**Reviewer Response**

**Manuscript Number: CAPR-24-0347**

Dear Dr. Dong,

Thank you for considering a revised version of our manuscript titled “Black raspberry modulates cecal and oral microbiome at the early stage of a dibenzo[def,p]chrysene-induced murine oral cancer model’. We sincerely appreciate the constructive feedback provided by you and the reviewers. We have conducted substantial revisions to address the comments adequately and are confident that these efforts have led to significant improvement in the clarity and accuracy of the manuscript. You will find the point-to-point responses to each comment in the following sections. All the modifications to the manuscript are included verbatim beneath each response with important changes underlined. We have included a copy with track changes. We appreciate the opportunity to resubmit the manuscript and look forward to further correspondence with you and/or the reviewers. We believe that our revised manuscript is now suitable for publication in Cancer Prevention Research.

Best regards,

Dr. El-Bayoumy

**Editor Comments**

1. **Please add the Prevention Relevance Statement to the manuscript text in between the Abstract and Introduction sections.**

We have included the prevention relevance statement between the Abstract and Introduction:

*“Our work clearly demonstrates the modulatory impact of Black Raspberry (BRB) on both gut and oral microbiomes within a dibenzo[def,p]chrysene (DBP)-induced oral squamous cell carcinoma (OSCC) mouse model and paves the way for future research examining a causal role of BRB-microbiota interactions at different stages of disease progression.”*

1. **Please be sure that the accession number in the Data Availability Statement is correct. I looked it up and not receive any results in the SRA.**

We correctly listed the SRA accession; however, it is currently set to be released automatically upon publication. We have made the data available to reviewers with the following private link: Bioproject PRJNA1142100: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1142100?reviewer=fbjc5pm7hpg7q1m1fvrmqj8j7>.

1. **Please move the author contributions section to the Acknowledgements. It can also be removed, as what is in the SmartSubmit submission form is sufficient.**

We have moved the author’s contributions to the Acknowledgements:

*“****J. Zhao****: investigation, methodology, formal analysis, visualization, data curation, writing—original draft, writing—review and editing.* ***Y-W. Sun****: conceptualization; DNA isolation from oral tissues, and writing original draft.* ***K-M. Chen****: LC-MS/MS analysis of DNA adducts derived from DBP.* ***C. Aliaga****: animal handling, carcinogen treatment, diet preparation, animal sacrifice, oral, cecal and tissue collection.* ***J.E. Bisanz****: Conceptualization, formal analysis, methodology, validation, supervision, writing–original draft, writing–review and editing.* ***K. El-Bayoumy****: Conceptualization, formal analysis, funding acquisition, methodology, writing–original draft, project administration, writing–review and editing. JEB is supported by NIGMS R35 GM151045 and funds provided by the Huck Institute for the Life Sciences. KEB: This study is supported in part by NCI R01 CA173465.”*

1. **The correct order for manuscript sections is as follows: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figure Legends, Figures. Please note that Figures should be uploaded in separate Figure files for revised manuscripts, not embedded in the main text.**

We have removed “Author’s Contributions” section and reordered other sections accordingly.

1. **Please do not put a figure caption on the figure itself just the figure number (ex. “Figure 1”). Please note that all figure legends should be listed together in one section.**

All figure legends have been listed together below References.

1. **Each supplementary item must be contained in its own supplementary data file. Please modify.**

We have generated two supplementary data files separately.

**Figures and Supplementary Figures with only one panel do not need to be labeled as panel "A". Please modify Figure S1.**We have removed panel “A” from Figure S1.

1. **Re: email requesting code availability received September 4, 2024**In the interest of reproducibility and rigor, we have made a Github repository which includes all source code and processed data for manuscript analyses. It is available at https://github.com/isuzjc/BRB2024. This repository will facilitate interested parties to replicate/extend analyses without any concerns over availability of data or methods. Unfortunately, because of the complexity of the project and multiple data types, this did not cleanly import into Code Ocean, and we would request that our highly detailed Github repository be accepted in lieu.

