathways [51,52]. The necessity of dietary zinc is illustrated by the long list of symptoms that occur with zinc deficiency, including hair loss, compromised wound healing, diarrhea, and impotence, to name a few [53].

The immune-activating properties of the nutraceutical immune blend IB was compared to the five ingredients to establish which ingredients contributed to the overall activity of the blend and to look for synergistic effects where the IB would act more strongly than the sum of each ingredient's contribution. The rationale for performing the work presented here was to document the immune activation and modulation by each ingredient and compared this to the IB at matching doses to evaluate whether and to what extent each ingredient contributes to the efficacy of the IB.

2. Materials and Methods

2.1. Reagents

Roswell Park Memorial Institute 1640 medium, penicillin–streptomycin 100×, interleukin-2 (IL-2), phosphate-buffered saline (PBS), and bacterial lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Monoclonal antibodies toward immune cell markers included anti-CD69 fluorescein isothiocyanate (clone FN50, cat# 11-0699-42), anti-CD56 phycoerythrin (clone CMSSB, cat# 12-0567-42), and anti-CD3 peridinin chlorophyll protein (clone OKT3, cat# 67-0038-42), were obtained from Thermo-Fisher (Waltham, MA, USA). The anti-CD25 monoclonal antibody conjugated to Brilliant Violet-421 (clone 2A3, cat# 564033) and heparin vacutainer tubes were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Customized Bio-Plex Pro™ human cytokine arrays were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

2.2. Test Products

The nutraceutical blend Quick-Start was supplied by the manufacturer LifeSeasons Inc., (Kaysville, UT, USA), along with the 5 ingredients, elderberry extract (Elder), EpiCor (Sacc), Natramune (Hemi), vitamin C (VitC), in the form of liposomal ascorbic acid of the brand PurewayC, and zinc gluconate (Zinc). Stock solutions of each test product were prepared fresh on the morning of each day of testing to ensure no degradation of bioactive compounds with storage in liquid medium. The dosing for this study aimed to compare the magnitude of an immune-activating event of a single ingredient to the blend, such that the dose of the IB and the dose of an ingredient would match the amount of that particular

ingredient in the IB. The percentage of each ingredient in the final formulation of IB is shown in Table 2, along with the doses of each ingredient.

Table 2. Test products compared in this project.

Name	Abbreviation	% in Immune Blend *	Dose in Assays (mg/mL) **
Immune Blend	IB	100%	1.000
ElderMune	Elder	3.36%	0.033
Natramune hemicellulose extract	Hemi	16.8%	0.168
Saccharomyces cerevisiae	Sacc	16.8%	0.168
PureWay Vitamin C	VitC	33.6%	0.338
Zinc gluconate	Zinc	0.50%	0.005

* The remaining 29% of the product includes 21% Gum acacia. There are also very small amounts of vitamin D, olive leaf, Andrographis, and below 0.4% of a probiotic. ** mg/mL refers to mL of cell culture medium in the assay.

2.3. Immune Cell Activation

Peripheral venous blood was drawn from healthy human donors upon written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board, Federalwide Assurance 2603. The blood was drawn into heparin vacutainer vials, and the peripheral blood mononuclear cells (PBMC) were isolated using Lympholyte Poly (Cedarlane Labs, Burlington, NC, USA) by centrifugation for 35 min at $400\times g$. The PBMC were washed twice in PBS, counted, and adjusted to establish cultures with a cell density at 10^6/mL, using Roswell Park Memorial Institute 1640 medium containing the antibiotics penicillin and streptomycin and 10% heat-inactivated fetal calf serum.

Serial dilutions of test products were added, and cultures were then incubated at 37 °C, 5% CO_2 for 2 and 24 h. The highly inflammatory LPS from *Escherichia coli* was used as a positive control for immune-cell activation (5 µg/mL). In parallel, IL-2 was used as a positive control for natural killer (NK)-cell activation at a concentration of 100 IU/mL. Untreated negative control cultures consisted of PBMC exposed to phosphate-buffered saline in the absence of test products. All treatments, including each dose of a test product and each positive and negative control, were tested in triplicate. After 24 h, blood cells were isolated from each culture well and stained for 15 min with fluorochrome-labeled antibodies: 5 µL CD3, 1.6 µL CD25, 3 µL CD56, and 5 µL CD69 antibodies. PBMC were then fixed using 0.5% formalin. The fluorescence intensities for CD3, CD25, CD56, and CD69 were measured by flow cytometry, using an Attune NxT acoustic-focusing flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis utilized gating on forward/side scatter to gate on lymphocytes versus monocytes, where the lymphocyte population was subsequently gated into CD3 + CD56- T lymphocytes, CD3 + CD56+ NKT cells, and CD3-CD56+ NK cells. Evaluation of CD25 and CD69 expression was then performed on each subset.

