Author's response to reviews

Title: Escherichia coli strains of phylogenetic group B2 and D and bacteriocin production are associated with advanced colorectal neoplasia

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Author's response to reviews: see over
Dear Sirs,

Thank you for your kind comments to our article "Escherichia coli strains of phylogenetic group B2 and D and bacteriocin production are associated with advanced colorectal neoplasia".

Detailed responses to the comments/questions are written at the base of this document.

We have made corrections required by the reviewers and we send our new version of the manuscript. Changes made in the manuscript are highlighted with the coloured (red) text.

The final text was also edited by an English native speaker.

Kind regards,

Darina Kohoutova, MD, Ph.D.
Answers to Reviewer 1:

**Major compulsory revisions:**

1) Results, 2nd paragraph - Please clarify, as *E. coli* is also a member of the Enterobacteriaceae family.

All the groups tested had similar numbers of bacteria from the *Enterobacteriaceae* family (*E. coli* + other bacteria from this family), but the groups tested differed in the numbers of *E. coli* strains. This is important because *E. coli* possesses some specific virulence factors (responsible for adhesion, invasion, etc) and further *E. coli* genotype B with its „pks“ island can lead to chromosomal aberrations (which is not known for other bacteria from the *Enterobacteriaceae* family).

2) Results 3rd paragraph – while it is true that a significantly higher frequency of co-coproduction of colicin and microcins was detected in patients with CRC compared to that in patients with CRA, for the controls the value was comparable to that from patients with CRC. The authors must comment these results.

A trend towards significantly higher co-production of colicins and microcins in the healthy controls, when compared to the patients with CRA, was revealed, p=0.065; see Table 1 for details.

We do think, that in healthy subjects there could be an intermicrobial competition (for nutrition, etc) in the large bowel conveyed as a high simultaneous production of microcins and colicins. We can hypothetize, that if the production of antimicrobial, antiapoptotic and potentially antineoplastic bacteriocins decreases in healthy individuals, this could co-initiate the development of a non-advanced colorectal neoplasia. With the growth, development and progression of a non-advanced neoplasia into an advanced one, higher production of bacteriocins is again identified and this could help the macroorganism in combating the colorectal cancer. Our hypothesis needs further investigation especially in the field of potential antineoplastic properties of the bacteriocins and clarification of their interaction with the eukaryotic host.
3) Results, 4th paragraph, the authors found a low prevalence of the A and B1 groups and a significantly higher prevalence of the D group. The authors should provide some information regarding the B2 group.

Similar frequency of *E. coli* phylogroup B2 was found in all the groups tested; see Table 1 for details. However a trend towards the statistically significant difference in the frequency of *E. coli* phylogroup B2 was revealed between the patients with N-A CRA and the patients with A CRA: N-A: 4/17 (24%), A: 31/60 (52%); p=0.054.

4) Discussion, last paragraph, paragraph 6, second line from bottom up, as well as elsewhere, it should be stated that the E. coli strains are potentially more virulent.

*We added this statement.*

5) Page 7, 1st paragraph, the authors describe the pks genomic island of E. coli strains from the B2 phylogenetic group however, in the presented study, strains from this group were not more prevalent among patients with CRC compared with the controls.

Despite the incidence of *E. coli* B2 genotype did not differ between the whole groups tested, we found a significantly higher frequency of B2 genotype in the patients with right-sided colorectal carcinoma when compared to the patients with left-sided colorectal carcinoma. We also identified a trend towards significantly higher incidence of B2 genotype in the patients with advanced CRA when compared to the patients with non-advanced CRA. Therefore we hypothesize that there is an involvement of *E. coli* B2 genotype in the progression of a non-advanced colorectal neoplasia into an advanced one. Further we think, that *E. coli* B2 phylogroup could play a key role in the pathogenesis of the right-sided colorectal cancer. Both results were discussed in our Discussion accordingly.

6) Page 7, 2nd paragraph, 2nd line – the authors should clarify »mutagenic E. coli B2«.
Cuevas-Ramos et al (Ref. 21) describes in his paper that „We show that a single, short exposure of cultured mammalian epithelial cells to live pks(+) E. coli at low infectious doses induced a transient DNA damage response followed by cell division with signs of incomplete DNA repair, leading to anaphase bridges and chromosome aberrations. Micronuclei, aneuploidy, ring chromosomes, and anaphase bridges persisted in dividing cells up to 21 d after infection, indicating occurrence of breakage-fusion-bridge cycles and chromosomal instability. Exposed cells exhibited a significant increase in gene mutation frequency“. Secher et al (Ref. 22) shows following: „Mammalian cells exposed to live pks+ bacteria exhibit DNA-double strand breaks (DSB) and undergo cell-cycle arrest and death. Here we show that cells that survive the acute bacterial infection with pks+ E. coli display hallmarks of cellular senescence: chronic DSB, prolonged cell-cycle arrest, enhanced senescence-associated β-galactosidase (SA-β-Gal) activity, expansion of promyelocytic leukemia nuclear foci and senescence-associated heterochromatin foci.“ According to both above mentioned quotations we dare to call E coli genotype B2 as a mutagenic one.

7) The authors should discuss the relevance of bacteriocin production in relation to the antineoplastic effect of colicins; the relevance of co-production of colicins and microcins in patients with CRC and the relevance of higher prevalence of potentially more virulent E. coli strains from patients with advanced neoplasia.

