Protected biofactors and antioxidants reduce the negative consequences of virus and cold challenge while enhancing performance by modulating immunometabolism through cytoskeletal and immune signaling in the jejunum

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ABSTRACT The aim of this study was to evaluate the effectiveness and mechanism of action of 2 feed additives in reducing the impacts of virus and temperature stressors. We determined the effects of protected biofactors and antioxidants (P(BF+AOx)), and protected biofactors and antioxidants with protected organic acids and essential oils (P(BF+AOx)+P(OA+EO)) on the immune and metabolic health of Ross 308 broiler chickens. These biofactors and antioxidants were derived from vitamins, and Aspergillus niger, Aspergillus oryzae and Bacillus subtilis fermentation extracts. All Ross 308 chickens were exposed to a double-dose of live bronchitis vaccine at d 0 and environmentally challenged by reducing the temperature from 32°C to 20°C at d 3 for 48 h. Control birds were fed without feed additives in the diet. Performance data and jejunum samples were collected to evaluate the effects of these treatments on growth, cytokine expression, and protein phosphorylation via kinome peptide array. ANOVA was used for statistical analysis of the performance and gene expression data (p-value of 0.05), and PIIKA2 was used for statistical evaluation and comparison of the kinome peptide array data. The P(BF plus;AOx) and P(BF+AOx)+P(OA+EO) treatments significantly increased bird weight gain and decreased feed conversion. The kinome peptide array data analysis showed increased activity of cytoskeletal, cell growth and proliferation proteins, and metabolic signaling in the jejunum of P(BF+AOx)+P(OA+EO) treated chickens. There was a significant decrease in IL-6 gene expression in the jejunum of P(BF+AOx)+P(OA+EO) samples compared to control at d 15. P(BF+AOx)+P(OA+EO) treatments in the jejunum showed strong immunomodulatory effects, perhaps to control inflammation. P(BF+AOx)+P(OA+EO) improves gut health via growth and metabolic signaling in the jejunum while inducing stronger immunomodulation.

Key words: immunometabolism, kinome peptide array, protected biofactors and antioxidants, cold stress, gut health

INTRODUCTION

Poultry is the most widely consumed meat in the world (OECD and Food and Agriculture Organization of the United Nations, 2021) and broiler chickens are the predominant source of this product (OECD/FAO, 2022; OECD and Food and Agriculture Organization of the United Nations, 2021). Per 2018 estimates, poultry production and exports are set to match both in the United States and worldwide (OECD/FAO, 2022) to match the increasing demand for poultry meat. It is projected that global poultry consumption will increase to 152 million tonnes or more from 2021 to 2030 (OECD and Food and Agriculture Organization of the United Nations, 2021). The quality and formulation of feed is important to meeting market demands and producers’ goals of increasing average daily gains (ADG) and reduced feed conversion ratio (FCR) of broilers; especially following government restrictions on antibiotics in many countries, and addressing consumers concerns about animal welfare. However, there are factors that may not be within the control of the producer that can induce biological stress responses and negatively impact health and growth. Poultry researchers have developed many intervention strategies to boost birds’ immune response to infections and stressors (Swaggerty et al., 2019). Temperature stress can have severe performance and health related effects on commercial broilers (Zhang et al., 2016). While in much of the world heat stress is

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the predominant concern, in northern broiler producing nations, cold stress is often a more severe production limiting issue (Hangalapura et al., 2003). Meanwhile respiratory infections, the most predominant of which in many locations is infectious bronchitis virus (IBV), can degrade performance directly or due to secondary infections (Jackwood and De Wit, 2013) resulting in energy being redirected to mucosal immune response and away from growth. Feed ingredients play an important role in the regulation of birds’ biological responses to unfavorable events as it is the source of energy and metabolites birds require for all cellular and system processes and determines the robustness of immune responses and growth. These immune responses require the activation of key metabolic pathways, thus the concept of immunometabolism is a critical consideration in poultry nutrition (Arsenault and Kogut, 2015). Feed additives and formulations can act to improve poultry responses to environmental and immune challenges, and we can improve these responses by understanding their mechanisms of action. Herein, we compared the effects of feed additives on the immune and metabolic health of birds that have been challenged with IBV and environmental cold stress, using performance metrics, the kinome peptide array technique and gene expression. The kinome peptide array uses species-specific and process-specific kinase target peptides printed on a glass array; these target peptides can be phosphorylated by active kinases in the biological samples (Arsenault et al., 2011; Daigle et al., 2014; Arsenault and Kogut, 2015). The phosphorylation of these peptides can be measured and visualized to determine changes in signaling cascades in a sample which may alter biological functions and activities (Arsenault and Kogut, 2015). The objective of this study was to evaluate and compare the immunometabolic effects and mechanisms of action of 2 feed additives, protected biofactors and antioxidants (P(BF+AOx)), and protected biofactors and antioxidants with protected organic acids and essential oils (P(BF+AOx)+P(OA+EO)) in broilers exposed to an early life cold stress and immune stimulation; and to identify their mechanism of action in the broiler gut. Previous research showed (P(BF+AOx)) induced changes in the immunometabolic profile of both liver and jejunum samples (Bortoluzzi et al., 2021). This paper focuses on the differences and similarity between (P(BF+AOx)) and (P(BF+AOx)+P(OA+EO)) treatments in the jejunum, with more emphasis on the kinomic profile.

MATERIALS AND METHODS

Birds, Housing, and Treatments

At the hatchery, 1,080 one-day old male Ross × Ross 308 chickens were vaccinated against Marek’s disease (HVT). The trial was conducted at the experimental station of Jefo Nutrition Inc., in Saint-Hyacinthe, QC, Canada. The feeding program was divided into 2 phases: starter (0–14 d) and grower (14–35 d). The feed formulation was corn and soybean meal based (Table 1); feed additives were mixed separately in the feed. The experiment consisted of 3 treatment groups: treatment 1; control, treatment 2; P(BF+AOx), and treatment 3; P(BF+AOx)+P(OA+EO). Treatments 2 and 3 were feed additives that contained mixtures of protected Biofactors and Antioxidants (P(BF+AOx)) (Jefo Nutrition Inc.) given to chickens from d 1 to 14. The experimental diets follow the NRC guidelines (NRC, 1994) for all experimental diets.