2.4. Production of Cytokines, Chemokines, and Growth Factors

After 2 and 24 h of incubation, the supernatants were harvested from the PBMC cultures described above. The levels of 27 cytokines and chemokines were quantified using Bio-Plex protein arrays (Bio-Rad Laboratories Inc.) and utilizing xMAP technology (Luminex, Austin, TX, USA). The following analytes were tested: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. In brief, during manufacturing, the 27 types of magnetic beads (one type for each analyte) were dyed internally to produce a signature for each bead type, pre-coated with capture antibodies toward analytes, and mixed, allowing for simultaneous quantification of the 27 analytes. The testing of cytokine levels in the culture supernatants was performed in 96-well plates by adding magnetic beads to supernatants and incubating for 60 min,

after which the beads were washed on a magnet to remove unbound proteins. Biotinylated detection antibodies were added, and the samples were incubated for 45 min and washed to remove unbound detection antibodies. Streptavidin-PE was added and incubated for 10 min, and the beads were washed again to remove unbound streptavidin-PE. The fluorescence intensity of the beads was documented using a MagPix microplate reader, and the mean fluorescence intensity of the analytes in each sample was calculated using the xPonent software (Luminex, Austin, TX, USA).

2.5. Statistical Analysis

Average and standard deviation for each data set was calculated using Microsoft Excel. Statistical analysis of in vitro data was performed using the two-tailed, independent *t*-test. A statistical trend was set at $p < 0.10$, statistical significance was set at $p < 0.05$ and a high level of significance at $p < 0.01$.

3. Results

3.1. Early-Responding Cytokines

The immune blend (IB) showed rapid and highly selective immune cell-activating properties (Figure 1). From the results, the peripheral blood mononuclear cells (PBMC) treated with the IB were actively secreting multiple pro-activating cytokines already at 2 h, reaching statistical significance ($p < 0.05$) for Interleukin-8 (IL-8, Figure 1B), and a high level of statistical significance ($p < 0.01$) for Interleukin-6 (IL-6, Figure 1A), Macrophage inflammatory protein-1 alpha (MIP-1α, Figure 1C), and tumor necrosis factor-alpha (TNF-α, Figure 1D). Among the five ingredients, the *Saccharomyces* extract (Sacc) contributed to this early response for all four cytokines, and the increase at 2 h reached statistical trends for IL-8 and TNF-α when compared to untreated control cultures ($p < 0.1$, Figure 1B,D). Vitamin C (VitC) contributed to the 2 h increase in IL-8 production, reaching statistical trends ($p < 0.1$, Figure 1B). The plant-based hemicellulose extract (Hemi) induced increased production of all four cytokines, where the increase reached a statistical trend ($p < 0.1$, Figure 1C) for MIP-1α, statistical significance ($p < 0.05$, Figure 1D) for TNF-α, and a high level of significance ($p < 0.01$, Figure 1B) for IL-8. Neither elderberry nor zinc contributed to this early response at 2 h; both ingredients triggered a mild decrease in IL-8 and MIP-1α at 2 h (Figure 1B,C), reaching statistical significance ($p < 0.05$) for elderberry. The magnitude of the 2 h induction of IL-8, MIP-1α, and TNF-α by the IB was at least two-fold stronger than any of the contributing ingredients.

After 24 h, the reduction in the levels of IL-8 triggered by elderberry and zinc (Figure 1B) reached a high level of significance ($p < 0.01$) when compared to untreated control cultures. At both 2 and 24 h, the magnitude of IL-6, MIP-1α, and TNF-α induced by the IB was lower than the levels induced by the LPS control.

The synergistic effect of the ingredients induced higher cytokine levels than what could be expected from contributions of each immune-activating ingredient. This was particularly clear for TNF-α production after 24 h (Figure 1D, right), where the IB triggered five-fold higher levels than the strongest single ingredient Sacc.

3.2. Late-Responding Cytokines

The early immune cell activation by the IB resulted in a second wave of cytokine production, seen after 24 h of cell culture (Figure 2). The late-responding cytokines showed no increase at 2 h but showed increased levels after 24 h, including Interleukin-1 beta (IL-1β), Interleukin-10 (IL-10), Interleukin-17A (IL-17A), and Interferon gamma-induced protein 10 (IP-10). The IB-induced levels of IL-1β at both 2 and 24 h and of IL-10 at 24 h remained below those induced by the positive control, LPS. There was no significant difference between the IB-induced levels of IL-17A or IP-10 compared to the LPS-treated control cultures. The IB-induced increased levels of IL-10 (Figure 2B) and IP-10 (Figure 2D) reached statistical trends ($p < 0.1$), and the levels of IL-1β (Figure 2A) and IL-17A (Figure 2C) reached high levels of significance ($p < 0.01$) when compared to untreated control cultures. Only one

ingredient, Sacc, also caused increases in these four cytokines, but to a much lesser degree. The Sacc-induced increase in IL-1β (Figure 2A) and IP-10 (Figure 2D) reached statistical significance ($p < 0.05$), and the increased levels of IL-10 (Figure 2B) and IL-17A (Figure 2C) reached high levels of significance ($p < 0.01$) when compared to untreated control cultures. If the increased levels of these cytokines were induced only by the ingredient Sacc, then the cytokine levels of the IB and Sacc should be of similar magnitude; however, the data showed that the IB induced at least two-fold higher levels of these cytokines than Sacc, suggesting that impacts from the other four single ingredients contributed to a robust effect by the IB above that of Sacc alone.

