（1）The title is misleading because it is not the “*F. nucleatum*-derived butyric acid” that promotes tumorigenesis but the F. nucleatum-induced imbalance in microbiome-derived butyric acid levels

Response:

We concur with the reviewer's suggestion and have accordingly revised the title of our manuscript.

（2）the corresponding sentence in the abstract is also of unclear wording: “We discovered that when we gavaged mice with *F. nucleatum*, the butyrate-producing bacteria in the treatment group reduced, showing that F. nucleatum can regulate the quantity of butyric acid, the intestinal metabolite.”. Less ambiguous would be “showing that F. nucleatum can compete with butyrate-producing bacteria leading to deregulated quantity of butyric acid, the intestinal metabolite”

Response:

Thank you for your valuable advice. After further language polishing, we have revised this sentence.

（3）page 11: “Fusobacterium was found in high abundance”. What Fig 1A shows is rather a low abundance compared to other bacteria families; however, the relative difference between normal and cancer may be high. However, this cannot be appreciated in the figure due to its scale; the author should add a zoom-in area, which magnifies the scale for the case of fusobacteria and clostridia.

Response：

We gratefully thank the reviewer’s comment. We have added enlarged regions of Fusobacteria and Clostridia in new Figure 1.

（4）p 12: “It indicates that F. nucleatum is crucial to the occurrence and progression of colorectal cancer.” I suggest to modify to “is strongly associated with ”

Response:

Thank you for your advice. After further language polishing, we have revised this sentence and highlighted it in yellow.

（5）Legend to Figure 2: the authors need to explain what is shown in part A and B of the Figure.

Response:

We are grateful for the reviewer's suggestion. In response, we have provided an explanation in the manuscript's legend and highlighted these details in yellow for clarity.

（6）Legend to Fig 3: the authors need to explain what each bar stands for; each individual mouse that was treated?

Response:

We sincerely thank the reviewer for their careful and responsible attention. In response,

we have included an explanation in the manuscript's legend and highlighted these sections in yellow.

（7）p 13: Shown is the change in the amount of total short chain fatty acids (SCFAs) in the F. nucleatum-treated group, but then the further analysis focusses only on butyrate; the authors should state or show whether the other SCFAs are also diminished to the same extent

Response:

We are grateful for the reviewer's suggestion. Analysis of Figure 4C reveals that various short-chain fatty acids (such as propionic acid, acetic acid, valeric acid, etc.) also exhibit varying degrees of reduction in the group treated with Fusobacterium nucleatum. Further comparison of the 16s rDNA results indicates a significant decrease in the abundance of butyric acid-producing bacteria in this group. Consequently, our subsequent research will be centered on the analysis of butyrate salts.

（8） p 14: please comment on the choice of HCT116 and DLD-1 cells as an in vitro model to test the effects of butyrate

Response：

Thank you for your advice. Both HCT116 and DLD-1 cells are classified as colorectal adenocarcinoma cells and carry k-ras mutations. Research has demonstrated that oncogenic activation of the k-ras allele heightens cell sensitivity to butyrate-induced apoptosis. This finding underpins our decision to select these two cell lines. Relevant literature supporting this includes the study 'Oncogenic Ras promotes butyrate-induced apoptosis through inhibition of gelsolin expression.'

（9） p 14 and 15: “sodium butyrate's inhibitory action diminished when sodium butyrate co-treated with F. nucleatum, showing that F. nucleatum can suppress the efficacy of sodium butyrate”. The authors need to explain how this experiment was performed. Were bacteria added to the cells’ culture medium? How can you exclude that culture medium exhaustion by the rapidly growing bacteria was not the cause of the observed effect? Later, for the description of Figs 6, 7 and 8, the same issue applies, but here the authors mention the use of F. nucleatum supernatant. This needs to be well explained in the text and the figure legends.