Briefly, the protected biofactors and antioxidants P(BF+AOx) were derived from a complex of vitamins and fermentation extract of vitamin A, vitamin D₃, vitamin E, menadione, thiamine, riboflavin, niacin, pantothenic acid, vitamin B₆, biotin, folic acid, vitamin B₁₂, L-tryptophan, and fermentation extract of dried Bacillus subtilis, Aspergillus niger, and Aspergillus oryzae. The P(BF+AOx)+P(OA+EO) formulation comprised the P(BF+AOx) formulation plus organic acids (citric acid, malic acid, sorbic acid, fumaric acid) and essential oils (thymol, eugenol, and vanillin). The P(BF+AOx)+P(OA+EO) active compounds were microencapsulated in a matrix of triglycerides from hydrogenated edible oil (Jefo Nutrition Inc.) (Table 1).

Each treatment consisted of 12 replicate pens with 30 birds each. The birds were placed onto floor pens with new litter. Each pen was provided with supplemental heat, and ad libitum access to water and feed in mash form.

Challenge

On d 0, all the birds received a double-dose of live bronchitis vaccine (MILDVAC-Ma5) at the hatchery. On d 3 all the chickens were submitted to an acute cold stress for 48 h with temperature between 20 and 23°C (or 9 to 12°C below the thermoneutral temperature for this age) and returned to a normal temperature afterward (Bortoluzzi et al., 2021).

Sample Collection

At 7 and 15 d, jejunum samples were collected from 6 birds per experimental group to evaluate the expression of immune-related genes. Jejunum samples from 3 of the d 15 birds per experimental group were harvested and immediately flash frozen in liquid nitrogen to preserve kinase enzymatic activity and stored at −80°C prior to further processing. Samples were shipped overnight on dry ice to the Kinome Center at University of Delaware, for kinome peptide array analysis.

Gene Expression

Jejunum samples were evaluated for expression of immune-related genes, according to Kogut and Arsenault (2015). Briefly, the mRNA was isolated from 25 mg of tissue using the RNeasy Plus mini kit (Qiagen, Hilden, Germany). The total isolated mRNA was eluted with 50 μL of RNase-free water and stored at −80°C for
SUPPLEMENTS REDUCE EFFECTS OF CHALLENGE

Table 1. Starter (1−21 d) and grower (21−35 d) diets formulation, and formulated energy and nutrient composition.

| Ingredient, % | Starter control | Starter treatment | Grower control | Grower treatment |
|---------------|-----------------|------------------|----------------|-----------------|
| Corn          | 30.6            | 30.6             | 34.0           | 34.0            |
| Soybean meal, 48% CP | 26.0          | 26.0             | 18.3           | 18.3            |
| Wheat         | 31.0            | 31.0             | 34.3           | 34.3            |
| DDGS          | 5.0             | 5.0              | 5.0            | 5.0             |
| Animal fat    | 2.8             | 2.8              | 4.4            | 4.4             |
| Monocalcium phosphate | 0.98         | 0.98             | 1.01           | 1.01            |
| Calcium carbonate | 2.13         | 2.13             | 1.73           | 1.73            |
| NaCl          | 0.31            | 0.31             | 0.28           | 0.28            |
| L-lysine HCl  | 0.315           | 0.315            | 0.310          | 0.310           |
| DL-Methionine, 99% | 0.305        | 0.305            | 0.245          | 0.245           |
| L-threonine   | 0.090           | 0.090            | 0.045          | 0.045           |
| Choline, 60%  | 0.076           | 0.076            | 0.076          | 0.076           |
| L-Valine      | 0.259           | 0.259            | 0.076          | 0.076           |
| L-Tryptophane | 0.029           | 0.029            | 0.024          | 0.024           |
| Vitamin-Mineral Premix\(^1\) | 0.15          | 0.15             | 0.15           | 0.15            |
| Sodium bicarbonate | -             | -                | 0.04           | 0.04            |
| P(BF+AOx)\(^2\) or P(BF+AOx)+P(OA+EO)\(^3\) | -          | 0.015 or         | 0.015 or       |
| Formulated energy and nutrient composition | | | | |
| ME Kcal/Kg   | 2,950           | 2,950            | 3,097          | 3,097           |
| Crude Protein, % | 20.5          | 20.5             | 17.5           | 17.5            |
| Fat, %        | 5.34            | 5.34             | 6.98           | 6.98            |
| Lysine, %     | 1.200           | 1.200            | 1.003          | 1.003           |
| Thr, %        | 0.797           | 0.797            | 0.639          | 0.639           |
| Met+Cys, %    | 0.938           | 0.938            | 0.810          | 0.810           |
| Non phytate phosphorus, % | 0.410        | 0.410            | 0.440          | 0.440           |
| Total Ca, %   | 1.11            | 1.11             | 0.95           | 0.95            |
| Na, %         | 0.15            | 0.15             | 0.15           | 0.15            |

\(^1\)Supplied per kg of diet: vitamin A, 10,005 IU; vitamin D₃, 3,000 IU; vitamin E, 30 IU; vitamin K, 2.55 mg; vitamin B₁₂, 15 mg; biotin, 201 mg; thiamine, 3 mg; riboflavin, 6 mg; pantothenic acid, 14.1 mg; pyridoxine, 3.6 mg; niacin, 49.95 mg; folic acid, 1 mg; Zn, 100; Fe, 49.5 mg; Cu, 15 mg; I, 0.09 mg; Se, 0.45 mg; Mn, 100 mg.

\(^2\)Supplied per kg of diet: vitamin A, 900 IU; vitamin D₃, 450 IU; vitamin E, 12 IU; vitamin K, 0.135 mg; vitamin B₁₂, 0.00525 mg; biotin, 0.03 mg; thiamine, 0.9 mg; riboflavin, 1.35 mg; pantothenic acid, 3 mg; pyridoxine, 0.75 mg; niacin, 12 mg; folic acid, 0.3 mg.

\(^3\)The P(BF+AOx)+P(OA+EO) formulation comprised the P(BF+AOx) formulation plus 0.01% organic acids (citric acid, malic acid, sorbic acid, fumaric acid) and essential oils (thymol, eugenol, and vanillin) microencapsulated in a matrix of triglycerides from hydrogenated vegetable oil.

qRT-PCR analysis. RNA was quantified and the quality evaluated using a spectrophotometer (NanoDrop Products, Wilmington, DE).