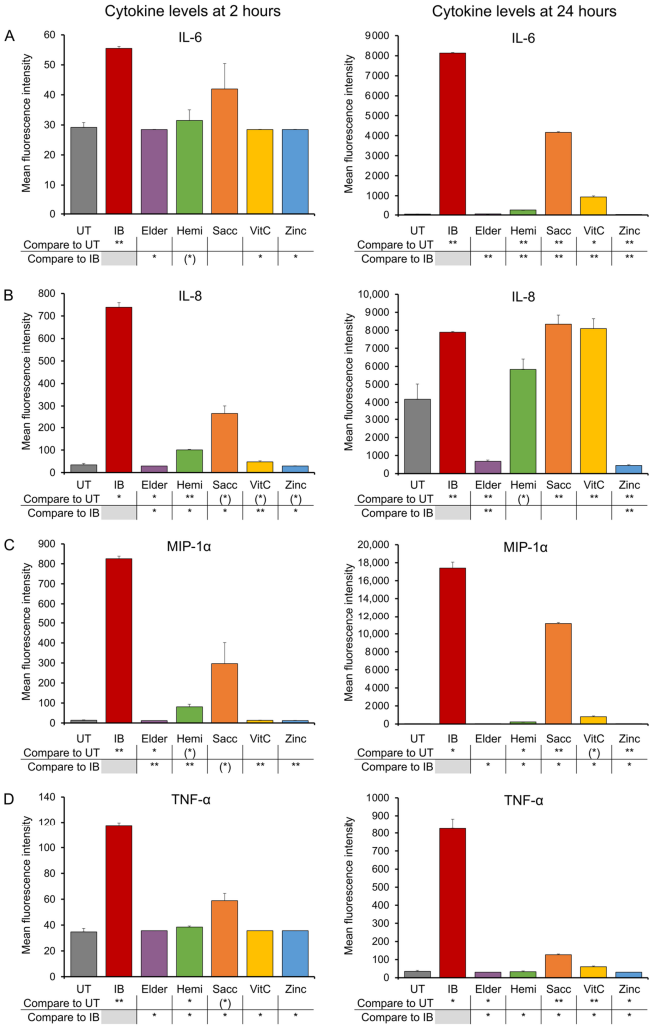

Figure 1. Early-responding cytokines in culture supernatants from 2 h (**left** panel of graphs) and 24 h PBMC cultures (**right** panel of graphs): untreated control cultures (UT), cultures treated with the immune blend (IB), elderberry extract (Elder), hemicellulose extract (Hemi), *Saccharomyces cerevisiae* fermentate (Sacc), vitamin C (VitC), and zinc gluconate (Zinc). (**A**). Interleukin-6 (IL-6). (**B**). Interleukin-8 (IL-8). (**C**). Macrophage inflammatory protein-1 alpha (MIP-1α). (**D**). Tumor necrosis factor-alpha (TNF-α). The cytokine levels for the LPS-treated control cultures at 2 h were 153 ± 25 for IL-6, 640 ± 61 for IL-8, 5791 ± 1067 for MIP-1α, and 394 ± 27 for TNF-α. The cytokine levels for the LPS-treated control cultures at 24 h were 9865 ± 386 for IL-6, 7793 ± 1934 for IL-8, 18,312 ± 257 for MIP-1α, and 901 ± 60 for TNF-α. For each test product, results are shown as the average ± standard deviation of duplicate samples. In the table below the graph, statistical significance at different doses is indicated by asterisks, when $p < 0.10$: (*), $p < 0.05$: *, and $p < 0.01$: **.