**Reviewer #1**

1. **The authors use the word saliva for the oral samplings. Saliva or oral rinses are liquid samples that are obtained through some sort of expectoration, which is impractical in rodents. In the referenced article, the collection technique will certainly sample the exfoliated cell compartment almost more than any true fluid component, as judged by the photos of the sampling technique. Therefore, the term saliva would not be the correct nomenclature for the samples derived from the mice. This may seem trivial, but there is an entire literature on the adequacy of swab obtained samples from other sites and their appropriate use. For example, male anal "pap smears" are collected by a variety of techniques that require validation for genetic and exfoliated histology. This needs to be clarified, renamed even oral samples or something similar because there is not simple guarantee they represent saliva only, with small portions of cell sediment. Also, in human studies care is taken to describe which components are being sampled with oral collection techniques-- saliva, salivary rinse, oral cdx brush or other brushings, periotron collections, incisional biopsy, etc.**

We fully agree with the reviewer’s recommendation, and based on the technique used in our study which was adapted from the literature, we now renamed “saliva” samples as “oral” samples collected in this study as listed below:

*“Oral and cecal samples were processed with a ZymoBIOMICS 96 MagBead DNA Kit (D4308) according to the manufacturer’s instructions.”*

*“Mouse oral and cecal samples were collected after the last dose of carcinogen administration.”*

*“After the collection of oral samples, the oral tissues were harvested to examine the levels of DNA adducts derived from DBP (DBPDE-dA). ”*

*“The number of observed ASVs ranged from a median of 48.7 [25-111] in the oral microbiota which was significantly lower relative to that in the gut microbiota with 125.5 [77-179] ASVs”*

1. **Were the cecal samples collected by identical techniques with the same swabs? This is hard to tell from the article. If not, please explain the differences in the techniques as they could result in significant differences in the analytic parameters for the microbiomics performed.**

The collection of the cecal samples was different and the method utilized for collection is now included in the revised manuscript (*cf.* Materials and Methods – Animal Experiments) as: Following animal sacrifice and opening the abdominal cavity, the cecum was collected, then opened and its contents was collected by using a sterile spatula to scrape it into a sterile tube on the same day.

1. **Sex as a biologic variable is not addressed by using only female animals--any rationale for this choice?**

We have now included the rationale for selecting female mice in the revised manuscript. The rationale for this choice is described as follows (*cf*. Materials and Methods and Discussion Sections): Our previous carcinogenesis and mechanistic studies on the role of tobacco constituent DBP and the chemopreventive effects of BRB were performed in female mice. In these studies, we showed that the histological changes (hyperplasia, dysplasia, carcinoma *in situ*, OSCC) and molecular characteristics identified in the oral cavity of female mice following DBP treatment mimic those found in human OSCC further emphasizing the significance of utilizing our female mouse model as a realistic platform for further mechanistic studies reported in the current manuscript. Specifically, we aimed to examine the shift of the microbiome at early stage before tumor development with the ultimate goal of discovering biomarkers for early detection. Nevertheless, the lack of information in male mice is considered a limitation which we discussed in the revised manuscript (*cf.* Discussion Section).

1. **When do histologic changes occur in this model, and in what percent of animals?**

The following information has already been published by our team, but to respond to the reviewer’s comment, we briefly include it in the “Introduction” section of the revised manuscript. In this model, the tobacco constituent DBP induces OSCC in female mice. At the dose of DBP used in this study, we anticipate that at termination of the bioassay, the incidence of each histological type as reported by us (Chen *et al*. Cancer Prev Res 2020) as: Dysplasia/papilloma (33.3%), carcinoma *in situ* (13.3%), OSCC (40%). No histological changes were observed in the first 6 weeks; however, at 15 and 22 weeks, mice developed dysplasia/papilloma and carcinoma *in situ*, respectively. As described above at termination, all histological types were observed in the mouse oral cavity.

**Reviewer #2**

**Major comments:**

1. **Animal Study Design: In the in vivo section, it states that a total of 20 mice (n=5 per group) were used. However, the rationale for sac’ing two mice per group at the 5th week is not mentioned, nor is it indicated in Figure 1. The rationale for this would need to be included.**

At the end of week 5, our animal biologist was able to sacrifice 2 mice per group in one day and carefully, to avoid contamination (tedious process) collect oral and cecal samples as well as oral tissues for the analysis of DNA damage and microbiome analysis. At the beginning of week 6, 3 mice were sacrificed per group in one day and collection of samples was performed. Based on our previous studies, maximum DNA damage was observed at 5 weeks. Furthermore, dietary changes can rapidly alter the microbiome within days and thus, a duration of 5 weeks is sufficient for observing a shift in microbiome following BRB administration. Therefore, we combined the microbiome data generated at both timepoints as indicated in Figure 1a.