The PCR was performed using the TaqMan fast universal PCR master mix and one-step RT-PCR master mix reagents (Applied Biosystems, Waltham, MA). Normalization was carried out using 28S rRNA as a housekeeping gene, and the corrected cytokine mean change in mRNA levels were calculated as follow: mean 40−Ct/slope of the standard curve of the target cytokine/slope of the standard curve of the 28S gene\(^\ast\)differential factor of the 28S gene (Arsenault and Kogut, 2015). Jejunum samples were tested for IL-6 and IL-10. The prime and probe sets used in the qRT-PCR are reported in Bortoluzzi et al. (2021).

**Kinome Peptide Array**

The kinome peptide array was performed as described by (Johnson et al., 2019). Forty (40) mg of samples were lysed using bead-based homogenization in 100 μL of lysis buffer containing protease and phosphatase inhibitors. The lysed tissue samples were incubated on ice and then spun down in a refrigerated microcentrifuge at 14,000 g for 10 min at 4°C. An aliquot of supernatant was mixed with 10 μL of activation mix containing ATP as the phosphate group donor. Eighty μL of the supernatant-activation mix solution was applied to the peptide microarray. The custom-designed peptide arrays were obtained from JPT Peptide Technologies (Berlin, Germany), based on our sequence designs. A 25 × 60 mm, glass lifter slip was then applied to the microarray to sandwich and disperse the applied lysate.

Microarrays were then incubated in a humidity chamber at 40°C and 5% CO$_2$. Arrays were then placed in a 50 mL centrifuge tube containing phosphate-buffered saline (PBS)—1% Triton, to remove the lifter slip from the microarray surface. Arrays were then submerged in 2M NaCl-1% Triton and agitated for a minimum of 30 s. This process was then repeated with fresh 2M NaCl-1% Triton. Arrays were given a final wash in double distilled water with agitation.

Array slides were submerged in phosphospecific fluorescent ProQ Diamond Phosphoprotein Stain (Life Technologies, Carlsbad, CA) in a large dish and placed on a shaker table at 50 rpm for 1 h. Arrays were then placed in a new dish and submerged in destain solution (20% acetonitrile (EMD Millipore Chemicals, Billerica, MA) and 50 mM sodium acetate [Sigma Aldrich, St. Louis, MO]) for 10 min with agitation at 50 rpm. This process was repeated 2 times. A final wash was performed with double distilled water. The arrays were spun dried. Arrays were then scanned using a Tecan PowerScanner microarray scanner (Tecan Systems, San Jose, CA) at 532 to 560 nm with a 580 nm filter to detect dye fluorescence to collect the array image.
Kinome Peptide Array Data Analysis

Images were gridded using GenePix Pro software, and the spot intensity signal collected as the mean of pixel intensity using local feature background intensity calculation with the default scanner saturation level. The resultant data was then analyzed by the PIIKA2 peptide array analysis software (http://saphire.usask.ca/saphire/piika/index.html) (Trost et al., 2013). Briefly, the resulting data points were normalized to eliminate variance due to technical variation, for example, random variation in staining intensity between arrays or between array blocks within an array. Variance stabilization normalization was performed. Using the normalized data set comparisons between treatment and control groups was performed, calculating fold change and a significance P-value. The P-value was calculated by conducting a one-sided paired t test between treatment and control values for a given peptide. The resultant fold change and significance values were then used to generate higher order analysis (heatmaps, hierarchical clustering, principal component analysis, pathway analysis, etc.).

The kinome peptide array analysis was performed in triplicate for each group per tissue. A total of 9 samples, 3 per group were used for kinome peptide array analysis. Three treatment (cold stress and IBV vaccine challenged broilers given feed additives) vs. control (cold stress and IBV vaccine challenged broilers without feed additives) combinations (Table 2) were used to generate kinome profiles of these samples.

As described by Perry et al. (2020), post PIIKA2 analysis was performed using the following online databases and tools; STRING database (Szklarczyk et al., 2019) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and KEGG color and search pathways (Kanehisa and Sato, 2020), PhosphoSitePlus (Hornbeck et al., 2008, 2009), Uniprot (The UniProt Consortium, 2021), and Venny 2.1 (Oliveros, 2007).

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Statistical Analysis

A two-way ANOVA was used for statistical analysis of the performance data via the SAS software (SAS 9.4). The data were tested for normality and homogeneity. Nonparametric data were submitted to the Kruskal-Wallis test (P < 0.05). A one-way ANOVA and Tukeys post-hoc test was used for statistical analysis of the gene expression data via the JMP software (JMP Pro 16). All P-values lower than or equal to 0.05 were considered statistically significant. P-values greater than 0.05 but less than or equal to 0.1 indicate a trend to statistical significance. The European Production Efficiency Factor (EPEF) was calculated as described by Bortoluzzi et al., 2021, using the formula: body weight (Kg) × % survivability × 100/FCR × trial duration in days.

RESULTS

Effects of Treatments on Performance

Both P(BF+AOx) and P(BF+AOx)+P(OA+EO) fed birds showed significant increase in BWG compared to control (Table 3), with the exceptions from 0 to 21 d

Table 3. Growth performance of control and antioxidant + biofactors treated birds.

| Measurement | Control | P(BF+AOx) | P(BF+AOx)+P(OA+EO) | SEM | P-value |
|-------------|---------|-----------|--------------------|-----|---------|
| BWG 0–7 d   | 92b     | 95a       | 98a                | 1.33| 0.002   |
| F1 0–7 d    | 118     | 117       | 1.226b             | 0.86| 0.02    |
| FCR 0–7 d   | 345     | 359b      | 359b               | 3.68| 0.007   |
| BWG 0–14 d  | 456     | 469       | 470                | 3.51| 0.54    |
| F1 0–14 d   | 1.322   | 1.306     | 1.31               | 0.04| 0.19    |
| FCR 0–14 d  | 770b    | 799b      | 783ab              | 6.96| 0.007   |
| BWG 0–21 d  | 1069    | 1096      | 1088               | 7.2 | 0.55    |
| F1 0–21 d   | 1.389b  | 1.372b    | 1.385ab            | 0.01| 0.02    |
| FCR 0–21 d  | 1367    | 1422      | 1393ab             | 11.43| 0.002   |
| BWG 0–28 d  | 2042    | 2107      | 2085               | 14.44| 0.73    |
| FCR 0–28 d  | 1.545a  | 1.516b    | 1.533a             | 0.01| 0.001   |
| BWG 0–35 d  | 2,051a  | 2,136a    | 2,108a             | 20.15| 0.008   |
| F1 0–35 d   | 3,281   | 3,390     | 3,363              | 24.93| 0.16    |
| FCR 0–35 d  | 1.686a  | 1.647b    | 1.663ab            | 0.01| 0.03    |
| EPEF2       | 358.4   | 373.03    | 370.13             | 5.76 | 0.22    |
| Survival rate | 0.98   | 0.97      | 0.97               | 0.01| 0.35    |

Boldface pathways are discussed in further detail in this paper.