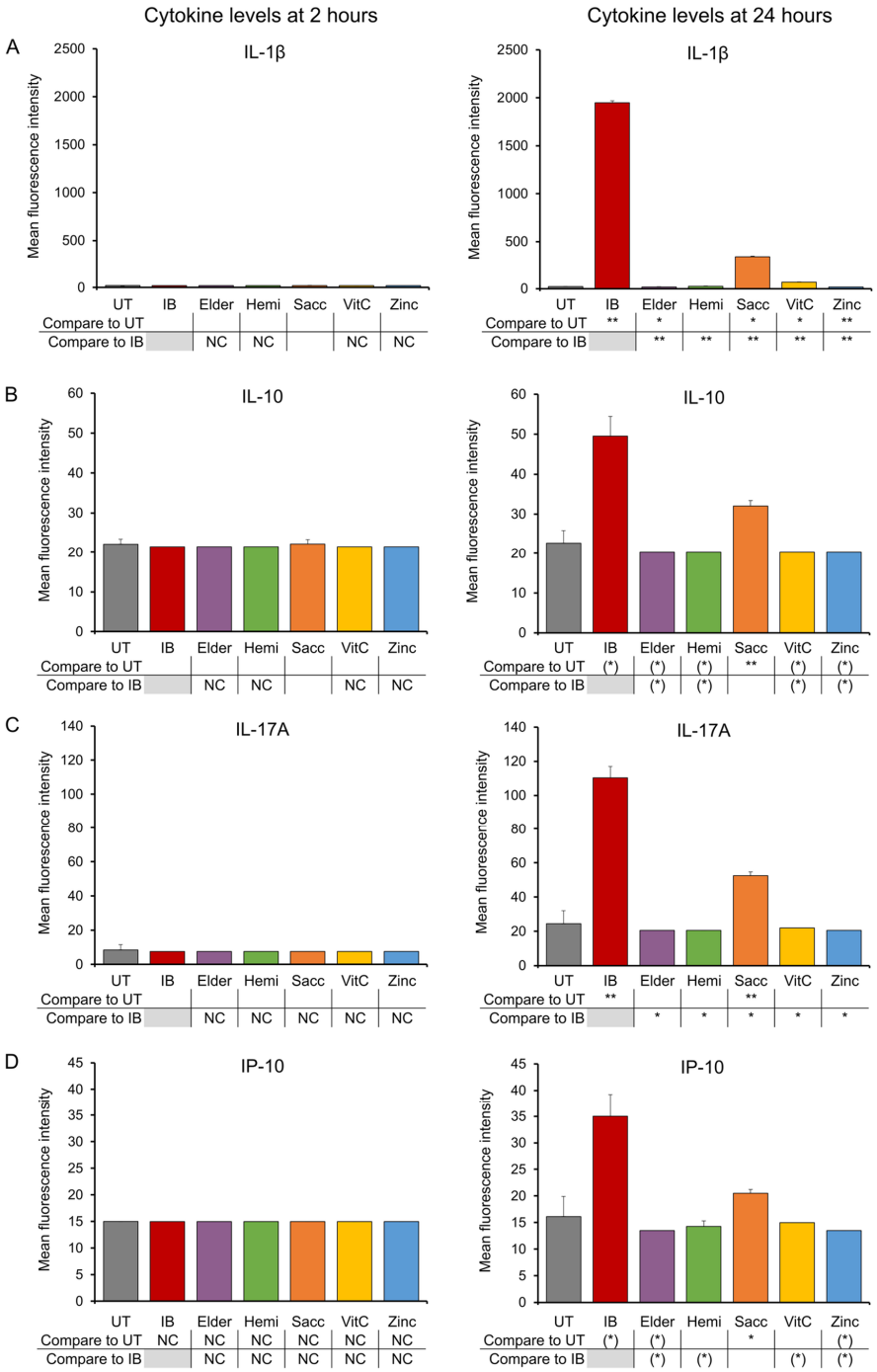

Figure 2. Late-responding cytokines in culture supernatants from 2 h (**left** panel of graphs) and 24 h PBMC cultures (**right** panel of graphs): untreated control cultures (UT), cultures treated with the immune blend (IB), elderberry extract (Elder), hemicellulose extract (Hemi), *Saccharomyces cerevisiae* fermentate (Sacc), vitamin C (VitC), and zinc gluconate (Zinc). (**A**). Interleukin-1 beta (IL-1β). (**B**). Interleukin-10 (IL-10). (**C**). Interleukin-17A (IL-17A). (**D**). Interferon-gamma-inducible protein 10 (IP-10). The cytokine levels for the LPS-treated control cultures at 2 h were 76 ± 21 for IL-1β, 22 ± 1 for IL-10, 11 ± 2 for IL-17A, and 17 ± 1 for IP-10. The cytokine levels for the LPS-treated control cultures at 24 h were 4175 ± 172 for IL-1β, 311 ± 46 for IL-10, 112 ± 4 for IL-17A, and 39 ± 1 for IP-10. For each test product, results are shown as the average ± standard deviation of duplicate samples. In the table below the graph, statistical significance at different doses is indicated by asterisks, when $p < 0.10$: (*), $p < 0.05$: *, and $p < 0.01$: **.

3.3. Growth Factors

The early activation by the IB also induced increased levels of the growth factor granulocyte colony-stimulating factor (G-CSF, Figure 3A), known for its effects on stem cell mobilization during reparative functions. Both the ingredients Hemi and Sacc contributed to this early effect, where G-CSF levels were highly significantly increased ($p < 0.01$) in cultures treated with Hemi, and the effect of Sacc did not reach statistical significance. After 24 h, the G-CSF levels were further increased, primarily due to the effects of Sacc but also with contributing effects from Hemi and VitC.