1. **In Vivo vs. In Vitro Correlation: As noted in the results, no correlation between the in vivo and in vitro studies. Further evaluation using RNA-seq, based on taxonomical classification, maybe recommended to better understand the underlying mechanisms (e.g. TLR2-4, NOD2, AMPK, GPR41-43, TP53, EGFR, CCND1, ATM).**

The reviewer raises an important point highlighting the differential observations between in vivo and in vitro results. It is important to emphasize that *in vivo* studies, mice received BRB in the diet in the form of freeze-dried powder and *in vitro* cultures were treated with BRB extract; it has been reported that the extract lacks certain active components found in the powder and this difference may, in part, explain the lack of correlation between *in vivo* and *in vitro* studies. Furthermore, the in vitro experiments assess direct interactions between compounds and microbes in pure cultures, while in vivo assays capture more complex host-microbe and microbe-microbe interactions within a dynamic community. Both in vitro and in vivo results indicate that BRB impacts the microbiome during the early stages of oral cancer development. Further, DBP has no effect on the microbes either in vivo or in vitro. Together, the in vivo and in vitro assays offer a comprehensive understanding of both direct and indirect effects, sufficiently elucidating BRB's role in microbiome modulation during early OSCC development. Although RNA-seq analysis can provide insights into the function of specific bacteria/hosts, these discovery-based methods would require significant experimental design and validation to demonstrate the mechanism which are beyond the scope of this manuscript.

Please see our improved interpretations of these findings in the manuscript.

*“Next, we turned to investigate the effect of DBP. Consistent with in vivo studies, there were no significant effects for any tested strain treated with DBP (Fig. 4b).”*

*“Countering in vivo observations, after 72 hours of incubation, the BRB group showed minor increases in growth (Fig. S2). This suggests that while A. muciniphila may utilize BRB substrates in vitro, the mechanism of inhibition in vivo may involve host-microbe and microbe-microbe interactions not captured in mono-culture.”*

*“Contrasting to high baseline A. muciniphila levels in our in vivo models, the control group in the previous research only had <0.01% A. muciniphila in the community (36), further suggesting that BRB may indirectly impact A. muciniphila through complex host-microbe and microbe-microbe interactions in vivo.”*

1. **A. muciniphila and OSCC Development: The observed alteration in \*A. muciniphila\* following BRB treatment and its potential role in OSCC development remains unclear. Investigating immune cell fractions (e.g., using CIBERSORT) could potentially help to elucidate the mechanisms involved.**

The reviewer raises an important consideration of the role of host immunity in mediating host-microbe interactions and carcinogenesis. Our study focused on the microbial perspective of the effect of BRB on a DBP-induced OSCC model. Our results support the hypothesis that BRB significantly alters both oral and cecal microbiomes. While investigating immune cell fractions could elucidate how BRB impacts host immune functions in this model, it does not directly establish a causal relationship or directionality between *A. muciniphila* and cancer development. We included these as potential future investigations that may directly determine the role of A. muciniphila in OSCC development:Top of FormBottom of Form

*“The alteration in A. muciniphila following BRB treatment was observed; however, its specific influence on OSCC development remains unclear. To gain insights into the mechanism involved, it will be necessary to isolate the representative strains and employ further animal models to examine causality at different stages of disease during the multi-step carcinogenesis process from normal, dysplasia, carcinoma in situ, and OSCC.”*

**Minor comments:**

1. **Study Groups: Consider adding more study groups to explore different concentrations of the BRB and DBP combination for a more comprehensive analysis.**

We thank the reviewer for the suggestions for exploring different concentrations of the BRB and DBP combination. The concentrations used in this experiment were selected based on findings from previous studies. We have added a detailed rationale for our choice of concentrations to the manuscript.

*“Extensive previous studies conducted in our laboratory have tested several doses of DBP and different dietary levels of BRB. Previous evidence had indicated that 24 nmol, 3 times per week of DBP administration could induce maximum DNA damage at 5-6 weeks without causing any histological changes (26) and 5% was the optimal level of BRB to protect against tumor development in murine models (20).”*

1. **Gender Ratio in Oral Cancer: The study notes the 2:1 male-to-female ratio in oral cancer progression, but the rationale for including female mice is unclear. Clarification is needed to effectively correlate pre-clinical findings with clinical outcomes.**

Please refer to our response to Reviewer 1, Comment #3.