1Abbreviations: BWG, body weight gain; F1, feed intake; FCR, feed conversion ratio.

2European Production Efficiency Factor, body weight (Kg) × % survivability × 100/FCR × trial duration in days.

a,bP < 0.05.
Table 4. The top 20 list of KEGG pathways in P(BF+AOx)+P(OA+EO) treated jejunum relative to control.

| KEGG pathways | Observed protein count | False discovery rate |
|---------------|------------------------|----------------------|
| MAPK signaling pathway | 56 | 1.02E-39 |
| Pathways in cancer | 65 | 6.09E-37 |
| PI3K-Akt signaling pathway | 55 | 1.46E-35 |
| Insulin signaling pathway | 37 | 4.48E-31 |
| Ras signaling pathway | 41 | 3.20E-28 |
| Central carbon metabolism in cancer | 27 | 1.61E-26 |
| Proteoglycans in cancer | 36 | 3.83E-25 |
| Focal adhesion | 36 | 4.58E-25 |
| Hepatitis B | 32 | 1.02E-24 |
| Neurotrophin signaling pathway | 30 | 1.08E-24 |
| Insulin resistance | 29 | 2.17E-24 |
| MicroRNAs in cancer | 32 | 2.80E-24 |
| ErbB signaling pathway | 26 | 2.91E-23 |
| Kaposi’s sarcoma-associated herpesvirus infection | 33 | 5.32E-23 |
| Rap1 signaling pathway | 34 | 8.14E-23 |
| EGFR tyrosine kinase inhibitor resistance | 25 | 1.18E-22 |
| Osteoclast differentiation | 28 | 7.25E-22 |
| Acute myeloid leukemia | 23 | 1.42E-21 |
| Chemokine signaling pathway | 31 | 3.93E-21 |
| AMPK signaling pathway | 27 | 4.08E-21 |

Boldface pathways are discussed in further detail in this paper.

1Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx)+P(OA+EO).

2The significantly phosphorylated peptides generated from the t test performed by PIIKA2 were entered into the STRING database. The list of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were downloaded and analyzed for common and/or relevant immune or metabolic pathways.

and to 0 to 28 d, where P(BF+AOx)+P(OA+EO)-fed birds were not significantly different from control or P(BF+AOx) treated birds (Table 3). P(BF+AOx) showed statistically significant reduction in FCR compared to control from 0 to 7, 0 to 21, 0 to 28, and 0 to 35 d, while P(BF+AOx)+P(OA+EO) showed a statistically significant reduction only from 0 to 7 d with no change in FCR when compared to control or P(BF+AOx) from 0 to 14 and 0 to 35 d. There was no effect of treatment on feed intake (FI) across all groups. The same can be said for survival rate and European production efficiency factor (EPEF).

**Treatment Effects on Signaling Profile**

Functional analysis of the phosphorylation data showed that both treatments altered the immunometabolic profiles of the jejunum via MAPK signaling which encompasses growth, inflammatory and cell cytoskeleton signaling (Tables 4 and 5). To understand the effects and changes these treatments induced in the signaling cascades within the broiler jejunum compared to challenge alone, the list of all significantly phosphorylated proteins for each treatment vs. control pair was entered into STRING database. The lists of KEGG pathways were then extracted from the STRING database and sorted by false discovery rate (FDR) in ascending order. The lists of top 20 KEGG pathways for each treatment compared to control are reported in Tables 4 and 5. Each treatment significantly altered several different biological pathways of the respective tissues. Many of the pathways shown in the lists are immunometabolic pathways that are downstream of growth receptors and are important in the regulation of inflammation, cell communication, cell cytoskeleton, and metabolism during stress response. More specifically, both jejunum samples showed pathways involved in cytoskeleton regulation and cell growth such as Rap1 signaling, Ras signaling, and focal adhesion signaling (Table 4).

There were differences in P(BF+AOx)+P(OA+EO) and P(BF+AOx) signaling. The signaling pathways **Cellular Senescence** and **Relaxin Signaling** were observed in the top 20 KEGG pathways of the jejunum.
P(BF+AOx) but not in P(BF+AOx)+P(OA+EO) (Figure 1, Tables 4 and 5). These two pathways are linked via TGF-beta receptor signaling pathway which is characterized by the secretion of inflammatory cytokines and growth receptors signaling. In the top 20 KEGG pathways of P(BF+AOx)+P(OA+EO), we observed Insulin resistance, chemokine and AMPK signaling Pathways that were not observed in the top 20 of jejunum P(BF+AOx) (Figure 1). Chemokine signaling involves the stimulation of cytokine production and cell growth factors via MAPK and Jak-STAT signaling. Insulin resistance and AMPK signaling both involve the regulation of energy metabolism via mTOR and PI3K signaling. Besides the differences in the number of proteins involved in MAPK signaling between the 2 treatments, we also observed more protein counts in P(BF+AOx)+P(OA+EO) KEGG pathways than P(BF+AOx) KEGG pathways (Tables 4 and 5). These differences between treatments show stronger immunometabolic effects of P(BF+AOx)+P(OA+EO) in the jejunum.

