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Effect of black soldier fly larvae (*Hermetia illucens*) meal supplementation on the adaptive immunity in vaccinated mice[1]

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ABSTRACT.- Rodrigues RR, Alves MLF, Galvão CC, Bilhalva MA, Ferreira MRA, Santos FDS, Júnior CM, Quatrin PHDN, Aguirres CL, Nava DE, Gonçalves RS, Nörnberg SD, Deschamps JC, Conceição FR. **Effect of black soldier fly larvae (*Hermetia illucens*) meal supplementation on the adaptive immunity in vaccinated mice.** *Pesquisa Veterinária Brasileira 46:e07694, 2026.* Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário, Pelotas, RS 96160-000, Brazil. E-mail: rodrigues.rr@ufpel.edu.br

With the increasing demand for protein-rich foods and the challenges associated with traditional sources, including those used in animal farming, this study investigated the effects of a 10% supplementation of black soldier fly larvae meal (BSFLM) on the adaptive immunity of mice immunized with recombinant chimeras composed of the alpha (CPA), beta (CPB), and epsilon (ETX) toxins of *Clostridium perfringens*. The results revealed that the inclusion of BSFLM in the diet did not compromise feed palatability, feed conversion ratio (FCR, 27.2 < 30.4), or animal weight gain (< 24.8 g). The vaccines induced a humoral immune response, and immunoglobulin levels remained unaffected by supplementation. Conversely, BSFLM significantly stimulated the transcription of mRNA for both anti-inflammatory (IL-10) and pro-inflammatory (IL-12 and IFN-γ) cytokines in stimulated splenocytes. These findings suggest that BSFLM supplementation improves vaccine-induced immune responses, especially cell-mediated ones, indicating potential for more efficient and sustainable animal production methods.

INDEX TERMS: Black soldier fly larva, insect meal, recombinant vaccine, immunomodulation.

RESUMO.- [**Efeito da suplementação alimentar com larvas de mosca soldado negra (*Hermetia illucens*) na imunidade adaptativa de camundongos vacinados.**] Com o aumento da demanda por alimentos ricos em proteínas e os desafios associados às fontes tradicionais, incluindo aquelas utilizadas na criação de animais, este estudo investigou os efeitos da suplementação de 10% de farinha de larvas da mosca soldado negra (BSFLM) sobre a imunidade de camundongos imunizados com quimeras recombinantes compostas pelas toxinas alfa (CPA), beta (CPB) e épsilon (ETX) de *Clostridium perfringens*. Os resultados revelaram que a inclusão de BSFLM na dieta não comprometeu a palatabilidade do alimento, a taxa de conversão alimentar (FCR; 27,2 < 30,4) ou o ganho de peso dos animais (< 24,8 g). As vacinas induziram uma resposta imunológica humoral, e os níveis de imunoglobulinas permaneceram inalterados pela suplementação. Por outro lado, a BSFLM estimulou significativamente a transcrição de mRNA para citocinas anti-inflamatórias (IL-10) e pró-inflamatórias (IL-12 e IFN-γ) em esplenócitos estimulados. Esses achados sugerem que a suplementação com BSFLM melhora as respostas imunológicas induzidas por vacinas, especialmente as mediadas por células, indicando potencial para métodos de produção animal mais eficientes e sustentáveis.

TERMOS PARA INDEXAÇÃO: Larva da mosca soldado negra, farinha de inseto, vacina recombinante, imunomodulação.

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INTRODUCTION

The growth of livestock farming, coupled with the increasing global demand for animal feed supplements in this sector, has been driving the search for alternative protein sources (Boland et al. 2013). Numerous challenges associated with traditional ingredient production primarily revolve around those based on soybean meal and fish oil, both of which serve a pivotal role in protein supply for animal feed (Tippayadara et al. 2021). Among the constraints are the elevated expenses associated with soybean production and apprehensions stemming from the unregulated expansion of agriculture to fulfill said demand (Silva et al. 2021), alongside the finite character of piscine-derived products and the significant ecological concerns associated with excessive fishing (Cashion et al. 2017).

To maintain high production levels while also addressing the requirements for animal health, it is necessary to promote efforts to implement healthier, more sustainable, and cost-effective diets in the livestock sector (Foley et al. 2011). In this challenging scenario, converting organic waste, such as livestock-generated manure, into protein- and fat-rich biomass using insects, such as black soldier fly larvae (*Hermetia illucens*; BSFL), becomes a promising option. The inclusion of BSFL in animal supplementation emerges as a system capable of promoting a circular economy by reducing and eliminating organic matter waste (Wang & Shelomi 2017).

Among other advantages, animal supplementation with black soldier fly larvae meal (BSFLM) is recognized for its exceptionally rich lipid profile in essential fatty acids (Sprangers et al. 2017). With its balanced amino acid profile, adequate calcium content, high digestibility, and functional properties, it represents a potential protein source in diets when there are digestion issues and low feed intake (Crosbie et al. 2020). Lauric acid content can account for up to 70% of the saturated fatty acids, depending on the animal diet formulation (Surendra et al. 2016). In the small intestine, evidence associated with lauric acid and antimicrobial peptides indicates antimicrobial activity (Sprangers et al. 2017, Spranghers et al. 2018, Wu et al. 2018). Furthermore, the larvae's exoskeleton also contains chitin, which possesses immunostimulatory properties and promotes beneficial intestinal microbes (Elieh Ali Komi et al. 2018).

The occurrence of diverse diseases, including those induced by bacteria and viruses, is prevalent within breeding environments. Such diseases not only exert a detrimental impact on productivity through compromised feed conversion and diminished animal weight gain but can also culminate in the abrupt mortality of entire herds. For example, some pathogenic species within the *Clostridium* genus are lethal to production animals, including ruminants, pigs, horses, and poultry (Uzal et al. 2016), making vaccination the primary immunoprophylactic measure. In particular, vaccination against the alpha (CPA), beta (CPB), and epsilon (ETX) toxins of *Clostridium perfringens* is the best prophylactic measure against necrotic enteritis, gas gangrene, and enterotoxemia, the main diseases caused by this species (Ferreira et al. 2016).

The significance of incorporating BSFLM into animal diets is emphasized by substantial evidence indicating its non-compromising effect on the health of production animals. To date, the nutritional advantages of BSFLM have been extensively investigated and demonstrated in studies involving swine (Crosbie et al. 2021), poultry (Marono et al. 2017), fish (Li et al. 2019), and cattle (Astuti & Wiryawan 2022). However, the effect of supplementation on vaccine-induced adaptive immunity remains largely unknown. The present study investigated the impact of BSFLM supplementation on both humoral and immune mediator responses in mice immunized with an experimental recombinant vaccine.