Response:

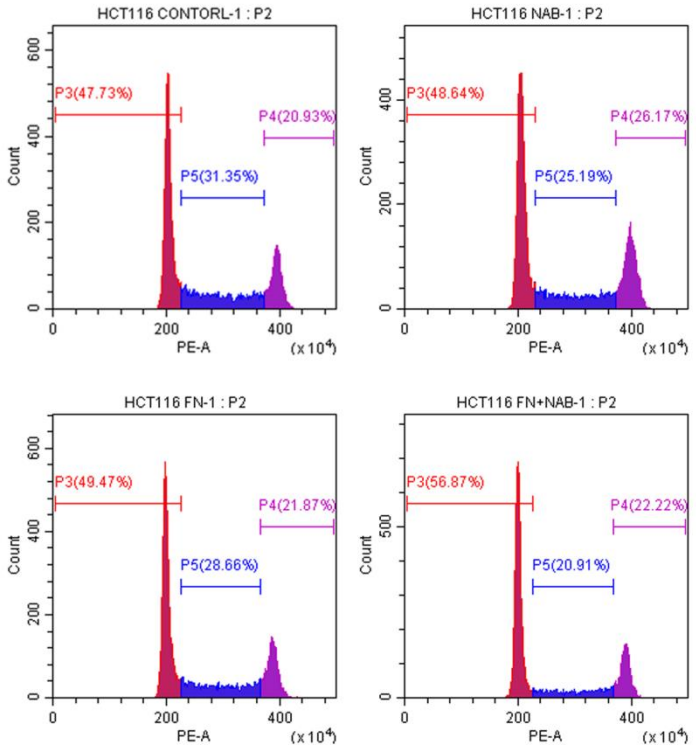
This experiment entails the concurrent addition of sodium butyrate and *Fusobacterium nucleatum* to the cell culture medium, followed by the detection of relevant proliferative and apoptotic proteins after 24 hours. Given the 24-hour processing time, we observed that the color of the culture medium had not fully turned yellow, thus enabling us to discount any results attributable to the depletion of the medium. In some subsequent experiments, we utilized bacterial culture supernatants. This approach was based on studies indicating that bacteria themselves can influence the detection of ATP, ROS, and membrane potential. Consequently, we shifted our focus to bacterial metabolites and discovered that they, too, can produce a similar effect.

References：

Duan X, Huang X, Wang X, Yan S, Guo S, Abdalla AE, Huang C, Xie J. l-Serine potentiates fluoroquinolone activity against Escherichia coli by enhancing endogenous reactive oxygen species production. J Antimicrob Chemother. 2016 Aug;71(8):2192-9. doi: 10.1093/jac/dkw114. Epub 2016 Apr 26. PMID: 27118777.

Cao K, Lai F, Zhao XL, Wei QX, Miao XY, Ge R, He QY, Sun X. The mechanism of iron-compensation for manganese deficiency of Streptococcus pneumoniae. J Proteomics. 2018 Jul 30;184:62-70. doi: 10.1016/j.jprot.2018.06.004. Epub 2018 Jun 18. PMID: 29913266.

Below is the cell cycle analysis chart depicting the results following the co-treatment of cells with *Fusobacterium nucleatum* and sodium butyrate:



（10） p 18, In the discussion, the authors should use ‘we discovered that’ instead of “was discovered” to distinguish their results from those reported by others in the literature.

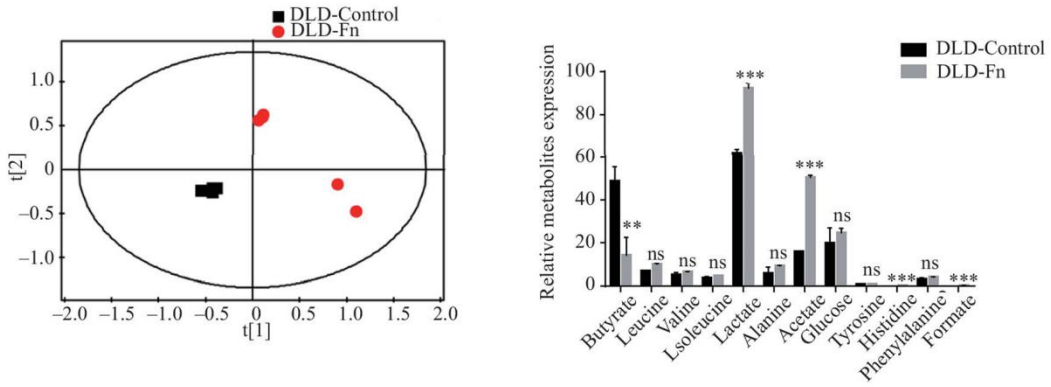
Response:

Thank you for your valuable advice. After further language polishing, we have revised this sentence.