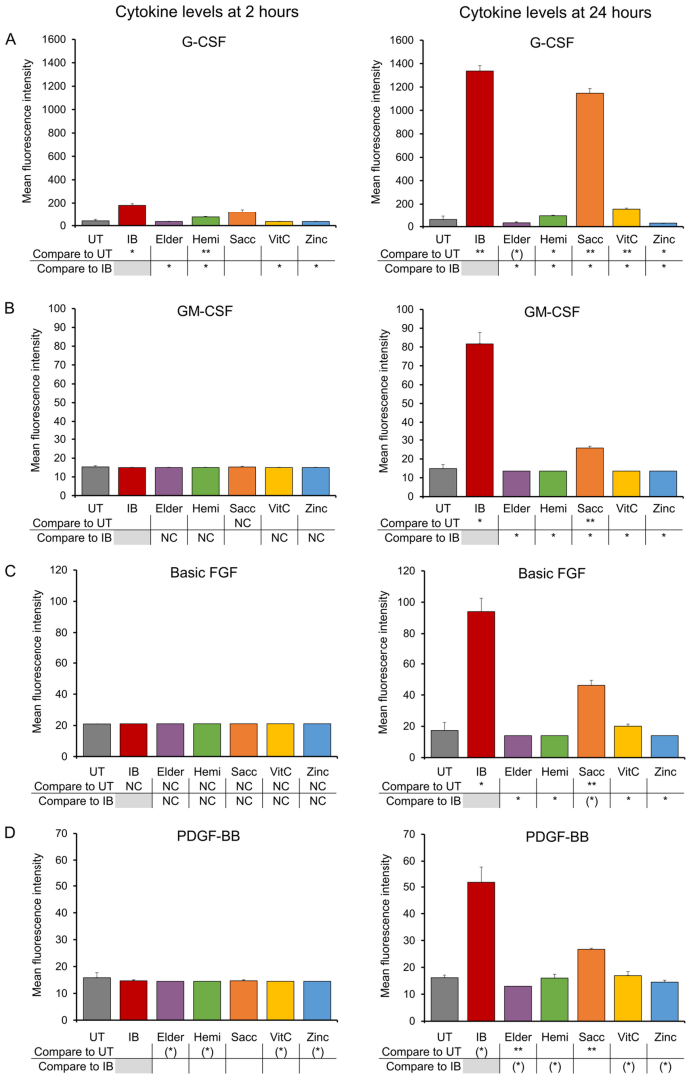

Figure 3. Growth factors in culture supernatants from 2 h (**left** panel of graphs) and 24 h PBMC cultures (**right** panel of graphs): untreated control cultures (UT), cultures treated with the immune blend (IB), elderberry extract (Elder), hemicellulose extract (Hemi), *Saccharomyces cerevisiae* fermentate (Sacc), vitamin C (VitC), and zinc gluconate (Zinc). (**A**). Granulocyte colony-stimulating factor (G-CSF). (**B**). Granulocyte-macrophage colony-stimulating factor (GM-CSF). (**C**). Basic fibroblast growth factor (Basic FGF). (**D**). Platelet-derived growth factor BB (PDGF-BB). The cytokine levels for the LPS-treated control cultures at 2 h were 708 ± 132 for G-CSF, 17 ± 1 for GM-CSF, 29 ± 2 for Basic FGF, and 19 ± 1 for PDGF-BB. The cytokine levels for the LPS-treated control cultures at 24 h were 1518 ± 390 for G-CSF, 46 ± 2 for GM-CSF, 89 ± 7 for Basic FGF, and 45 ± 3 for PDGF-BB. For each test product, results are shown as the average ± standard deviation of duplicate samples. In the table below the graph, statistical significance at different doses is indicated by asterisks, when $p < 0.10$: (*), $p < 0.05$: *, and $p < 0.01$: **.

In contrast, none of the other three growth factors tested were increased at 2 h but showed increased levels after 24 h. The increased levels of G-CSF, Basic FGF, and PDGF-BB in the IB-treated cultures were in similar ranges as the LPS-induced levels of these growth factors, whereas the GM-CSF levels in the IB-treated cultures exceeded that of the LPS control. The IB-induced increased levels at 24 h reached a statistical trend ($p < 0.1$) for PDGF-BB (Figure 3D) and significance ($p < 0.05$) for GM-CSF (Figure 3B) and Basic FGF (Figure 3C). The increase triggered by the IB was at least two-fold higher than any of the single ingredients.