MATERIALS AND METHODS

Ethical approval. The experiment was conducted in accordance with the recommendations of the National Council for the Control of Animal Experimentation (CONCEA). Protocol number 2549 was approved by the Ethics Committee for Animal Experimentation at the "Universidade Federal de Pelotas" (UFPel).

Chimeric recombinant vaccines. Two recombinant chimeric antigens (rEBA and rBAE), composed of the alpha (CPA), beta (CPB), and epsilon (ETX) toxins from *Clostridium perfringens*, were used for vaccine formulation. The genes encoding rEBA and rBAE were synthesized with optimized codons for expression in *Escherichia coli* (Epoch Life Science, USA), cloned into the pET28a vector (Biobricks) (Rodrigues et al. 2023), and used to transform *E. coli* BL21 (DE3) (Sigma-Aldrich, USA).

The chimeric and individual antigens (rCPA, rCPB, and rETX) were expressed and purified as previously described (Moreira et al. 2016), with some modifications. In summary, protein expression was induced by using 0.5 mM IPTG over 3 hours in Luria Bertani (LB) medium supplemented with 50 µg/mL of kanamycin at 37 °C and 200 RPM on an orbital shaker. Cells were collected via centrifugation (7,000 g; 10 min; 4 °C) and resuspended in a lysis buffer consisting of 0.2 M NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole, and 50 mg/mL of lysozyme. The suspension was kept under gentle agitation at 37 °C for 1 hour. After this step, the cells underwent the process of sonication (10 cycles of 15 seconds at 60 kHz) and were then centrifuged (7,000 g; 10 min; 4 °C). The pellets were subjected to washes with 0.2%, 0.4%, and 0.8% N-Lauroylsarcosine, and the sediments were suspended in a buffer containing urea (0.2 M NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole, and 8 M urea).

The proteins were purified by Ni^{2+} affinity chromatography using the low-pressure liquid chromatography system ÄKTAprime (GE Healthcare) from solubilized inclusion bodies. Similar to the expression levels, the eluted fractions were verified by 12% SDS-PAGE and Western blot using a monoclonal anti-6xHis antibody (Sigma-Aldrich, USA), and subsequently dialyzed against phosphate-buffered saline (PBS) with 0.05% Triton X-100. Finally, the proteins were quantified using the BCA kit (Sigma-Aldrich, USA) and stored at 4–8 °C until further use.

For the formulation of recombinant vaccines, 50 µg of purified antigen (rEBA or rBAE) were adsorbed onto 15% aluminum hydroxide (v/v) for 18 hours at room temperature with gentle agitation, resulting in a final volume of 0.5 mL.

Animal experimentation: supplementation and vaccination. Forty Balb/C mice (female, 4-6 weeks old) were randomly distributed into four experimental groups (n = 10 each): (1) vaccination with rEBA without supplementation; (2) vaccination with rEBA and supplementation with BSFLM; (3) vaccination with rBAE without supplementation; (4) vaccination with rBAE and supplementation with BSFLM. The supplemented groups were provided with commercial feed (Presence, Laboratórios) enriched with 10% (w/w) of BSFLM (ad libitum) throughout the experiment. The non-supplemented groups received only commercial feed (Table 1). The mice and the feed were weighed periodically throughout the experiment to determine feed consumption and body weight (BW). The animals were weighed on days 0, 9, 23, and 30, and the average feed consumption was recorded every seven days. BW was included in the feed conversion ratio (FCR).

Table 1. Mean weight gain and feed conversion ratio (FCR) of animals supplemented or not with black soldier fly larva meal

Group	Initial weight (g)	Final weight (g)	FCR
rEBA	20.2	23	27.3
rEBA + BSFLM	20.5	23.2	27.2
rBAE	21.2	23.7	29.7
rBAE + BSFLM	22.3	24.8	30.4

BSFLM = black soldier fly larvae meal; The initial and final weight refers to the data collected on days 0 (initial) and 33 (final) of the experiment; FCR is the ratio of the total feed consumption to the individual's weight gain.

$$FCR = Feed\ intake / Body\ weight\ gain$$

$$Individual\ feed\ intake = Replicate\ weight\ gain / Number\ of\ birds\ per\ replicate$$

Individual body weight gain (BWG) was calculated as the average BW at the end of the period minus the average BW at the beginning of the period.

The rEBA and rBAE vaccines were administered subcutaneously on days 0 and 14. Blood samples were collected via the submandibular route on days 0, 14, and 25, and serum samples obtained by centrifugation (3,000 × g, 7 min) were stored at -20 °C until use.

Humoral immune response evaluation. Humoral immune response was evaluated by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Rodrigues et al. 2021). Briefly, microplates Nunc-Immuno Micro Well MaxiSorp (Sigma-Aldrich, USA) were coated with rCPA, rCPB or rETX (200 ng/well) in carbonate-bicarbonate buffer pH 9.6 and incubated at 4 °C for 18 h. Between each next step, the plates were washed three times with PBS-T (50 TS, microplate washer, 241 BioTeck). The plates were blocked with 5% skim milk powder in PBS-T, and the serum samples were diluted in PBS-T (1:200) and added to the plate (duplicate). The IgG horseradish peroxidase (HRP) anti-mouse conjugate (Sigma-Aldrich, USA) was diluted 1:5000 and added to the plate. Subsequently, orthophenylenediamine (OPD), citrate-phosphate buffer (0.2 M, pH 4.0, and 0.02% H_2O_2) and the enzymatic reaction were stopped after 15 minutes with 50 µl of 2 N sulfuric acid. Afterward, the absorbance of each well in the plate was measured at 492 nm using a microplate reader (Biochrom 251 EZ Read 400). To calculate the optical density (OD), the absorbance obtained from the reference well (without serum) was subtracted from the absorbance obtained from the well containing the serum sample. This procedure was performed to eliminate background noise before statistical analysis.

IgG total, IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA anti-rCPA, anti-rCPB, and anti-rETX concentrations in the sera of rEBA or rBAE-immunized mice were determined through indirect ELISA. In brief, following the sensitization, blocking, and well incubation steps with serum samples (as detailed above), the plates were incubated for 1 hour with 100 µL per well of anti-isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA) (Sigma-Aldrich, Darmstadt, Germany) diluted in PBS-T (1:2000). Afterward, the plates were incubated with HRP-conjugated anti-specific immunoglobulin antibodies (1:4000) (Sigma-Aldrich, USA). Both incubation phases occurred at room temperature and were separated by washing steps. The OD was obtained and read as previously described.