**Effects of P(BF+AOx)+P(OA+EO) Compared to P(BF+AOx)**

To determine the impact of P(BF+AOx)+P(OA+EO) in chickens compared to P(BF+AOx), PIIKA2 comparisons were ran using P(BF+AOx)+P(OA+EO) as the treatment and P(BF+AOx) as the control as shown in Table 2. After further analysis as previously described, the top 20 KEGG pathways for jejunum

**Figure 1.** Schematic of immunometabolic pathways processes induced by feed additives in the jejunum. This schema illustrates the different immunometabolic pathways altered by each feed additive and the signal transduction cascades these pathways have in common that lead to changes in key immunometabolic processes. The pathways in this schema were derived by comparing the list of top 20 KEGG pathways for each feed additive in the jejunum for differences.
samples are reported in Table 6. Table 6 shows the signaling pathways due to significant changes in phosphorylation by P(BF+AOx) + P(OA+EO) treatment in the jejunum compared to P(BF+AOx). Most of these pathways are part of the MAPK cell growth and energy metabolism signal transduction cascades previously mentioned except for the Toll-like receptor signaling pathway, an innate immune pathway that plays a critical role in recognizing pathogens (Table 6). It is important to remember that the activation status of each protein in these pathways cannot be assumed by the information provided in Tables 4–6, and Figure 1. Detailed analysis of the phosphorylation sites of each protein is necessary to fully understand the effects of these treatments on the activation status of these proteins and to determine the immunometabolic mechanism of action in the jejunum.

**Effects on Cytoskeleton Regulation**

The lists of top 20 KEGG pathways for each group showed that the treatments induced significant changes in cell motility, cell migration and cytoskeletal regulation compared to control (challenge alone) (Tables 4 and 5). Therefore, regulation of actin cytoskeleton, a pathway that includes the major cytoskeleton and cellular junction proteins was further analyzed. Analysis of the regulation of the actin cytoskeleton pathway for each treatment showed that P(BF+AOx) + P(OA+EO) treatment positively affects actin and myosin contractility, although the P(BF+AOx) treatment groups showed significant changes in cytoskeleton regulation proteins compared to control, the phosphorylation status of proteins like RHOA, ROCK, PAK, MLCK and others did not indicate significant changes in actin contractility and polymerization (Table 7). The direct comparison of P(BF+AOx) + P(OA+EO) to P(BF+AOx) revealed that, P(BF+AOx) + P(OA+EO) treatment induce increased activity of cytoskeletal regulatory proteins in the jejunum of broilers (Supplementary 1). Increased activation of microtubule regulators like SOS-1, Rac1, MEK2, IRSp53, and PPP2c-alpha was observed only for P(BF+AOx) + P(OA+EO) jejunum, supporting that P(BF+AOx) + P(OA+EO) induces more cytoskeletal changes in the jejunum.

**Effects on Immunoregulation and Cell Death**

The list of top 20 KEGG pathways for each treatment contained several pathways that involve signaling through cytokine receptors and the activity of many inflammatory proteins associated with immune response and regulation (Tables 4–6). Therefore, the effects of phosphorylation on the proteins involved in inflammatory and immune pathways like the Chemokine, T cell receptor signaling pathway, Fas and TNF signaling pathways were analyzed (Supplementary 1). Activated MAPKs can induce increased activity of cytoskeletal regulatory proteins. The list of top 20 KEGG pathways for each treatment contained several pathways that involve signaling through cytokine receptors and the activity of many inflammatory proteins associated with immune response and regulation (Tables 4–6). Therefore, the effects of phosphorylation on the proteins involved in inflammatory and immune pathways like the Chemokine, T cell receptor signaling pathway, Fas and TNF signaling pathways were analyzed (Supplementary 1).

### Table 6. The top 20 list of KEGG pathways in the jejunum P (BF+AOx)+P(OA+EO) treatment groups relative to P(BF+AOx).

| Pathway                                      | Observed protein count | False discovery rate |
|----------------------------------------------|------------------------|----------------------|
| MAPK signaling pathway                       | 60                     | 6.58E-42             |
| Pathways in cancer                          | 72                     | 3.47E-41             |
| PI3K-Akt signaling pathway                  | 56                     | 1.92E-34             |
| Insulin signaling pathway                   | 37                     | 1.26E-29             |
| Central carbon metabolism in cancer         | 29                     | 5.02E-28             |
| Hepatitis B                                 | 36                     | 7.32E-28             |
| MicroRNAs in cancer                         | 35                     | 3.65E-26             |
| Ras signaling pathway                       | 39                     | 8.61E-25             |
| Neurotrophin signaling pathway              | 31                     | 1.18E-24             |
| Proteoglycans in cancer                     | 36                     | 6.29E-24             |
| Kaposi’s sarcoma-associated herpesvirus     | 35                     | 9.69E-24             |
| MAPK signaling pathway                      | 60                     | 6.58E-42             |
| Pathways in cancer                          | 72                     | 3.47E-41             |
| PI3K-Akt signaling pathway                  | 56                     | 1.92E-34             |
| Insulin signaling pathway                   | 37                     | 1.26E-29             |
| Central carbon metabolism in cancer         | 29                     | 5.02E-28             |
| Hepatitis B                                 | 36                     | 7.32E-28             |
| MicroRNAs in cancer                         | 35                     | 3.65E-26             |
| Ras signaling pathway                       | 39                     | 8.61E-25             |
| Neurotrophin signaling pathway              | 31                     | 1.18E-24             |
| Proteoglycans in cancer                     | 36                     | 6.29E-24             |
| Kaposi’s sarcoma-associated herpesvirus     | 35                     | 9.69E-24             |

**Boldface pathways are discussed in further detail in this paper.**

1. Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx) + P(OA+EO).
2. Protected biofactors and antioxidants P(BF+AOx).
3. The significantly phosphorylated peptides generated from the t test performed by PHKA2 were entered into the STRING database. The list of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were downloaded and analyzed for common and/or relevant immune or metabolic pathways.

