Answer to Reviewer 2

As extra feed additives, add extra costs to the administered diets, a cost-benefit analysis should be presented to substantiate their use in feed for Nile Tilapia.

> Thank you for pointing out this gap in the evaluation of the performance data. We have calculated the feed cost and the specific feed cost for the trial, and the results have been incorporated into Table 1. Based on these data, the application of the tested materials as feed additives increased the specific cost by 1.4% (EXP-I) and 5.6% (EXP-A), respectively, compared to the control commercial feed (COM), and by 0.91% (EXP-I) and 5.05% (EXP-A), respectively, compared to the feed containing a commercial feed additive. However, the production cost of *Nannochloropsis gaditana* CCMP526 is expected to decrease with commercialization, as the current price was calculated based on its use as an experimental material. Nevertheless, we conclude that the potential advantages offered by the feed additives could compensate for the observed increases in feed cost.

Lines 171-175: regarding the stress trial, why have authors chosen "five minutes" as the interval time for the air exposure, and not two, three, four, or six minutes? Is there a referred specific protocol for this? As fish have been returned back to the water after the air-exposure, have fish survived after this "5-minute air exposure"?

> There are no established or widely accepted protocols for conducting air exposure stress experiments with tilapia. In a similar study (Zahran et al. 2021), one minute of air exposure was applied. We selected an exposure duration of five minutes to evaluate the effects of a common stressor while minimizing unnecessary suffering for the fish. Following air exposure, the fish were returned to water and over-anesthetized prior to sampling. All stressed fish individuals were sampled, and this procedure has been better clarified in Section 2.5 in revised version of the manuscript.
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> In Laos, it is common practice to expose tilapia to air during transportation and weighing. These fish have adapted to environments with low dissolved oxygen through evolution and have developed the ability to breathe air, enabling them to survive out of water for extended periods (Guan et al. 2017; Fan et al. 2022).
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> Zahran et al. (2021): *Nannochloropsis oculata* enhances immune response, transcription of stress and cytokine genes in Nile tilapia subjected to air exposure stress. *Aquaculture Reports*, 21, 100911.
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> Guan et al. (2017): Cooling combined with hyperoxic CO2 anesthesia is effective in improving the air exposure duration of tilapia. *Scientific Reports*, 7, 14016
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> Fan et al. (2022): Carbon dioxide anesthesia: A potential application to improve the air exposure duration of tilapia. *Iranian Journal of Fisheries Sciences*, 21(1), 82-92.

Lines 100-112: The Initial and Final Stocking Density of fish in the cages, are somehow low (< 1 kg/m-3). This does not correspond to real commercial fish farming conditions, where stocking densities range between 10-20 kg/m-3. However, this is not a crucial drawback for the experimental design and credibility of the experimental outcome

> Thank you for pointing this out. The experimental design was specifically structured to obtain sufficient fish biomass for evaluating production parameters and conduct the stress experiment. As such, the results were not intended for direct comparison with production data from typical farm conditions.

Lines 144-145 (Table 2): There is a drawback (in the experimental design), as far as the protein and amino acid content of the experimental feeds, is concerned: the COM diet contains 40% fishmeal and 36% soybean meal. In the rest of experimental diets, soybean meal is diminished by half (18% of the diet) and fishmeal is increased by 10-13%. Does this increase in fishmeal, compensate for the loss of amino acids supplied by soybean meal? (which has been halved compared to the COM diet). This is clearly acknowledged in the Discussion Section (lines 296-301) and is good that the authors realize the negative side-effect of this specific experimental drawback.

> Thank you for your question and comments. Variations in the fishmeal-to-soybean meal ratio are reflected less in amino acid composition and more in differences in nutrient digestibility or fatty acid profiles or enteritis caused by the soybean. The feeds were supplemented with premix containing synthetic amino acids to satisfy the requirement.

Specific comments referring to line numbers, tables or figures that point out inaccuracies within the text or sentences that are unclear.

> By some error in the submission system, the entire Figure 4 was missing from the manuscript. The figure with presentation of the immune gene expression data have been included into the revised version of the manuscript.

Reviewer 3:

Line 2-3: It is recommended to specify the specific types of additives in the title.

> Thank you for your suggestion. The title has been changed to "Effects of feed additives (*Nannochloropsis gaditana and Hermetia illucens*) on growth and expression of antioxidant and cytokine genes in Nile tilapia (Oreochromis niloticus) subjected to air exposure stress "

Line 16: "Oreochromis niloticus" should be in italics, and the same applies to the other Latin names.

> All Latin names in the article have now been written in Italics.

Line 33: The notation of $p<0.05$ here is incorrect. The original text means that there is no significant difference, which should be $p>0.05$. Moreover, p-values are generally not required in the abstract.

> That p-value has been removed from the abstract.

Line 38-39: The statement " we recommend the inclusion of any of the studied additives " in the conclusion is contradictory to the significant advantages of the EXP-A group in the results. It should clearly prioritize the recommendation of *Nannochlorophsis gaditana*.

> The last sentences of the abstract have been modified prioritizing the best performing additive. "Based on our results, we recommend the inclusion of any of the tested additives at the evaluated doses to enhance the non-specific immune response of Nile tilapia. Additionally, *Nannochloropsis gaditan*a at a 3.5% inclusion level can be used to further improve antioxidant defence capacity."

Line 80: Please confirm whether a comma is needed between "European seabass [16] and "yellow catfish (*Pelteobagrus fulvidraco*)."

> A comma has been added between "European seabass" and "yellow catfish". Also, Latin names of rainbow trout *(Oncorhynchus mykiss*) and European seabass (*Dicentrarchus labrax*) have also been added.