Spleen cell culture. The mice from all groups were euthanized on day 28 of the experiment, and their spleens were collected and macerated. Spleen cells (2×10^6) were cultured in 1 mL RPMI 1640 medium (Gibco, Grand Island/NY, USA) containing 10% fetal bovine serum (FBS, Gibco), antibiotics and antifungal agents (10.000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B, Gibco) in 24-well plates (Kasvi, Taiwan, China), and incubated for 24 h at 37 °C in an incubator containing 5% CO_2. After incubation, the medium was replaced, and the cells were stimulated with 10 µg rCPA, 10 µg rCPB, 10 µg rETX, 10 µg concanavalin A (ConA, Sigma), and RPMI 1640 medium, and further incubated for 18 h under the same conditions as above. ConA and medium were used as positive and negative controls, respectively. Following incubation, the supernatant was discarded, cells were collected with TRIzol® reagent (Sigma-Aldrich, USA), and stored at -70 °C until RNA extraction.

RNA isolation, cDNA synthesis, and qPCR. The RNA was extracted using the TRIzol method, according to the manufacturer's instructions. Approximately 400 ng of RNA was used for the synthesis of cDNA, and the reaction was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City/CA, USA) according to the manufacturer's instructions. A quantitative polymerase chain reaction (qPCR) was performed on a CFX96TM Real-Time System platform (Bio-Rad, Hercules/CA, USA) using specific primers for β-actin, GAPDH, IL-4, IL-10, IL-12, and IFN-γ. The primer sequences used for this reaction have been previously described (Cardona et al. 2003, Dummer et al. 2014). The reaction efficiency for each primer pair was calculated using a two-fold dilution series on a cDNA sample, and the standard curves were represented as the semi-log regression line plot of the C_t value vs. log of the relative input cDNA concentration, as described by Bustin et al. (2009). A 97.9%–106.9% efficiency was considered acceptable and primers with efficiencies within these limits were included. β-Actin was used as an endogenous reference gene. The qPCR reactions were performed using 1 µL of cDNA (synthesized from 400 ng of RNA), 6.25 µL of GoTaq® qPCR Master Mix (Promega, Madison/WI, USA), 0.25 µM of each primer, and 4.25 µL of RNAse-free water (Sigma-Aldrich, USA), in a total reaction volume of 12.5 µL. The temperature conditions were: denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. All the samples were analyzed in triplicate. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to determine the relative amount of mRNA for each gene with β-actin as the reference gene, according to the method described by Livak & Schmittgen (2001).

Statistical analysis. All statistical analyses were conducted using GraphPad Prism software, version 7 (GraphPad, San Diego). A one-way repeated measures analysis of variance (ANOVA) was performed to identify differences in the effect of BSFLM supplementation on feed consumption and feed conversion ratio (FCR) over time. The relative cytokine mRNA transcription levels in each experimental group were analyzed using an unpaired t-test, and the impact of BSFLM supplementation on the humoral immune response was assessed using the non-parametric Kruskal-Wallis test with Dunn's post hoc comparisons. Significant *p*-values are indicated as follows: $*p < 0.05$.

RESULTS

Production of recombinant antigens

Recombinant versions of the alpha (CPA), beta (CPB), and epsilon (ETX) toxins, previously validated in vaccine antigen studies (Moreira et al. 2016, Rodrigues et al. 2021), were employed in the construction of two trivalent chimeras, rEBA (rETX-rCPB-rCPA) and rBAE (rCPB-rCPA-rETX). Bioinformatics tools were used for *in silico* analyses to refine antigens and construct chimeras (Rodrigues et al. 2021).

Remarkably high expression of the chimeras was achieved, duly confirmed through SDS-PAGE analysis (Fig. 1). Additionally, a heightened degree of purity was achieved after the affinity chromatography purification process (Fig. 2). Truncated fragments of the recombinant chimeras were observed in the Western blot (Fig. 3).

Average consumption and weight of animals

In all groups, the average daily feed consumption was 4.5 g per animal (± 0.27), and there was no significant difference ($p > 0.05$) between the consumption of supplemented or commercial feed (Fig. S1). At the end of the experiment, the average body weight of the subjects and the feed conversion ratio across all experimental groups showed no statistically significant differences ($p > 0.05$), as detailed in Table 1.

Throughout the monitoring period, mice fed diets both with and without BSFLM supplementation exhibited robust health and displayed no observable pathologies or physical anomalies. The data on daily feed consumption per animal consistently met the established daily nutritional requirements for adult mice (Bachmanov et al. 2002). Although discrepancies in feed consumption were documented across the experimental groups, it is plausible that these variations may be attributed to the handling period, spanning from days 7 to 24, which potentially induced stress in the subjects as a consequence of factors such as the collection of biological samples, alterations in enclosure conditions, physical restraint, and the administration of vaccines (Fig. S1).

Humoral immune response

Total IgG antibodies against all three toxins were induced by both chimeras, with a more robust response of total IgG anti-rCPA and anti-rCPB induced by rBAE. Regardless of the antigen evaluated, supplementation with BSFLM did not alter the humoral immune response ($p > 0.05$) (Fig. 4).

When determining the subclasses of IgG (IgG1, IgG2a, IgG2b, and IgG3), IgA, and IgM against CPA, CPB, and ETX, no statistical differences were observed in relation to the supplementation provided to the animals.

The rBAE chimera induced higher levels of anti-rCPA and anti-rCPB antibodies for all evaluated isotypes (Fig. 5 and 6). In contrast, the rEBA chimera generated higher levels of anti-rETX antibodies for the IgG1, IgG2a, IgG2b, and IgA isotypes (Fig. 7). Compared to the other immunoglobulins, IgG1 and IgM showed stronger levels of response against rCPA, rCPB, and rETX for both rEBA and rBAE.