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### Table 7. Effect of phosphorylation changes on major cytoskeleton regulation proteins.

| Protein name | P(BF+AOx) + P(OA+EO) | P(BF+AOx) |
|--------------|----------------------|-----------|
| Calmodulin   | Inactive             | Inactive  |
| CAMK         | Inactive             | Active    |
| EZ           | Inactive             | Inactive  |
| FAK          | Inactive             | Inactive  |
| MLCK         | P                    | D         |
| MYPT1        | Inactive             | Inactive  |
| PAK          | Inactive             | D         |
| Prolin 2     | D                    | No change |
| PYK2         | Active               | No change |
| RHOA         | Active               | Active    |
| ROCK         | Active               | No change |
| Stathmin     | No change            | Inactive  |

1. The phosphorylation status of proteins in this table was determined by entering each protein’s respective Uniprot accession into phosphosite, finding the annotation of the site of interest and accounting for the phosphorylation fold change (increased or decreased) of that site. Active denotes increased phosphorylation of an inducing site or decreased phosphorylation on an inhibitory site. Inactive denotes decreased phosphorylation of an inducing site or increased phosphorylation on an inhibitory site. P denotes that the function of the site is unknown and the data shows increased phosphorylation. D denotes that the function of the site is unknown and the data shows decreased phosphorylation. No change denotes there were no statistically significant difference observe between treatment and control for that protein.
2. Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx) + P(OA+EO).
3. Protected biofactors and antioxidants P(BF+AOx).
Table 8. Effect of phosphorylation changes on major immunoregulation and cell death proteins.

| Protein name | P(BF+AOx)+P(OA+EO)1 | P(BF+AOx)1
|--------------|-----------------------|-----------------------|
| AP-1/C-Fos   | P                     | No change             |
| B-AARRESTIN  | P                     | P                     |
| BLNK         | Inactive              | Inactive              |
| BTK          | Inactive              | Active                |
| Caspase 3    | Active                | No change             |
| Caspase 6    | No change             | Inactive              |
| C-JUN        | No change             | Active                |
| JNK          | Active                | Active                |
| MEKK3        | Active                | Active                |
| NFAT         | Active                | Active                |
| P38          | Active                | Active                |
| PERK         | Active                | Inactive              |
| SMAD2/3      | No change             | Inactive              |
| STAT1        | Active                | Active                |
| ZAP-70       | Active                | Active                |

1The phosphorylation status of proteins in this table was determined by entering each protein’s respective Uniprot accession into phosphosite, finding the annotation of the site of interest and accounting for the phosphorylation fold change (increased or decreased) of that site. Active denotes increased phosphorylation of an inducing site or decreased phosphorylation on an inhibitory site. Inactive denotes decreased phosphorylation of an inducing site or increased phosphorylation on an inhibitory site. P denotes that the function of the site is unknown and the data shows increased phosphorylation. D denotes that the function of the site is unknown and the data shows decreased phosphorylation. No change denotes there were no statistically significant difference observe between treatment and control for that protein.

Table 9. Phosphorylation changes in major cytokines and immune receptors in jejunum P(BF+AOx)1 at d 15.

| Uniprot accession | Protein name | Human site | Chicken site | FC | P-value |
|-------------------|--------------|------------|--------------|----|---------|
| P29460            | Interleukin-12 subunit beta: IL-12B; | Y314       | Y304         | -1.06939 | 0.01631 |
| P40189            | Interleukin-6 receptor subunit beta: IL-6R-beta; | S782       | S757         | 1.08012 | 0.01624 |
| Q8N5C8            | TAK1-binding protein 3; TAB-3; | T404       | T403         | 1.07588 | 0.03082 |
| Q12933            | TNF receptor-associated factor 2: Tumor necrosis factor type 2 receptor-associated protein 3; | S11        | S11          | -1.05706 | 0.02792 |
| P58753            | Toll interleukin-1 receptor domain-containing adapter protein; TIR-domain-containing adapter protein; | Y86        | Y77          | -1.09435 | 0.00018 |
| Q15455            | Toll-like receptor 3; | Y585       | Y584         | -1.11321 | 0.02742 |
| Q15455            | Toll-like receptor 3; | Y1755      | Y1754        | -1.08145 | 0.0378 |
| Q9N9R7            | Toll-like receptor 8; | S939       | S948         | 1.12636 | 0.00881 |
| Q9UK55            | TRAF2 and NCK-interacting protein kinase; | S764       | S730         | -1.06118 | 0.03767 |
| Q9N9R17           | X-linked interleukin-1 receptor accessory protein-like 2; IL-1-RAPL-2; | S343       | S317         | -1.08169 | 0.00005 |

Boldface pathways are discussed in further detail in this paper.

*Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx).

Table 10. Phosphorylation changes in major cytokines and immune receptors in jejunum P(BF+AOx)+P(OA+EO)1 at d 15.

| Uniprot accession | Protein name | Human site | Chicken site | FC | P-value |
|-------------------|--------------|------------|--------------|----|---------|
| P29460            | Interleukin-12 subunit beta: IL-12B; | Y314       | Y304         | -1.05492 | 0.01014 |
| P40189            | Interleukin-6 receptor subunit beta: IL-6R-beta; | S782       | S757         | 1.08541 | 0.01395 |
| P39897            | TGF-beta receptor type-1; TGFR-1; | T190       | T190         | 1.33268 | 0.00001 |
| Q8N5C8            | TAK1-binding protein 3; TAB-3; | T404       | T403         | 1.10401 | 0.00002 |
| Q8N5C8            | TAK1-binding protein 3; TAB-3; | T404       | T403         | 1.10401 | 0.00002 |
| Q7L0              | TLR4 interactor with leucine rich repeats; | T753       | T721         | 1.05601 | 0 |
| Q9Y4K3            | TNF receptor-associated factor 6; Interleukin-1 signal transducer; | Y353       | Y379         | -1.11472 | 0.00000 |
| P58753            | Toll interleukin-1 receptor domain-containing adapter protein; TIR-domain-containing adapter protein; | Y86        | Y77          | -1.1929 | 0 |
| Q15399            | Toll-like receptor 1; Toll interleukin-1 receptor-like protein; TIL; | Y691       | Y704         | 1.08534 | 0.00059 |
| Q6R5N8            | Toll-like receptor 13; | S200       | S208         | -1.16332 | 0.00023 |
| Q15455            | Toll-like receptor 3; | Y585       | Y584         | -1.25531 | 0 |
| Q60602            | Toll-like receptor 5; Toll interleukin-1 receptor-like protein 3; | Y798       | Y800         | 1.04 | 0.04634 |
| Q9NYK1            | Toll-like receptor 7; | S601       | S608         | 1.29188 | 0 |
| Q8N9R7            | Toll-like receptor 8; | S939       | S948         | 1.07154 | 0.0482 |
| Q98881            | Interleukin-2-inducible T-cell kinase; Tyrosine-protein kinase Lyk; | Y512       | Y511         | 1.14335 | 0.00001 |

Boldface pathways are discussed in further detail in this paper.

*Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx).
Table 11. Cytokine expression in the jejunum.

| Cytokine | Control | P(BF+AOx) | P(BF+AOx)+P(OA+EO) | SEM | P value |
|----------|---------|-----------|--------------------|-----|---------|
| IL-6     | 10.61   | 8.81b     | 7.53b              | 0.82| 0.05    |
| IL-10    | 7.87    | 6.12      | 7.75               | 0.63| 0.12    |

Boldface pathways are discussed in further detail in this paper.

1Corrected cytokine means of IL-6 and IL-10 expression in jejunum samples at d 15.

2From each experimental group samples of N = 6 kinome were collected for analysis.

Effect on Metabolic Signaling

Based on the result of our analysis, P(BF+AOx)+P(OA+EO) showed increased phosphorylation of IL-6R. However, the cytokine gene data only showed a significant decrease in IL-6 gene expression (P < 0.05) in the jejunum P(BF+AOx)+P(OA+EO) samples compared to control at day 15 (Table 11). No significant trends were observed for IL-10 expression, however, the pairwise comparison using Tukey’s HSD showed a trend (P = 0.101) of decreased IL-10 expression in P(BF+AOx) compared to P(BF+AOx)+P(OA+EO).

DISCUSSION

The performance data (Table 3) showed that treatment with both P(BF+AOx) and P(BF+AOx)+P(OA+EO) decreased FCR significantly. Performance data should be considered in the context that comparisons were made to a challenge (cold stress and IBV) control. Thus, an increase in performance suggests a decrease in the stress effects of the physiological and environmental challenge. Keeping in mind that the control in this study was a double-dose of viral vaccine and cold stress challenge, we wanted to understand the immunomodulatory effects of changes in phosphorylation on key pathways. Therefore, signaling profiles of treatment groups were determined by detailed analysis of immunoregulatory, metabolic, cell growth, and cell cytoskeleton pathways that were observed in our KEGG analysis (Tables 4–7).

The kinome peptide array results showed significant changes in actomyosin contractility and focal adhesion. Specifically, jejunum samples collected from P(BF+AOx)+P(OA+EO)−treated birds showed changes in the phosphorylation of ROCK, RHOA and MLCK which are orchestrators of actin and myosin contraction (Wu et al., 2010; Valencia-Expósito et al., 2016; Jin and Blikslager, 2020; Table 7 and Supplementary 1). The phosphorylation status of RHOA and ROCK indicate the inhibition of Myosin light chain phosphatase (MLCP), via phosphorylation by ROCK and the decrease phosphorylation of PAK attenuates its inhibition of Myosin light chain kinase (MLCK; Table 7), leading to actomyosin assembly and contraction. Another cytoskeleton signaling activity that was critically impacted by P(BF+AOx)+P(OA+EO) and P(BF+AOx) treatments was actin assembly, which involves polymerization and depolymerization of actin filaments; important for actin turnover which drives cell movement and shape. The decreased phosphorylation of profilin (Table 7), a driver of actin polymerization indicates its activation to facilitate the assembly of globular actin into filamentous actin (Sathish et al., 2004; Delorme et al., 2007; Zhang et al., 2011). Moreover, PAK which plays a key role in the activation of cofilin, the driver of actin depolymerization, was also less activated in P(BF+AOx)+P(OA+EO) jejunum (Callow et al., 2002). There were similar changes in focal adhesion signaling for both P(BF+AOx)+P(OA+EO) and P(BF+AOx) via Calmodulin, FAK and EZ. The overall difference in cytoskeletal regulation between P(BF+AOx)+P(OA+EO) and P(BF+AOx) was made clear via the changes in phosphorylation of MLCK, RHOA, ROCK and Profilin which were specific to P(BF+AOx)+P(OA+EO; Table 7 and Supplementary 1). Direct comparison of P(BF+AOx)+P(OA+EO) compared to P(BF+AOx) clearly showed that P(BF+AOx)+P(OA+EO) had induced critical changes in cell cycle regulation and growth via insulin signaling and AMPK signaling pathways. Additionally, many changes in phosphorylation of cell growth proteins were observed in the jejunum P(BF+AOx)+P(OA+EO) compared to P(BF+AOx).
like receptor signaling, chemokine signaling, Ras and Rap1 signaling, which involve signal transduction via T-cell receptor signaling. AP-1, JAK, STAT, NFAT, JNKK, MEKK3, P38, and ZAP-70 are some of the proteins involved in these pathways that were phosphorylated on their active sites for both treatments in the jejunum. P(BF+AOx)+P(OA+EO) induced more changes in the inflammatory profile in the jejunum because more changes were observed in the overall immune regulatory status. This includes changes in the cell death proteins, and linkers of adaptive and innate immunity proteins compared to control and P(BF+AOx); Table 8 and Supplementary 2). Increased activity of SOS, STAT, NF-kappa B, JNKK, IFN-R, TAB, TGFR, and more were observed in the jejunum samples of P(BF+AOx)+P(OA+EO) birds compared to control and P(BF+AOx); Table 8 and Supplementary 2). Increased activity of apoptotic proteins were observed in the jejunum samples of P(BF+AOx)+P(OA+EO) treated birds, specifically, caspases 3, 6, and compared to P(BF+AOx). Apoptosis is used to suppress pro-inflammatory responses such as necrosis and promote the release of anti-inflammatory factors by triggering phagocytosis of inflamed cells (Köröskényi et al., 2011; Szondy et al., 2017). The increased activity of apoptotic proteins in P(BF+AOx)+P(OA+EO) treated samples indicates its anti-inflammatory characteristics.

The phosphorylation status of IL-6R of P(BF+AOx)+P(OA+EO) and P(BF+AOx) treated jejunum samples showed increased phosphorylation (Tables 9 and 10), Gibson et al. (2000), showed that stimulation of the glycoprotein 130 (gp130) subunit of the IL-6 receptor (Table 9) results in the internalization of the receptor which triggers downstream activities. This gp130 is involved in inflammatory, immune, and metabolic regulation induced by IL-6 or IL-6 family of cytokines (February, 2007; White and Stephens, 2011; Cron et al., 2016). The gene expression results in P(BF+AOx) treated samples in the jejunum did not show any significant differences in IL-6 expression (Table 11). Because gp130 is stimulated by ligands other than IL-6, the phosphorylation of gp130 receptor as observed in the kinome peptide array results indicate metabolic downstream signaling via this receptor in P(BF+AOx) jejunum (Bortoluzzi et al., 2021). Thus, the immunomodulatory and metabolic effects of P(BF+AOx) is strongly dependent on signaling via the immunomodulatory cytokine receptor, IL-6R.