This text was added to Discussion:

We do think, that in healthy subjects there could be an intermicrobial competition (for nutrition, etc) in the large bowel conveyed as a high simultaneous production of microcins and colicins. We can hypothetize, that if the production of antimicrobial, antiapoptotic and potentially antineoplastic bacteriocins decreases in healthy individuals, this could co- initiate the development of a non-advanced colorectal neoplasia. With the growth, development and progression of a non-advanced neoplasia into an advanced one, higher production of bacteriocins is again identified and also more virulent E. coli strains are present. We assume, that this situation could be beneficial for the host and might help the macroorganism in combating the colorectal cancer. Our hypothesis needs further investigation especially in the field of potential antineoplastic properties of the bacteriocins and clarification of their interaction with the eukaryotic host.
Minor compulsory revision:

Page 3 and elsewhere – Bacteriocins are not produced just by E. coli and the Enterobacteriacea– and these are designated colicins.

We are aware, that the bacteriocins are not produced just by E. coli strains, therefore we wrote into the text that „Bacteriocins are produced by Escherichia (E.) coli strains and related bacteria from the Enterobacteriaceae family [12,13]”, explicitely.

E. coli strains do not produce colicins only, but microcins also.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being Published

The text of our manuscript was edited by an English native speaker.

Statistical review: No, the manuscript does not need to be seen by a statistician.
Answers to Reviewer 2

Major Compulsory Revisions
In this manuscript the authors try to analyze whether there are differences in E. coli colonization of intestinal mucosa among the groups tested and to investigate bacteriocin production in patients with colorectal adenoma (CRA) and CRC. The study presented by Kohoutová et al. is interesting because some strains of E. coli have acquired the ability to produce toxins, which may participate in the process of carcinogenesis. However the work lacks depth and accuracy and it is unclear.

Minor Essential Revisions
4. The work must be improved with a more detailed description of the methodology and results.

We added the details to Methodology and Results.

5. Authors should insert a table with more detailed information of patients.

We added the information (sex, age of the patients) in “Supplementary material 1“.

6. Is not clearly described how the assay for the production of bacteriocins was performed and therefore should be more detailed.

This information was added to „Methods“ section.

7. Acronyms non-advanced (N-A) and advanced (A) are not present in methods.

This was added to Methods section.

8. Authors should insert a table containing the list of primers used in the work and the sources which they were taken from, if not original.

We inserted this information into the Table 3 and 4 as a “Supplementary material 2“.
These data were provided by Prof Smajs, our co-author and are original.

9. Authors should specify the total number of isolates used in this work in section “Method”. In addition, they should specify how many isolates were taken from each patient and each portion of the intestine. This clarification is essential in the determination of real size of the sample studied because isolates coming from the same intestinal district of the same patient should be subjected to genotypic analysis (es. PFGE) in order to determine clonality and the number of different strains present.

Together, 622 isolates were identified: 221 in the group of patients with CRA, 151 in the healthy controls and 250 in the patients with CRC.

Numbers of isolates in each segment of the large bowel of each patient were added into “Supplementary material 1“.

10. Although the authors have analyzed a significant number of patients and strains, they have not provided any indication about the relative abundance of virulent of E. coli strains within the total E. coli population for each patient.

Virulence factors (such as fimbriae 1, etc) were not assessed in all of E. coli strains isolated from our patients, however E. coli phylogroups (A, B1, B2 and D) were investigated. According to the literature, pathogenic strains usually belong to E. coli B2 or D phylogroup and these possess more virulent factors when compared to the commensal strains (Ref. 16, 18, 19). Information about the E. coli phylogroups is available in Supplementary material 1.

11. Authors should specify which data were statistically treated by each statistical test specified in the text.

The differences in bacteriocinogeny, colicinogeny and microcinogeny between the groups tested were assessed by non-paired t-test (as this was referred to the number of biopsies taken in each group: 90 vs 90 vs 60).
Fisher t-test was used for investigation of differences in production of each bacteriocin between the groups tested (as this was referred to the number of individuals in each group: 30 vs 30 vs 20 and the numbers were small).

Statistics was reviewed by our statistician.

12. Authors should modify labels of table 1 in order to specify the meaning of numerical values showed in columns. Table 1, also seems to show some errors because the labels of the first five rows refer to “frequency of strains” but the total number of strains considered is 240 and not 625 as specified in the text. If there were some reasons that led to a change in the sample size, such reasons should be explained in the text. Just in case that those ratios refer to the number of biopsies, authors should modify rows labels. In addition, data from row 6 to 10 are difficult to interpret and does not seem to be in line with each other. The authors should describe such data more precisely.

Thank you, this was really confusing.
We changed the legend and the labels in the Table 1.

13. In supplementary data table legend, authors should introduce the meaning for L and R in column “Localization” of CRC datasheet and N and A in CRA one.

Thank you, these explanations were added in “Supplementary material 1“.

14. In discussion, an explanation or hypothesis should be given in relation to results found in this work

We added our hypothesis at the end of the discussion (in agreement with the requirements of the second reviewer).

15. The statement in the discussion section “we are the first group to report the contribution of Escherichia (E.) coli with their specific phylogroups and bacteriocin production to the development of colorectal neoplasia” is little supported by the results obtained.
We do think, that nobody has investigated bacteriocinogeny and *E. coli* phylogroups in association with colorectal neoplasia so far.

We discussed the relationship of increasing bacteriocinogeny and virulence of *E. coli* strains with the progression of colorectal neoplasia.

We know that further research focused mainly on the antineoplastic properties and interaction between bacteriocins and eukaryotic cells is needed and we added this to our discussion.

16. Some orthographic errors should be revised and corrected

We corrected the errors and the text was edited by an English native speaker.

**Level of interest:** An article of limited interest

**Quality of written English:** Needs some language corrections before being published

The text was edited by an English native speaker.

**Statistical review:** Yes, and I have assessed the statistics in my report.