（11）In the discussion, it remains unclear whether the author propose that F. nucleatum outcompetes other butyrate-producing bacteria and thus leads to lower butyrate levels, or whether some kind of active degradation or consumption of butyrate by F. nucleatum is involved in the observed results.

Response:

Thank you for your insightful advice. In our laboratory, we treated DLD-1 colorectal cancer cells with *Fusobacterium nucleatum* for 24 hours and then collected the supernatant from the culture medium. Post freeze-drying, we analyzed the metabolic product differences in the culture medium using nuclear magnetic resonance (NMR). The NMR findings revealed a significant decrease in the extracellular concentration of butyrate following the *Fusobacterium nucleatum* treatment in colorectal cancer cells. This leads us to hypothesize that *Fusobacterium nucleatum* actively degrades butyrate. The figure below illustrates the results obtained from the nuclear magnetic resonance analysis.



（12）Language use: Several paragraphs in the method section are incomplete sentences taken from the lab protocol instructions. These need to be adapted to provide a coherent methodological description. Also, several errors such as the lack of capital letter or of a space between words are highlighted in the attached pdf file

Response:

We express our sincere gratitude to the reviewer for their meticulous and responsible attention. We have thoroughly reviewed the article and made the appropriate modifications in response.

1) the choice of HCT116 and DLD-1 cells as an in vitro model (I suggest to add in the results section at the beginning of paragraph: Sodium butyrate blocks the cell cycle in HCT116 and DLD-1 cells). rebuttal answer: Both HCT116 and DLD-1 cells carry k-ras mutations and research has demonstrated that oncogenic activation of the k-ras allele heightens cell sensitivity to butyrate-induced apoptosis.

Response:

Thank you for your advice. We have added and marked in red in the article.

2) p 14 and 15: The authors need to explain how this experiment was performed. “sodium butyrate's inhibitory action diminished when sodium butyrate co-treated with F. nucleatum, showing that F. nucleatum can suppress the efficacy of sodium butyrate”. (I suggest to add this information to the Material and Method section) rebuttal answer: This experiment entails the concurrent addition of sodium butyrate and Fusobacterium nucleatum to the cell

culture medium, followed by the detection of relevant proliferative and apoptotic proteins after 24 hours, when the color of the culture medium had not fully turned yellow, thus enabling us to discount any results attributable to the depletion of the medium. In some experiments, bacterial culture supernatants were utilized to test whether bacteria themselves can influence the detection of ATP, ROS, and membrane potential. Consequently, we shifted our focus to bacterial metabolites and discovered that they, too, can produce a similar effect.

Response:

We are grateful for the reviewer's suggestion.We have added experimental procedures to the materials and methods and marked them in red.

3) In the discussion, it remains unclear whether (..) some kind of active degradation or consumption of butyrate by F. nucleatum is involved in the observed results. rebuttal answer: When DLD-1 colorectal cancer cells were treated with Fusobacterium nucleatum for 24 hours, the culture medium revealed a significant decrease in the extracellular concentration of butyrate using nuclear magnetic resonance (NMR) after post freeze-drying. This leads us to hypothesize that Fusobacterium nucleatum actively degrades butyrate. Please also note that in the provided Word format, Figures 1 and 4 are not correctly reproduced or with some formatting error.

Response:

We are grateful for the reviewer's suggestion.We've already illustrated this in the discussion and highlighted it in red. Figures 1 and 4 have been revised.