3.4. Expression of CD25 and CD69 Activation Markers on Immune Cell Subsets

Immune cell activation was evaluated after 24 h in PBMC cultures, where the expression levels of the two activation markers CD25 and CD69 were evaluated on NK cells, NKT cells, T cells, and monocytes (Figure 4). Immune cell activation was evident in cultures treated with the IB, where the activation marker CD25 was increased on NK cells, NKT cells, and monocytes, with statistical significance ($p < 0.05$) for NK and NKT cells and a high level of statistical significance ($p < 0.01$) for monocytes compared to the expression levels of the same cell type in untreated control cultures. The IB-induced increase in CD25 expression on NK and NKT cells exceeded that of the LPS-treated control cultures, whereas the CD25 levels on monocytes were lower than in the LPS-treated cultures. In contrast, none of the five ingredients alone triggered similar levels of CD25 expression, and they only affected some of the cell types in the PBMC cultures. Elder triggered an increase in CD25 on NKT cells, reaching a high level of significance ($p < 0.01$, Figure 4C). Hemi caused a mild but statistically significant increase in CD25 on monocytes ($p < 0.05$, Figure 4G). Sacc showed a mild but highly significant ($p < 0.01$) increase in CD25 on T cells (Figure 4E), in contrast to the IB that had no effects on the T cell expression of CD25. Sacc triggered an increase in CD25 expression on monocytes ($p < 0.05$, Figure 4G). VitC caused significant ($p < 0.05$) increased levels of CD25 on NK cells (Figure 4A) and NKT (Figure 4C) cells and triggered a mild but highly significant ($p < 0.01$) decrease in CD25 levels on T cells (Figure 4E). Zinc triggered a mild but significant reduction in CD25 on T cells (Figure 4E).

The expression levels of the activation marker CD69 was also increased by the IB, especially on NK cells ($p < 0.05$), NKT cells ($p < 0.01$), and T cells ($p < 0.1$). The IB-induced increase in CD69 expression on NKT cells exceeded that of the LPS-treated control cultures, whereas the CD69 levels on other cell types were lower than in LPS-treated cultures. Hemi triggered a mild but highly significant ($p < 0.01$) increase in CD69 on NK cells (Figure 4B). Sacc increased the expression of CD69 on NK cells ($p < 0.05$, Figure 4B) and a mild but highly significant ($p < 0.01$) increase in CD69 on monocytes (Figure 4H), in contrast to the IB that had no effects on the monocyte expression of CD69. VitC caused a significant increase in CD69 expression on NK cells ($p < 0.05$, Figure 4B), NKT cells ($p < 0.05$, Figure 4D), T cells ($p < 0.01$, Figure 4F), and monocytes ($p < 0.1$, Figure 4H). Both Elder and Zinc triggered a very mild but significant increase in CD69 expression on NK cells (Figure 4B).