1. **Histopathological Examination: The collection of oral tissues should ideally include histopathological examination to establish the development of OSCC in mice.**

Please refer to our response to Reviewer 1, Comment #4.

1. **BRB Components: The manuscript does not clearly identify which specific component of BRB could be the key factor contributing to the observed effects, and further clarification is needed.**

The reviewer has rightly identified that BRB is a complex mixture. Our previous studies demonstrated that BRB powder mitigated DBP-induced damage by altering genetic and epigenetic markers. In the current experiment, we used BRB in its entirety to build upon our previous findings from a microbial perspective. While identifying specific BRB components contributing to the observed effects is indeed a valuable area for future research, it is outside the scope of our current focus on the relationship between the microbiome and BRB's protective effects. Please see our rationales for using the BRB powder:

*“Recently, we discovered that dietary black raspberry (BRB) powder significantly reduced tumor incidence by attenuating DBP-induced DNA damage, strengthening DNA repair, and regulating epigenetics (35).”*

*“Previous evidence had indicated that 24 nmol, 3 times per week of DBP administration could induce DNA damage without causing any histological changes (26) and 5% was the optimal level of BRB to protect against tumor development in murine models (20).”*

1. **Bacterial Impact: The statement regarding the impact of various bacteria on other diseases appears disconnected from the primary focus of this paper and may require recontextualization or removal.**

We appreciate the reviewer’s comment regarding the impact of various bacteria. We have revised the manuscript to remove references reflected diseases other than cancers.

*“Interestingly, Acutalibacter muris has been identified as a biomarker of colorectal cancer (64) and lung cancer (65), suggesting that BRB may mitigate cancer development through microbial modulation. On the other hand, the presence of dietary BRB resulted in significantly more abundant Eubacterium sp., Schaedlerella arabinosiphila, and Kineothrix sp.. Eubacterium callanderi has demonstrated anti-colorectal cancer properties both in vitro and in vivo, potentially mediated by metabolites such as butyrate and 4-aminobutanoic acid (66), whereas Kineothrix alysoides is known to produce butyrate (67), demonstrating that BRB may selectively promote butyrate-producers resulting in suppressed carcinogenic potential (68). Our in vitro experiments showed that BRB promoted the proliferation of the microbes potentially involved in polyphenol metabolism: two lactic acid bacterial strains (69) and two Bacteroidotas (62). Among them, Lactobacillus paracasei has been reported to exert anti-proliferative and apoptotic effects in human colon cancer cells (70,71) and cervix cancer cells (72,73), while Parabacteroides goldsteinii has been noted for its anti-inflammatory properties through its lipopolysaccharide (74), underscoring their potential therapeutic relevance.”*

1. **OSCC Background: Including additional symptoms or further details regarding OSCC would enhance the reader's understanding of the disease's significance, particularly in relation to the increase or decrease of specific bacteria.**

It is a great suggestion. We have incorporated (*cf.* Introduction) additional symptoms of OSCC and provided a description of the role of specific bacteria in cancer development.

*“When OSCC happens, oral functions including swallowing and speech are often adversely influenced (3). Additionally, many patients suffer from impaired life quality resulting from damaged facial functions after surgery.”*

*“Previous studies also demonstrated that periodontal pathogens Fusobacterium nucleatum and Porphyromonas gingivalis promote oral carcinogenesis (7,8). In the oral cavity, Porphyromonas gingivalis disrupts the equilibrium of the immunoinflammatory state and Fusobacterium nucleatum becomes opportunistically pathogenic, contributing to periodontal diseases (9,10).”*

1. **Abstract: The sentence, "While tobacco smoking is a risk factor in the development of oral squamous cell carcinoma (OSCC), only a fraction of smokers develops the disease and compelling evidence suggests that other factors such as disruption of the microbiome are associated with carcinogenesis; however, causality has not been established," would benefit from a clearer logical connection between the components.**

We acknowledge the reviewers' concern regarding the logical flow of the sentences. We have rephrased the relevant sections to enhance clarity and improve the logical connection between the sentences.Top of FormBottom of Form

*“While tobacco smoking is a risk factor in the development of oral squamous cell carcinoma (OSCC), only a fraction of smokers develop the disease. Compelling evidence shows that microbial dysbiosis is associated with carcinogenesis, suggesting that the microbiome may play a role in the cancer development of smokers.”*

1. **Introduction: While the introduction discusses the relationship between the oral microbiome and OSCC and mentions saliva, the rationale for including gut microbiota analysis in this study is not clearly articulated.**

We have improved our discussion of the relationship between oral and gut microbiota as below.