The gene expression results showed a decrease in IL-6 expression for P(BF+AOx)+P(OA+EO) jejunum samples (Table 11), however, the kinome peptide array results showed phosphorylation of the same IL-6 receptor gp130 subunit mentioned above (Table 10). These results suggest gp130 was stimulated by other ligands in the IL-6 family of cytokine like ciliary neurotrophic factor (CNTF) which acts on AMPK and insulin related signaling (White and Stephens, 2011). CNTF has also been shown to act on the AKT-mTOR-S6K signaling cascade (Ott et al., 2002). Therefore, despite the decreased expression of IL-6, the phosphorylation of gp130 in P(BF+AOx)+P(OA+EO) jejunum samples can be attributed to the metabolic effects of other ligands without inducing inflammation. Moreover, the increased activity of immune regulators in P(BF+AOx)+P(OA+EO) treated groups (Table 8 and Supplementary 2) indicate that this treatment improves immune responses independent of IL-6 and excessive pro-inflammatory signaling in the jejunum, perhaps via phagocytosis of apoptotic bodies of inflamed cells to reduce inflammation.

Moreover, there were changes in metabolic and growth signaling status of proteins in P(BF+AOx) treated birds and P(BF+AOx)+P(OA+EO) treated samples compared to control as observed in Table 12. We also observed critical changes in energy metabolism and cell growth when P(BF+AOx)+P(OA+EO) was compared to P(BF+AOx). Energy metabolism and cell growth are interdependent. As a cell undergoes cell growth and proliferation which involves mTOR, S6K, Sirt-1, Cdk2, Cdk6, and other anabolism proteins, it consumes a large sum of energy which can be generated via the signaling of AMPK, PGC-1 alpha, PPAR, PTEN, PFK, and other catabolism proteins (Supplementary 3). The proteins mentioned above showed changes in phosphorylation that led to increased activity in P(BF+AOx)+P(OA+EO) compared to P(BF+AOx) (Supplemental 3). This increase energy metabolism and consumption is true for immune regulation and response, and it is a key factor in immunometabolism. Thus, increase in energy metabolism is a result of and facilitates cell growth, immune signaling and cytoskeletal regulation as seen in Tables 7, 8, and 12 and

| PROTEIN | P(BF+AOx)+P(OA+EO) P(BF+AOx) |
|---------|-------------------------------|
| AMPK    | Active                         |
| AKT     | Active                         |
| HIF     | Active                         |
| IRR     | Inactive                       |
| LKB1    | No change                      |
| MTOR    | Active                         |
| PDK     | No change                      |
| PKC     | Active                         |
| PKC-D   | No change                      |
| PP2A    | Active                         |
| PTEN    | Inactive                       |
| S6K1    | Active                         |
| 4E-BP1  | Inactive                       |
| TSC2    | D                              |

1The phosphorylation status of proteins in this table was determined by entering each protein’s respective Uniprot accession into phosphosite, finding the annotation of the site of interest and accounting for the phosphorylation fold change (increased or decreased) of that site. Active denotes increased phosphorylation of an inducing site or decreased phosphorylation on an inhibitory site. Inactive denotes decreased phosphorylation of an inducing site or increased phosphorylation on an inhibitory site. P denotes that the function of the site is unknown and the data shows increased phosphorylation. D denotes that the function of the site is unknown and the data shows decreased phosphorylation. No change denotes there were no statistically significant difference observe between treatment and control for that protein.

2Protected biofactors and antioxidants with protected organic acids and essential oils P(BF+AOx)+P(OA+EO).

3Protected biofactors and antioxidants P(BF+AOx).
Supplementary 1, 2, and 3. Thus, these results suggest that P(BF+AOx)+P(OA+EO) had stronger effects on metabolism in the jejunum.

Before concluding, it is important to discuss the potential reasons AMPK and mTOR may both be active in the jejunum. The samples of P(BF+AOx)+P(OA+EO) treated birds. AMPK does not always directly phosphorylate mTOR to inhibit its activity (Garza-Lombó et al., 2018). Rather, AMPK phosphorylates tuberous sclerosis (TSC1/2) which phosphorylates and inhibits Rheb, preventing Rheb’s activation of mTOR (Inoki et al., 2003). Akt can also phosphorylate and inhibit TSC, and kinase peptide array results show the phosphorylation of Akt on its active site for both treatment groups in the jejunum and the decreased phosphorylation of TSC in jejunum P(BF+AOx)+P(OA+EO) treated samples. Additionally, there are several proteins that phosphorylate and activate mTOR. And mTOR may indirectly regulate AMPK via S6K activity (Dagon et al., 2012). Thus, with the many intermediary and regulatory proteins between AMPK and mTOR, and the homeostatic cross-talk between the two proteins, the straightforward inhibitory effect of AMPK on mTOR is not always achievable. In some cases, both AMPK and mTOR activity is required to maintain a balanced catabolic and anabolic signaling during immunometabolic regulation as these results show, because a lack of balance may lead to negative effects (Garza-Lombó et al., 2018).

In conclusion, P(BF+AOx)+P(OA+EO) induced significantly more changes in the immunometabolic signaling of broiler chickens compared to P(BF+AOx). Specifically, P(BF+AOx)+P(OA+EO) altered the cytoskeletal activity and programmed cell death in the jejunum to promote gut health thus having a more homeostatic effect. Increased growth performance due to each treatment compared to control may be linked to changes in cell growth and metabolic signaling, P(BF+AOx)+P(OA+EO) appears to induce a unique effect in the growth and metabolic signaling in the gut compared to the P(BF+AOx) treatment. P(BF+AOx)+P(OA+EO) and P(BF+AOx) significantly altered the immunoregulatory signaling via adaptive and innate immunity to maintain a homeostatic immune response to cold stress and viral challenge without excessive inflammation, while P(BF+AOx)+P(OA+EO) displayed a less inflammatory response overall.

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L. Lahaye and E. Santin are employees and Jefo Nutrition. All other authors declare no conflict of interest.

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