Cytokine mRNA transcription

To ascertain whether supplementation with BSFLM in mice vaccinated with rEBA and rBAE could enhance the relative mRNA transcription of the cytokines IL-4, IL-10, IL-12, and IFN-γ, splenocytes were stimulated with the recombinant proteins rCPA, rCPB, or rETX, which constitute the vaccine antigens of rEBA (Fig. 8) and rBAE (Fig. 9). For the comparison of cytokine levels generated, the increases in cytokine levels

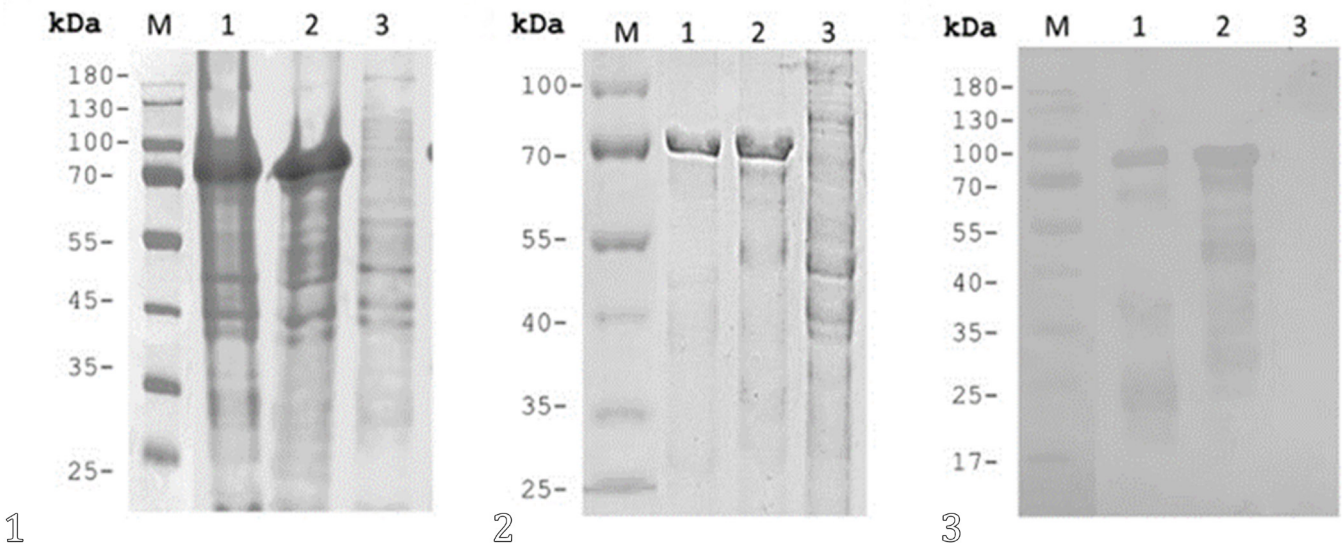

Fig. 1-3. SDS-PAGE and Western Blot of rEBA and rBAE. (**1**) SDS-PAGE: M = marker, 1 = *Escherichia coli* BL21 (DE3) after rEBA induction, 2 = *E. coli* BL21 (DE3) after rBAE induction, 3 = untransformed *E. coli* BL21 (DE3). (**2**) SDS-PAGE: M = marker, 1 = rEBA after purification, 2 = rBAE after purification, 3 = untransformed *E. coli* BL21. (**3**) Western Blot: M = marker, 1 = rEBA after purification, 2 = rBAE after purification, 3 = untransformed *E. coli* BL21.

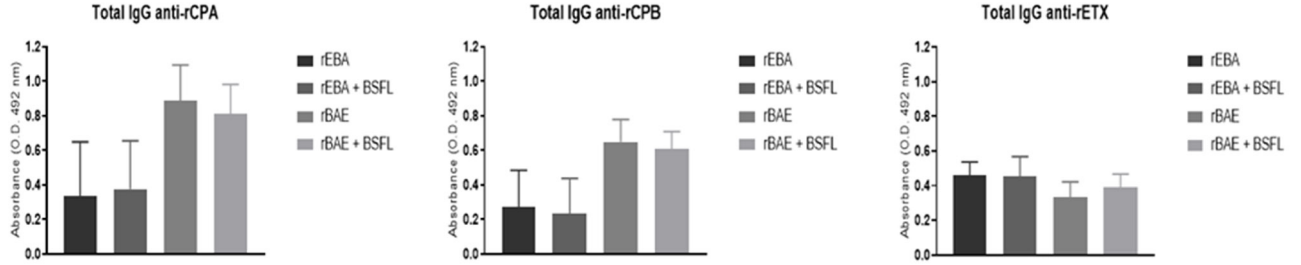

Fig. 4. Levels of total IgG antibodies in serum samples on day 25. Post-vaccination immunoglobulin levels between animals supplemented and not supplemented with black soldier fly larvae meal (BSFLM) are compared for each administered recombinant vaccine (rEBA and rBAE). No significant differences were observed in the levels of total IgG antibodies against rCPA, rCPB, and rETX in both groups ($p > 0.05$).

of each supplemented group were contrasted with those of their corresponding non-supplemented groups.

The spleen cells of mice from the supplemented group vaccinated with rEBA and stimulated with ConA showed a significant increase ($p < 0.05$) in mRNA transcription of IL-10 (2.2-fold), IL-12 (2.6-fold), and IFN-γ (4.3-fold). Similar trends were noted following stimulation with rCPA (IL-10: 14.6-fold; IL-12: 15-fold; IFN-γ: 84.7-fold), rCPB (IL-10: 41.3-fold; IL-12: 40-fold; IFN-γ: 28-fold), and rETX (IL-10: 5.28-fold; IL-12: 8.8-fold; IFN-γ: 5.45-fold). Interestingly, these cells, when stimulated with ConA, rCPA, and rETX, showed a significant increase in mRNA transcription levels of IL-4 in the non-supplemented group compared to the BSFLM-supplemented group.

Splenocytes from the rBAE-supplemented and vaccinated group exhibited a robust response to ConA stimulation, showing a significant increase in IFN-γ transcription levels ($p < 0.05$). Upon stimulation with rCPA, there was a moderate increase in IL-10 mRNA transcription levels (1.5-fold) and a notable elevation in IFN-γ (189.3-fold), both statistically significant ($p < 0.05$). Stimulation with rCPB resulted in significant increases in mRNA transcription levels of IL-4 (9.7-fold), IL-10 (4.8-fold), and IFN-γ (28.4-fold), while elevated levels were observed for IL-10 (1.93-fold) and IFN-γ (5.8-fold) upon stimulation with rETX ($p < 0.05$). However, the effect on IL-12 showed distinct patterns, revealing an increase with ConA, rCPA, rCPB, and rETX stimuli within the non-supplemented group as opposed to the BSFLM-supplemented group.