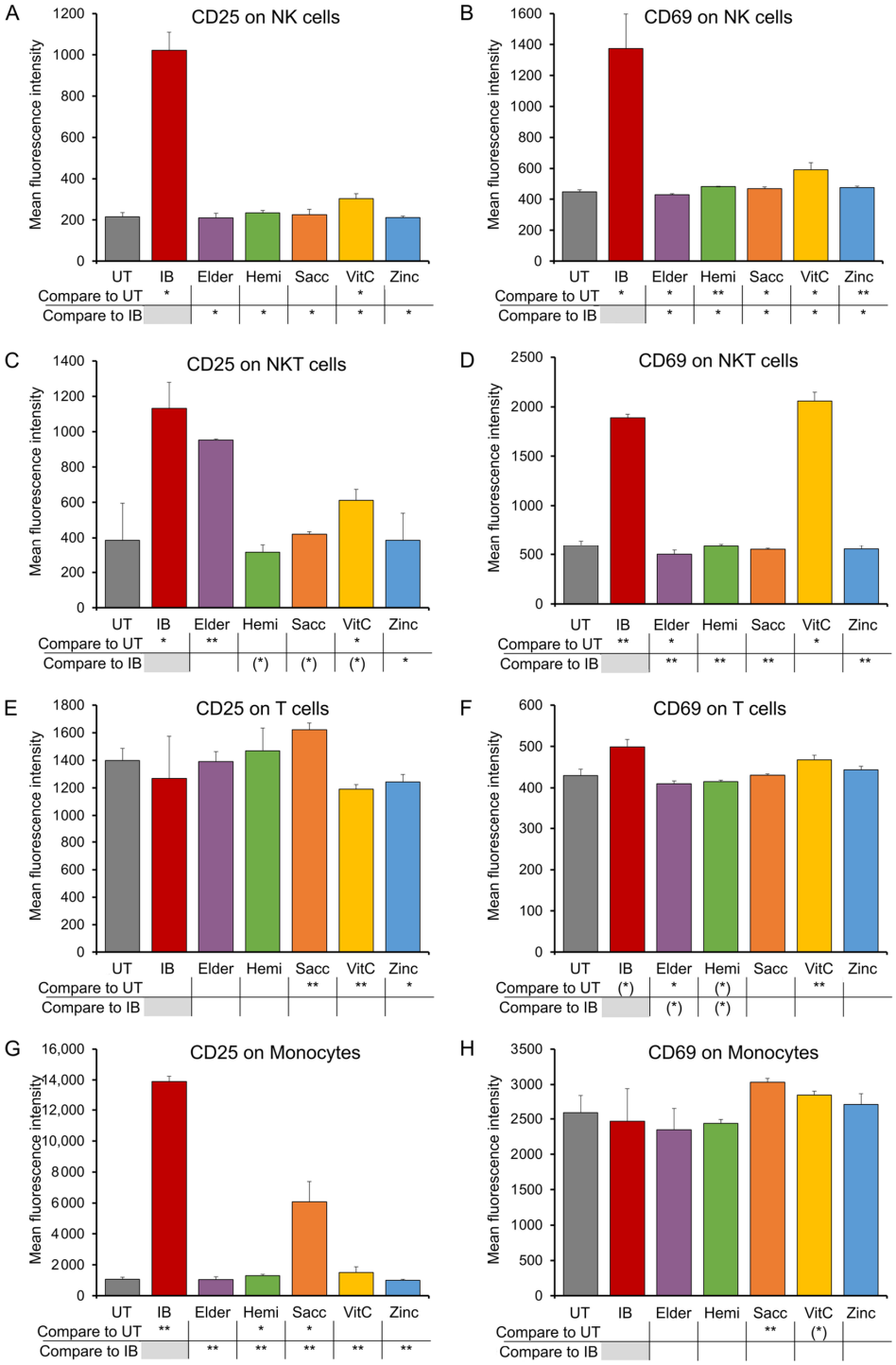

Figure 4. Expression levels of the two activation markers CD25 (**left** panel) and CD69 (**right** panel) on NK cells (**A**,**B**), NKT cells (**C**,**D**), T cells (**E**,**F**), and monocytes (**G**,**H**) in 24 h PBMC cultures: untreated control cultures (UT), cultures treated with the immune blend (IB), elderberry extract (Elder), hemicellulose extract (Hemi), *Saccharomyces cerevisiae* fermentate (Sacc), vitamin C (VitC), and zinc gluconate (Zinc). The levels of CD25 for the LPS-treated control cultures were 832 ± 56 for NK cells, 372 ± 98 for NKT cells, 1674 ± 57 for T cells, and 30,097 ± 1406 for monocytes. The levels of CD69 for the LPS-treated control cultures were 1901 ± 106 for NK cells, 798 ± 32 for NKT cells, 594 ± 12 for T cells, and 3570 ± 188 for monocytes. For each test product, results are shown as the average ± standard deviation of triplicate samples. In the table below the graph, statistical significance at different doses is indicated by asterisks, when $p < 0.10$: (*), $p < 0.05$: *, and $p < 0.01$: **.

4. Discussion

This work was conducted to document the effects on innate immune cells of a nutraceutical immune blend (IB) and its five ingredients: elderberry extract (Elder), a *Saccharomyces cerevisiae* fermentate (Sacc), a hemicellulose-rich plant extract (Hemi), vitamin C (VitC), and zinc gluconate (Zinc). We showed that the IB was highly bioactive and activated immune cells directly using an in vitro model of peripheral blood mononuclear cells, where multiple cell types are present in their natural proportions and are allowed to engage in crosstalk. The initial rapid activating effects were highly selective and showed increased production of several cytokines within 2 h, including the four immune-activating cytokines IL-6, IL-8, MIP-1α, and TNF-α in the cell cultures treated with the IB. These changes also occurred in cells treated with Sacc, VitC, and Hemi, though to a lesser extent relative to the cultures treated with the IB. This suggests that there may be synergistic effects between each of these ingredients, leading to at least a two-fold increase in cytokine production by immune cells activated by the IB, above those produced by single ingredients. This enhanced immune cell activation is likely due to collaborative signaling via different Pattern Recognition Receptors (PRRs) by the different ingredients. Recent research has solidified that Dectin-1 acts as a center for orchestrating complex collaborations with other PRRs, where the exact underlying mechanisms remain poorly understood [54]. Given the prominent role of Sacc in the early phase of the IB-mediated immune cell activation, we suggest a central role for Dectin-1-mediated immune cell activation as the initiating event, leading to the increased production of IL-1β, IL-6, and TNF-α.

The initial immune cell activation at 2 h resulted in a cascade, where the downstream events, documented after 24 h of immune cell cultures, were robust and had contributions from all five ingredients tested. After 24 h, the effect had widened, seen as increased production of additional cytokines, including IL-1β, IL-17A, and IP-10, as well as the anti-inflammatory cytokine IL-10, supporting the immune system to return to homeostasis. It is important that some pro-inflammatory cytokines, such as IL-1β, were absent in the early phase. IL-1b was produced later and detectable at 24 h, but this happened in conjunction with the anti-inflammatory cytokine IL-10, which plays a role in immune modulation by assisting in restoring the immune system back towards homeostasis. This suggests a selective immune-activating and -modulating process, unlike an immune-activating event by a pathogen or endotoxin where all pro-inflammatory cytokines are upregulated at 2 h.