*“The gut and oral microbiota constitute two of the highest biomass microbial ecosystems within the human body (50), characterized by diverse composition and ecological dynamics (51). Anatomically contiguous through the gastrointestinal tract and linked chemically via saliva and ingested food transit (52), the oral-gut microbiome axis has been implicated in various disease processes (53). In our study, we examined both oral and gut microbiota, at early time points prior to any morphological changes associated with cancer to elucidate their response to DBP-induced tumorigenesis.”*

To address this issue, we have added a rationale for including gut microbiota analysis in the Introduction section of the revised manuscript.

*“Given gut and oral microbiota highly connect to and interact with each other (38), the oral-gut microbiome axis is a potential key player in the development and prevention of OSCC.”*

1. **Incorporation of Previous Findings: Previous findings should be integrated into all sections to strengthen the establishment of results and hypotheses.**

We have integrated the important pertinent previous findings into the Introduction, Methods, Results, and Discussion sections as below which completement the more general previous findings found in the discussion.

Introduction:

*“We previously showed that DBP caused DNA damage and mutations in the murine oral cavity with profiles similar to those found in the p53 gene in human HNSCC (28). Our studies also showed that the p53 and COX-2 proteins were upregulated (26), while the expression of the tumor suppressor p120ctn protein was reduced in DBP-induced OSCC; p120ctn cooperates with EGFR to promote carcinogenesis (29,30).”*

*“Recently, we discovered that dietary black raspberry (BRB) powder significantly reduced tumor incidence by attenuating DBP-induced DNA damage, strengthening DNA repair, and regulating epigenetics (35).”*

Methods:

*“Animal studies were conducted based on a previously established murine model utilizing a relevant tobacco smoke constituent DBP to induce OSCC (26).”*

*“Previous evidence had indicated that 24 nmol, 3 times per week of DBP administration could induce DNA damage without causing any histological changes at early timepoint (5-6 weeks) (26) and 5% was the optimal level of BRB to protect against tumor development in murine models (20).”*

Results:

*“Although no histological changes at this early time point are observed in this model (26,35), DBPDE-dA was detected in the DBP group at the level of 0.495 ± 0.021 adducts per 106 deoxyadenosine (dA) by using an LC-MS/MS with stable isotope dilution assay, confirming the capacity of DBP to trigger DNA damage.”*

*“Consistent with our previous reports, the adduct was not detected in mice treated with DMSO (26,43).”*

*“Since the microbiome is associated with the development of oral cancer (5,6) and we have previously shown protective effects of BRB against DBP-mediated OSCC (35), we sequenced a total of 20 cecal samples which resulted in 611,815 reads after processing (30,590.8 ± 6,582.6 per sample (mean ± sd)).**”*

*“The doses were selected according to our previous studies showing that BRB had dose-dependent protections against DBP-induced DNA damage in human and rat oral cells (47,48).”*

Discussion:

*“Overall, this work extends our prior mechanistic investigations (35) for BRB modulation in DBP-induced OSCC, focusing on the microbial perspective.”*

1. **Title Revision: The title, "BRB exhibits minor but significant effects on oral microbiota," lacks clarity in conveying its intended meaning and could be rephrased for better precision.**

We have revised the title to enhance its precision.

*“BRB exhibits significant but limited effects on oral microbiota”*

1. **Bacterial Impact: The statement regarding the impact of various bacteria on other diseases appears disconnected from the primary focus of this paper and may require recontextualization or removal.**

We appreciate the reviewer’s comment regarding the impact of various bacteria. However, we believe that this reviewer mistakenly repeated Comment #5 above; please see our response to this comment.

1. **Food Intake: Adding a rationale for how food intake might influence disease development could provide a more comprehensive understanding of its role in OSCC.**

Treating cancers at late stage, including OSCC continues to be a major challenge and thus, development of safe and effective strategies for cancer prevention remains a desirable approach. Consumption of diets rich in fruits and vegetables may lower the risk of developing oral cancer and foods that contain agents known to inhibit the initiation and/or progression of the multi-step carcinogenesis process are favorable candidates for cancer prevention (El-Bayoumy *et al.* Chem Res Tox 2017; El-Bayoumy *et al.* Cancer Prev Res. 2020). The above information is now included in the Discussion section in the revised manuscript.