DISCUSSION AND CONCLUSION

Immunization with recombinant toxins derived from *Clostridium perfringens* has consistently exhibited the capability to elicit elevated levels of antibody titers across multiple animal species (Ferreira et al. 2016), rendering it a pertinent model for the evaluation of the influence of BSFLM supplementation on the immune response in vaccinated animals (Santos et al. 2021). In general, BSFLM acts as a functional mediator of various biochemical and nutritional parameters at the cellular level and stands out as a potential food source for both livestock and humans (Marono et al. 2017). Nevertheless,

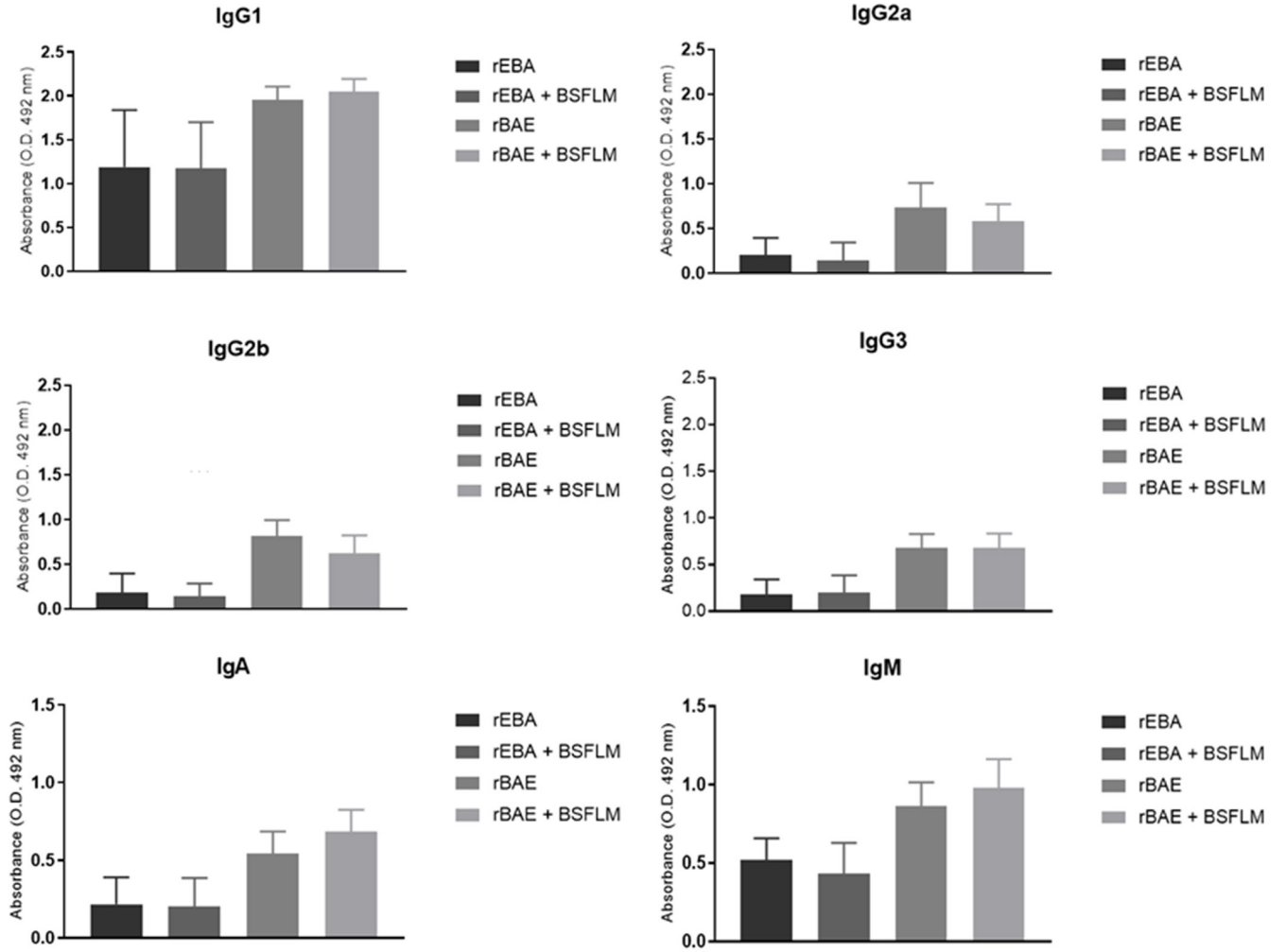

Fig. 5. Levels of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), IgA, and IgM against rCPA determined by indirect ELISA. Post-vaccination levels of immunoglobulin subclasses between animals supplemented and not supplemented with black soldier fly larvae meal (BSFLM) are compared for each administered recombinant vaccine (rEBA and rBAE). No significant differences were observed in the levels of anti-rCPA antibodies in both groups ($p > 0.05$).

the complete scope of its impact when integrated with vaccination remains predominantly uncharted. This is one of the issues to be addressed before considering large-scale production. Therefore, in this study, mice supplemented with BSFLM were evaluated for feed consumption rate, growth, and immune response following vaccination with different chimeric antigens against *C. perfringens*, previously developed and characterized by our group.

The inclusion of 10% of BSFLM in the animals' diet exhibited no discernible adverse effects on weight gain and feed consumption, aligning with assessments conducted for zootechnical performance in production species (Schiavone et al. 2018). To ascertain whether these observations imply the feasibility of substituting conventional diets with this supplementation without compromising or augmenting the antibody-mediated immune response elicited by recombinant vaccines, we conducted serological analyses.

Both recombinant chimeras successfully induced IgG responses specific to the three main *C. perfringens* toxins, confirming their immunogenicity. Higher IgG titers against CPA and CPB observed in animals immunized with rBAE, compared to those immunized with rEBA, were expected, given the previously demonstrated potency of this chimera. Despite these distinct immunogenic profiles, BSFLM supplementation did not alter total IgG or its subclasses (IgG1, IgG2a, IgG2b, and IgG3), nor IgA or IgM levels, indicating that the diet did not modulate the humoral response or affect the Th1/Th2 balance associated with antibody production. The predominance of IgG1 and IgM in both chimeras further supports a balanced activation of humoral immunity without evidence of polarization. However, potential effects of BSFLM on cellular responses cannot be excluded, as later evidenced by differential cytokine expression.

