For research article

Response to Reviewer 2 Comments

1. Summary

Thank you very much for taking the time to review this manuscript. Please find the detailed responses below and the corresponding revisions/corrections highlighted/in track changes in the re-submitted files.

2. Point-by-point response to Comments and Suggestions for Authors

Comments 1: Line nos-34-35- Decreased serum levels of TBA, BUN, and UA, while increasing the levels of DBIL, TBIL, and GSH-PX at day 3, followed by a decrease from day 5 onwards. – The abbreviations should be written in full form

Response 1: We are really appreciate for your time and helping us to improve the paper. We totally agree with this comment. Therefore, we put all abbreviations in the abstract into full form.

Line no: 34-36 "[decreased serum levels of total bile acid (TBA), blood urea nitrogen (BUN), and uric acid (UA), while increasing the levels of direct bilirubin (DBIL), total bilirubin (TBIL), and glutathione peroxidase (GSH-Px) at day 3, followed by a decrease from day 5 onwards.]"

Comments 2: Line no 50-51: Its approval for treating respiratory diseases in chickens caused by *Mycoplasma gallisepticum* and *Mycoplasma hyopneumoniae* highlights its practical significance- *Mycoplasma hyopneumoniae* is the pathogen of pigs so it should be removed, may be *Mycoplasma synoviae* could be added.

Response 2: Thank you for pointing this out. We totally agree with this comment. Therefore, we did more research, removed the Mycoplasma hyopneumoniae and added Mycoplasma synoviae instead.

Line no: 51-52 "[Its approval for treating respiratory diseases in chickens caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae* highlights its practical significance]"

Comments 3: Line no-85 CA concentration of 0.75g/L tilmicosin was ad- 85-What do you mean by CA concenteration

Response 3: Thank you for pointing this out. We totally agree with this comment. "CA concentration" was a typo, we corrected it as "Concentration".

Line no: 91-92 "[Concentration of 0.75g/L tilmicosin was administrated to the treatment group through drinking water at the beginning of the experiment and for the consecutive of three days.]"

Comments 4: Li Line no-87- , 10ten chickens – Correct and give in either in number or in word
Line no-87- 6six chickens - Correct and give in either in number or in word

Response 4: Thank you for pointing this out. We totally agree with this comment. Therefore, we changed these all into number form.

Line no: 94" [10 chickens from the treatment group and 6 chickens from the control group]"

Comments 5: It would be better if the authors justify why they have chosen Silkie chicken over meat type /egg type chickens.

Response 5: Thank you for pointing this out. We chose silkie chickens for this study due to their significance within the unique context of Chinese dietary culture. Silkie chicken is esteemed for its higher nutritional and medicinal value in Chinese cuisine, particularly in the preparation of nourishing soups. Consequently, silkie chickens hold considerable economic importance in China. Given the physiological differences among various chicken breeds, we decide to conduct a comprehensive investigation only into the effects of tilmicosin on silkie chickens.

Comments 6: The discussion part is very brief. The authors should add information about global usage of antibiotics in poultry with special reference to tilmicosin. Further discussion is needed how the accumulation of antibiotics in chicken tissues will negatively impact animal/public and environmental health. That is more important.

Response 6: Thank you for pointing this out. We totally agree with this comment. Therefore, we added information on global usage of antibiotic in poultry specially tilmicosin in line no-476-478. We also added discussion on the accumulation of antibiotics' impact on antibiotic resistance in line no-587-589.

Line no: 326-330 "[As a result, a large amount of tilmicosin was used; in 2023, tilmicosin and its derivatives contributed to 786 million dollars in the global market. Moreover, an average of 68 mg/PCU of antimicrobials were consumed by chicken, contributed 33% to the global increase in antimicrobial consumption]"
Line no: 393-398 "[Due to the widespread use of antimicrobials in poultry, which can accumulate in the environment via animal feces[41,42], there is a resultant concern regarding strain mutations and the increased antibiotic resistance of pathogens[43].]"

Comments 7: The points discussed in line nos 335-340 need to be elaborated in detail and the ill effects compared with other antibiotics whether tilmicosin have similar effects or more or less

Response 7: Thank you for pointing this out. We totally agree with this comment. Therefore, we elaborated those points in detail in line no 346-362.

Line no: 346-362 “The accumulation of TIM also disrupted the chickens' homeostasis, as seen in changes in blood biochemical parameters. In this study, we observed a decrease in blood urea nitrogen (BUN) and uric acid (UA). The kidneys play a crucial role in eliminating chemical waste products, including nitrogenous compounds and uric acid, from the bloodstream. An increase in blood urea nitrogen (BUN) and uric acid levels can signify a compromised renal function, where the kidneys fail to adequately filter urea and expel waste. Therefore, the observed elevation of BUN and uric acid levels following TIM treatment may suggest a potential improvement in renal function[25,26]. Moreover, hepatic function indicators such as ALP, DBIL, TBIL, and TBA showed fluctuations after TIM administration. Alkaline phosphatase (ALP), direct bilirubin (DBIL), total bilirubin (TBIL), and total bile acids (TBA) serve as critical biomarkers for assessing liver function, primarily secreted by the liver and typically maintained at relatively low levels in the bloodstream. In our experiment, we observed a decrease in ALP levels on days 5 and 20 in the TIM-treatment group, alongside a reduction in DBIL and TBIL on day 3, followed by an increase on day 7. Notably, TBA levels increased on days 0.16 and 9. These findings do not exhibit a clear pattern of fluctuation, suggesting a complex hepatic response to TIM in the host[27–29].

Comments 8: Regarding microflora the authors should add discussion regarding importance all four beneficial genera Bacteroidetes, Firmicutes, Euryarchaeota, and Proteobacteria and their correct ratios, as well as their beneficial effects also.

Response 8: Thank you for pointing this out. We totally agree with this comment. Therefore, we added more content on these four dominant in line no 364-384.

Line no 363-380: “[TIM not only caused physiological changes but also led to alterations in the composition of the gut microbiome. At the phylum level, the dominant taxa identified were Bacteroidetes, Firmicutes, Euryarchaeota, and Proteobacteria. Notably, the TIM-treatment group exhibited a significant increase in the relative abundance of Bacteroidetes on day 20, with a difference of 19.07%. Certain strains of Bacteroidetes are known to benefit the host by limiting pathogen colonization and enhancing immune responses[30,31]. In contrast, Firmicutes demonstrated a decrease in the TIM-treatment group, with the largest difference of 12.03% observed on day 9. Many Firmicutes are renowned for their butyrate-producing capabilities, converting fiber into butyrate, which is crucial for pathogen defense[32,33]. The reduction of Firmicutes in the TIM-treatment group may consequently lead to decreased butyrate levels, warranting further investigation. Euryarchaeota, which decreased by 10.62% in the TIM-treatment group, are generally considered commensal or beneficial organisms, performing immunomodulatory functions by promoting cytokine production[34]. Further exploration is required to elucidate the effects of Euryarchaeota on silkie chickens. Additionally, Proteobacteria increased by 3.31% on day 20 and 2.68% on day 120; however, given that Proteobacteria are recognized as biomarkers of disease in humans[35], this increase may have adverse implications for the health of the TIM-treatment group.]”