In addition, the IB induced the production of G-CSF, a growth factor involved in regenerative processes, stem cell mobilization, neurogenesis, and neuroplasticity [55,56]. The induction of G-CSF was also seen for the ingredient Sacc, with similar levels as the IB, suggesting that Sacc is the ingredient responsible for the induction of G-CSF. The Sacc-mediated enhanced induction of C-GSF may be influenced by the beta-glucan content in the fungal cell wall, which engages both C-type lectin and toll-like receptors.

The treatment of immune cells with the IB showed increased expression after 24 h for both the activation markers CD25 and CD69. This increase was highly significant on innate immune cell types including NK cells, NKT cells, and monocytes. VitC and Sacc were the main contributing ingredients to this increased expression, whereas Elder contributed to the increased expression of CD25 on NKT cells. In contrast, Elder triggered mild downregulation of CD69 on NKT cells.

This experimental model is relevant for predicting the effects of the IB consumption on some of the gut mucosal immune activity and communication. In the PBMC cultures, crosstalk between different cell types is allowed, including dendritic cells, monocytes/macrophages, NK cells, and T cells. A similar crosstalk happens in the gut immune tissue, where dendritic cells are involved in initiating immune-activating events [57]; these cells extend tentacles into the gut lumen and their cell membrane comes in direct contact with immune-modulating substances, even when those substances are not absorbed across the mucosal layer [58]. The part of a dendritic cell that is located inside the gut epithelium communicates the activating event to other cells (macrophages, NK cells, and T cells) [59,60], similar to what we observe in PBMC cultures, where dendritic cells communi-

cate the activating events to other cells within the culture. The gut mucosal dendritic cells express PRRs [1–3] that recognize beta-glucans and hemicellulose from the IB. This is likely one of the major initial events after the IB is consumed, leading to activation of mucosal macrophages, NK cells, and T cells and resulting in increased cytokine production [61]. The simultaneous contributions from Vit C and Zinc provide support for molecular mechanisms involved in the epigenetic regulation of immune activation.

The immune-activating properties of Sacc on CD69 expression and cytokine production are well known [10,14]. For the data presented here, Sacc was tested at a lower dose to match the dose of Sacc in the IB. This helped document synergistic effects between ingredients, since Sacc was tested at a sub-optimal dose.

The contributions to immune cell activation by the two ingredients Elder and Zinc were milder and more selective, where Elder supported increased expression of CD25 on NKT cells, and Zinc had a mild but highly significant effect on CD69 expression on NK cells. In contrast, both ingredients triggered a significant reduction in the pro-inflammatory cytokine IL-8, suggesting that these ingredients may help modulate the immune-activating, pro-inflammatory response triggered by other ingredients such as Sacc, as also suggested by other evidence of anti-inflammatory effects of elderberry extracts [62].

This in vitro work has direct clinical relevance, since we hypothesize that similar events can occur when the IB is consumed, thereby introducing it to mucosal membranes capable of activating innate immune cells in the mucosa. Further in vitro work is warranted to study the interaction between gut immune cells and gut epithelial cells. Given the integral role that Dectin-1-mediated immune activation plays in 'trained innate immune memory', this may also be explored using published models for Dectin-1-mediated trained immunity [6,63]. While in vitro work on mechanisms of action is a necessary starting point, there is also a need for clinical documentation in humans. Based on the rapid effects observed in vitro, a clinical study may document acute immune modulation, where healthy adults are tested following an established placebo-controlled, randomized, double-blinded, crossover study design, and each participant serves as his or her own control [11,64]. In a previous clinical trial on the IB ingredient Sacc, we documented rapid immune-modulating effects in a healthy population, including increased immune surveillance by NK and T cells and increased levels of IFN-γ [11]. Increased CD69 expression on NK cells reached statistical significance at 1 h post-consumption, and increased CD25 expression on NK cells was statistically significant at 2 h post-consumption. A similar clinical evaluation of acute effects of consuming the IB would allow documentation of the measurable effects on immune surveillance and priming as a result of the interaction between gut immune cells and gut epithelial cells, and downstream signals via the vagus nerve and the cytokine communication network.

Author Contributions: G.S.J. conceived the questions to be tested. G.S.J. wrote the research protocol and designed the study. S.V.M. compiled the literature search on the ingredients and drafted the overview tables. L.Y., I.I. and G.S.J. performed the data analysis. L.Y., D.C. and I.I. produced the figures and the Graphical Abstract. All co-authors participated in the preparation of the manuscript. All co-authors participated in the final edit of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was sponsored by LifeSeasons, LLC., a distributor of nutraceutical products.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Sky Lakes Medical Center Institutional Review Board (Federalwide Assurance 2603).

Informed Consent Statement: Written informed consent was obtained from the healthy blood donors from which venous blood samples were obtained for this work.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: All authors are employed in NIS Labs. The authors declare that this study received funding from LifeSeasons, LLC., a distributor of nutraceutical products. The funder was in support of the decision to submit the article for publication, but was not involved in the study design, collection, analysis, interpretation of data, or the writing of this article. The authors declare no other conflict of interest.

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