These findings are consistent with studies in other species, in which BSFLM supplementation did not produce measurable changes in humoral responses. In accordance with the findings of Crosbie et al. (2021), the administration of BSFLM as a protein source, at levels up to 50%, demonstrated no discernible impact on plasma concentrations of IgG and IgG1 elicited by the ovalbumin antigen (OVA). Similarly, in the study by Driemeyer (2016), supplementation with 3.5% BSFLM of total fat did not affect the assessed biochemical parameters (albumin, calcium, phosphorus, iron, IgA, IgG, and IgM) in non-vaccinated piglets. In fish, Li et al. (2019)

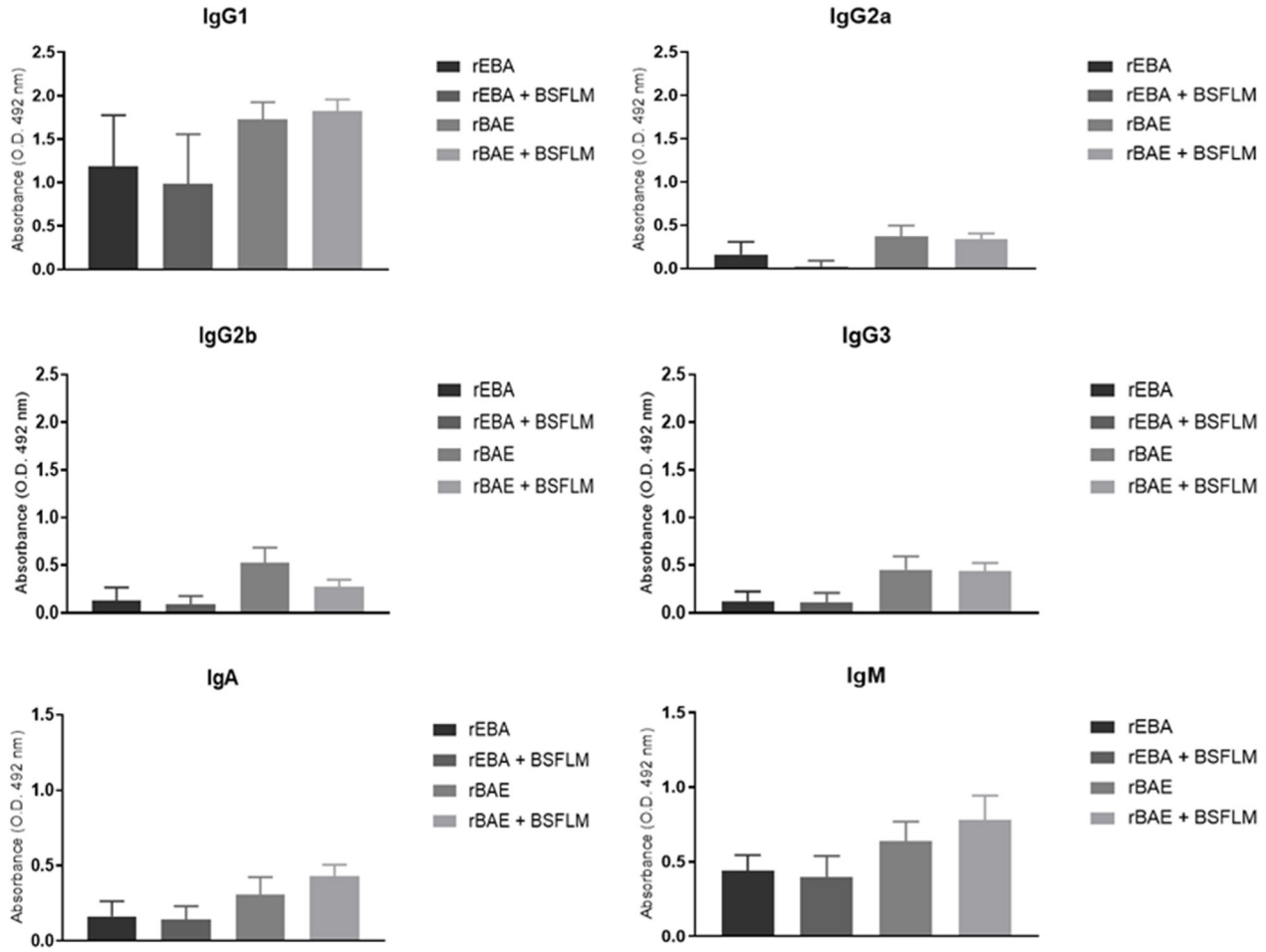

Fig. 6. Levels of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), IgA, and IgM against rCPB determined by indirect ELISA. Post-vaccination levels of immunoglobulin subclasses between animals supplemented and not supplemented with BSFLM are compared for each administered recombinant vaccine (rEBA and rBAE). No significant differences were observed in the levels of anti-rCPB antibodies in both groups ($p > 0.05$).