Line 118-119: The dissolved oxygen saturation in the water quality parameters is relatively low (41.5-66.6%), which may interfere with the stress response and metabolism of the fish. It is necessary to discuss the potential impact of this condition on the experimental results.

> Thank you for your suggestion. We have considered this observation when concluding our results. You are right that dissolved oxygen (DO) saturation in the water was often low during the rearing season. This is because, in early autumn, the region typically experiences a high number of cloudy days, which reduces the oxygen saturation levels in both the cages and in the reservoir itself. Tilapias are known for their resilience in low-oxygen environments, a trait that makes them suitable for intensive aquaculture systems. However, consistently low DO levels in tanks or cages can still pose significant challenges. Insufficient oxygen can stress the fish, reduce feed intake, slow growth, and increase susceptibility to diseases (El-Sayed, 2006). In our case, during the experimental period, however the oxygen level was relatively low, we considered adequate for those fish and were supported by the obtained growth performance and survival rate data.
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> During the air exposure period the fish suffered from hypoxia, which was indicated by the cancellation of the immunomodulatory effect of the feed additives present at the end of

feeding period (see Figure 3, c-d-g-h in the revised version of manuscript), as it is mentioned in the last paragraph of the Discussion (LINE 400-403, revised version of the manuscript). Similar results were obtained by Zahran et al, 2021 (reference No. 7 in the revised version of the manuscript), where one minute of air exposure cancelled the elevated expression of pro-inflammatory cytokines in tilapias treated with *N. oculata*. Because of this, we concluded that direct air exposure as a stressor was strong enough to have a negative effect on non-specific immune response, even if our fish were acclimatized to a relatively low oxygen level. Now it is mentioned in the last paragraph of the Discussion (LINES 403-405 in the revised version of the manuscript). Obviously, measuring stress markers in the blood or analysing the expression of stress-related genes would provide a clearer picture of the actual stress status of fish.

El-Sayed, A.-F. M. (2006). *Tilapia Culture*. CABI Publishing.

Line 128: It is necessary to supplement the name of the ethics review institution and the approval number.

The trial was conducted at the farming site of Aquatic Development Company Ltd., located in Nam Houm village, Vientiane, Lao PDR, which operates in accordance with the country's Good Aquaculture Practice (GAP) standards. Furthermore, the study was carried out with the active involvement of the Namxuang Aquaculture and Fisheries Development Center, (NAFDEC) which is operated by the Ministry of Agriculture and Forestry in Laos, this ensured that the research was conducted by the legal, professional, and ethical framework and standards of Lao PDR. The experiment and its results were presented at the highest national level at the LAO-HUNGARY-VIETNAM FRESHWATER AQUACULTURE WORKSHOP with Dr. Kaviphone Phouthavong, Deputy Director of the Department of Livestock and Fisheries as the chairperson.

The next information has been incorporated into the text.

“All procedures involving fish were conducted in accordance with the Law on Animal Husbandry and Veterinary (Revised) No. 08/NA, dated 11 November 2016, of the Lao People's Democratic Republic. All efforts were made to minimize the suffering of the fish.”

Line 295: Compared with "isoenergetics", "isoenergetic" is more appropriate and commonly used.

This correction has been made.

Line 306-308: It is necessary to conduct an in-depth explanation of why only the EXP - A group significantly upregulated antioxidant genes while the effects in other groups were not significant (such as the mechanism of action of specific components in *Nannochlorophsis gaditana*).

Thank you for pointing out this shortcoming about the regulation of antioxidant genes. The revised version of the manuscript has been supplemented with the following explanation in the second paragraph of the Discussion section:

“Microalgae of the genus *Nannochloropsis* are rich in valuable carotenoids like violaxanthin, zeaxanthin, astaxanthin or β-carotene [5]. These molecules can neutralize ROS by accepting electrons to their conjugated double bonds [30]. Beside this direct effect, they can activate the transcription factor Nrf2 (Nuclear factor erythroid 2-related factor 2), which increases the expression of genes *sod*, *cat* and *gpx* [31, 32]. This explains the significant increase in the expression of these genes measured in group EXP-A.”

30: Zuluaga M., Gueguen V., Pavon-Djavid G., Letourneur D.: Carotenoids from microalgae to block oxidative stress. *BioImpacts*, **2017**, 7(1), 1-3.
31: Kaulmann A., Bohn T.: Carotenoids, inflammation and oxidative stress – implications of cellular signaling pathways and relation to chronic disease prevention. *Nutrition Research*, **2014**, 34, 907-929.
32: Bohn T: Carotenoids and markers of oxidative stress in human observational studies and intervention trials: implications of chronic diseases. *Antioxidants*, **2019**, 8, 179.

Line 420-421: The original suggestion of "inclusion of any of the studied additives" contradicts the fact that the EXP-A group significantly outperforms the other groups in the data. It is recommended to be revised such as: "We recommend giving priority to supplementing 3.5% of *N. gaditana* to enhance the antioxidant defence capacity, and meanwhile, further studies are required to optimize the dosages of BSFL and commercial additives."

The last sentence of the Conclusion has been changed to “Based on our results, we recommend the application of any of the studied additives in the tested doses to enhance the innate immune response of Nile tilapia. In addition to its immunomodulatory properties, *N. gaditana* in 3.5% can be applied to increase the antioxidant defence capacity as well.”

In addition, it is a rather important issue that the expression of immune-related genes is not presented in the results section of the manuscript. Please check whether there is any omission of results.

Thank you for observing this mistake. We apologize for that. By some error in the submission system, the entire Figure 4 was missing from the manuscript. The figure with presentation of the immune gene expression data have been included into the revised version of the manuscript.