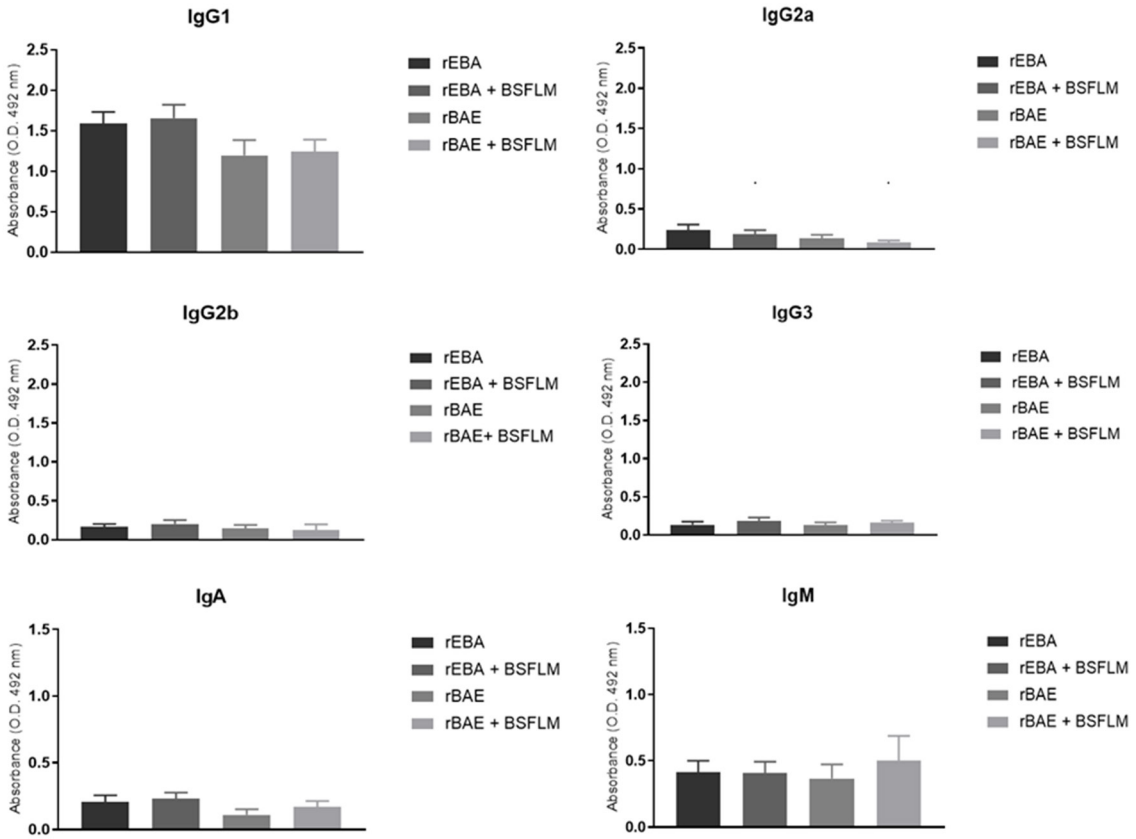

Fig. 7. Levels of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), IgA, and IgM against rETX determined by indirect ELISA. Post-vaccination levels of immunoglobulin subclasses between animals supplemented and not supplemented with black soldier fly larvae meal (BSFLM) are compared for each administered recombinant vaccine (rEBA and rBAE). No significant differences were observed in the levels of anti-rETX antibodies in both groups ($p > 0.05$).

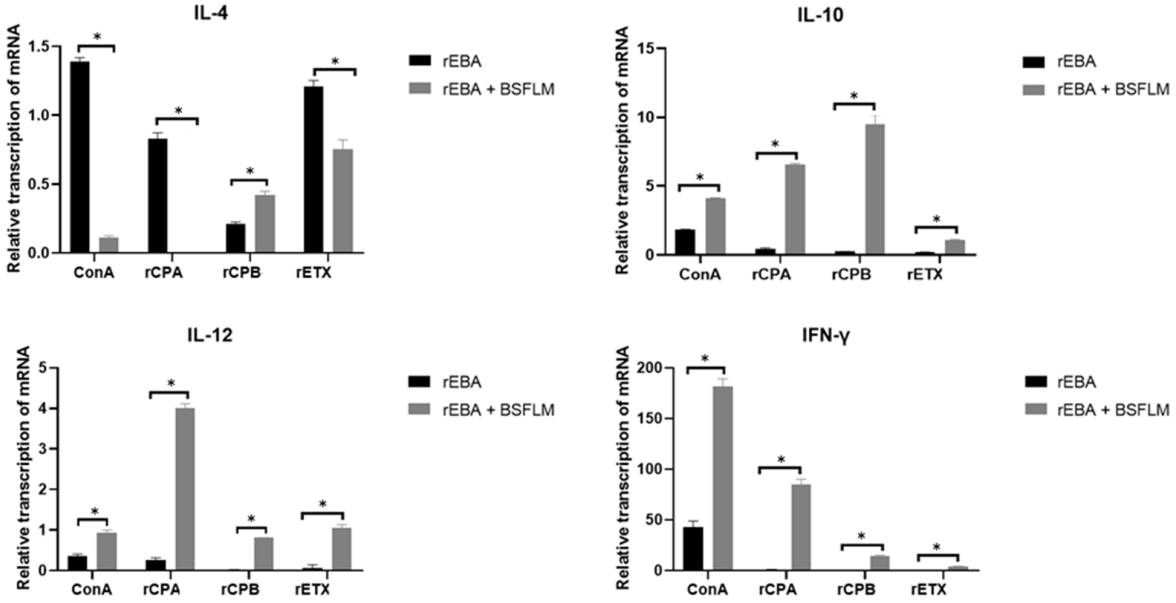

Fig. 8. qPCR cytokines transcription spleen cells from mice vaccinated with rEBA and supplemented with black soldier fly larvae meal (BSFLM). The data represent the mean ± SEM of IL-4, IL-10, IL-12, and IFN-γ mRNA transcripts in splenocytes from mice vaccinated with rEBA and supplemented with BSFLM, exposed to medium (RPMI 1640), and stimulated with ConA, rCPA, rCPB, and rETX. The relative mRNA transcription was determined using the comparative threshold cycle ($\Delta\Delta C_t$). Asterisks indicate a statistical difference (*$p < 0.05$) between the experimental groups

also reported no significant differences between groups fed BSFLM and those on a conventional diet in the antibody response against infectious pancreatic necrosis virus (IPNV).

In the cellular immune response, T cells produce a range of cytokines that play vital roles in immunity. IFN-γ is induced by cytokines such as IL-12 and is produced by Th1 CD4+ and CD8+ T cells, while the secretion of the cytokine IL-10 can be induced by IL-4 and is primarily produced by activated CD8+ cells (Rastogi et al. 2022). Overall, we observed that the production levels of some of these molecules, while elevated in certain groups, were not positively correlated with the stimulus induced by the supplement, specifically IL-4 stimulation in the groups that received rEBA and rBAE with or without BSFLM and IL-12 in the rBAE groups with or without BSFLM. However, IFN-γ and IL-10 exhibited higher transcription levels in mice that received BSFLM + rEBA or rBAE, and a greater stimulus of IL-12 detection induced by rEBA + BSFLM was observed. In a comparative analysis, BSFLM yielded a notably heightened increase in mRNA transcription levels of IFN-γ (5.45 < 189.3-fold), surpassing the increments observed for IL-10 (1.5 < 41.3-fold) and IL-12 (8.8 < 40-fold). This heightened stimulus was observed in response to three antigens (rCPA, rCPB, and rETX) included in both assessed vaccines. Therefore, when analyzing the main parameters of the cellular response obtained, it is important to highlight that the robust and remarkable expression of IFN-γ observed with the addition of BSFLM is a significant stimulus for a necessary cellular immune response in the control of diseases caused by viruses (e.g., influenza and hepatitis B) (Paillot et al. 2006, Rahimkhani et al. 2021), intracellular bacteria (e.g., tuberculosis and chlamydia) (Entrican et al. 2012, Zhu et al. 2018) and intracellular parasites (e.g., toxoplasmosis and malaria) (McCall & Sauerwein 2010, Ducournau et al. 2020). Thus, although BSFLM supplementation did not modulate the humoral response or the Th1/Th2 balance associated with antibody production, the differential cytokine expression observed suggests modulation at the cellular level. BSFLM supplementation could be strategically employed in immunization protocols targeting pathogens controlled predominantly by cellular immune responses.

In this context, it is worth noting that aluminum hydroxide was used as an adjuvant in this study. This salt is widely employed in vaccines and is known to primarily enhance the Th2 cell-mediated humoral immune response, without the need for large amounts of antigen (Moreira Jr et al. 2020, Pulendran et al. 2021). Furthermore, it has a history of safe use and the ability to promote immunological memory (Moyer et al. 2020, Freitas et al. 2021). Thus, the elevated IFN-γ transcription levels observed in vaccinated animals supplemented with BSFLM suggest that the supplement may have contributed to a greater balance between the Th1 and Th2 axes, mitigating the Th2 polarization typically induced by aluminum hydroxide and promoting a more balanced immune response.

Among the components that could explain the influence of BSFLM on the immune response, chitin and lauric acid stand out as immunomodulators of the cellular immune response (Lee et al. 2008, Mohana Devi & Kim 2014, Xing et al. 2017). Chitin plays a significant role as an immunostimulant for innate and adaptive responses (Elieh Ali Komi et al. 2018), while lauric acid and its metabolites have antimicrobial and anti-inflammatory properties (Mohana Devi & Kim 2014,

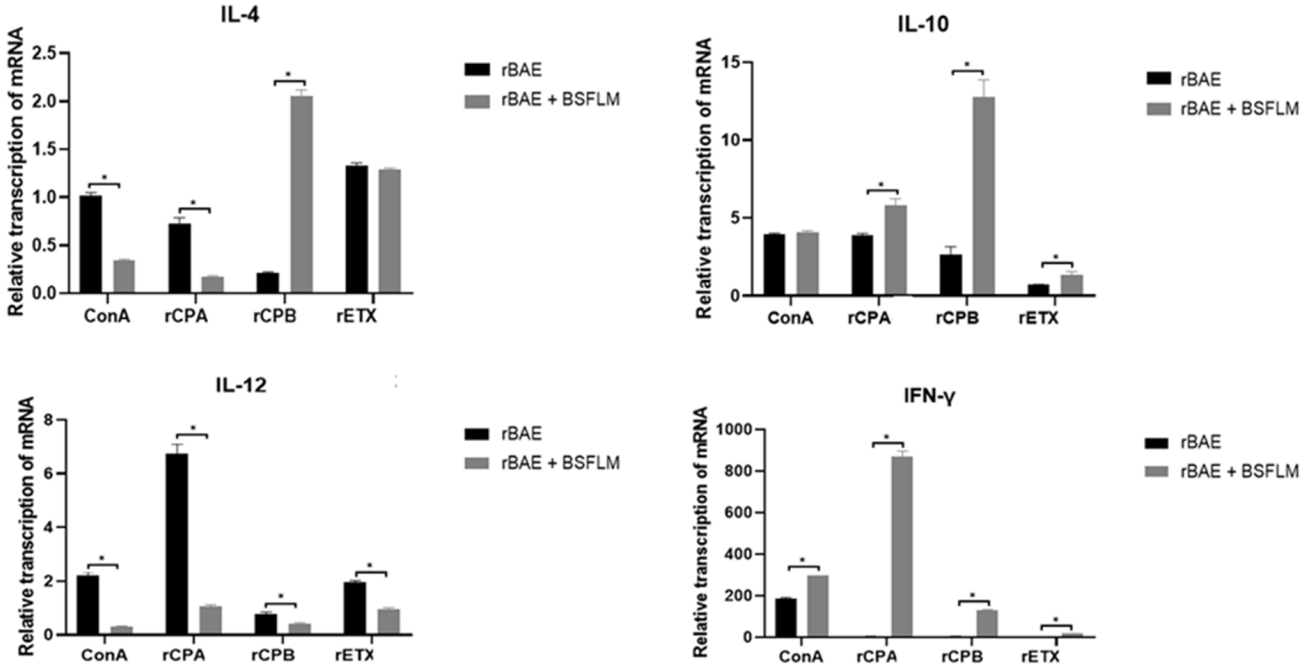

Fig. 9. qPCR cytokines transcription spleen cells from mice vaccinated with rBAE and supplemented with BSLFM. The data represent the mean ± SEM of IL-4, IL-10, IL-12, and IFN-γ mRNA transcripts in splenocytes from mice vaccinated with rBAE and supplemented with black soldier fly larvae meal (BSFLM), exposed to medium (RPMI 1640), and stimulated with ConA, rCPA, rCPB, and rETX. The relative mRNA transcription was determined using the comparative threshold cycle ($\Delta\Delta C_t$). Asterisks indicate a statistical difference (*$p < 0.05$) between the experimental groups.

Sprangers et al. 2017). Specifically, chitin can stimulate the production of IFN-γ and IL-10 while seemingly exerting a negative regulatory effect on the responses of other inflammatory mediators, such as IL-4 and IL-5 (Shibata et al. 2000, 2001, Da Silva et al. 2009, Nagatani et al. 2012). In contrast, lauric acid appears to stimulate the production of IL-10 (Lee et al. 2017). While not fully explored, these and other mechanisms associated with BSFLM may help explain the observed upregulation of both IFN-γ and IL-10 in the supplemented mice of both evaluated groups.

The findings of this study are particularly relevant to livestock production systems. The ability of BSFLM to promote a balanced immune response in mice suggests that, beyond its established nutritional benefits in poultry and swine (Driemeyer 2016, Schiavone et al. 2018), this ingredient could also enhance the efficacy of vaccines that rely on robust cellular immunity in production animals. Although physiological differences between rodents and production species may affect the bioavailability of BSFLM bioactive components, such as antimicrobial peptides, chitin, and medium-chain fatty acids, many immunological pathways responding to these compounds are conserved across species. The marked increase in IFN-γ and IL-10 expression observed in mice implies that similar modulatory effects could occur in production animals, potentially improving vaccine-mediated protection and resistance to infections. Controlled trials in livestock will therefore be essential to confirm the translational relevance of these results. Future studies should also investigate whether the immunomodulatory effect of BSFLM — particularly the striking upregulation of IFN-γ (5.45 to 189.3-fold) — is consistent across different antigens and vaccine formulations. These results position BSFLM not only as a sustainable nutritional resource but also as a potential dietary adjuvant capable of enhancing cellular immunity elicited by vaccines, with practical implications for animal health and sustainable production systems.

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