Dear Editors at the *World Journal of Gastroenterology*:

My coauthors and I would like to thank you for the opportunity to revise our paper. We appreciate the time and effort expended by the reviewers and editorial staff to assist us in the goal of making this paper worthy of publication. We have addressed the concerns of the reviewers (see below).

Again, we appreciate the opportunity to work with you in the editorial process.

Sincerely yours,

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Editor's comments:

We thank you for sending us the guidelines and for your helpful comments. We revised the abstract and references per the journal guidelines. We added our detailed methods to the manuscript body. We also incorporated study highlights and attached the proper documents mentioned in your instructions.

Answers to reviewer:

Reviewer #1:

1. **I think this manuscript is valuable to understand the association of Fusobacterium and colorectal cancer. The authors reviewed 90 articles systematically and comprehensively and found some reliable associations between Fusobacterium and colorectal cancer. This manuscript is well designed and written.**

Answer:

Thank you for evaluating our manuscript and for the kind comments. We agree that this review will shed light on the rapidly expanding literature linking *Fusobacterium* and colorectal cancer.

Reviewer #2:

1. **This is a broad review of literature dedicated to fusobacterium sequences found in colonic samples from patients with colonic cancer. The review will be definitively helpful for all interested in the topic, surely often cited and should be published. However the presentation is very subjective, the terminology blurring and the interpretation overextended. The presentation would be improved, if some especially marked overstatements will be corrected.**

Answer:

Thank you for the finding our review helpful and worth publishing. We made revisions to the manuscript body and the tables to improve the presentation and objectivity of the reviewed data. The changes are highlighted in the revised version of the manuscript.

2. **The title "FUSOBACTERIUM'S LINK TO COLORECTAL NEOPLASIA PLACED UNDER THE MICROSCOPE:" should be changed. I do understand that the authors are using the word "microscope" literarily. However, the metaphor is definitively wrong in the context of the manuscript. No data on microscopic appearance of the microbe-mucosa interactions are presented or discussed in the review. The only reference, which was using FISH with unspecific Fusobacteria probe[57] completely avoids the description of the observed histopathology of bacterial involvement and is not discussed by author in context.**

Answer:

We understand the reviewer's concern. We changed the title to the following:
"**FUSOBACTERIUM'S LINK TO SEQUENCED COLORECTAL NEOPLASIA: A SYSTEMATIC REVIEW AND FUTURE INSIGHTS**"

The new title is more concise and will reflect the content of the manuscript better, since most included studies use RNA sequencing to identify *Fusobacterium*.

3. **The authors are pretty reluctant in using medical terms. Also this bad habit is getting more and more spread as pure biologic studies are published in medical journals, the WJG is clinically oriented. The correct use of clinical terminology should be aimed at. The word pathogen was mentioned in the following sentences: Fusobacterium is one of the most cited bacterial pathogens Oral Fusobacterium consist mainly of the species Fusobacterium nucleatum (Fn), an adherent [12], invasive [13], and proinflammatory [14, 15] pathogen Fn is classified into subsp. animalis, fusiforme, nucleatum, polymorphum, and vincentii [20]. F. Varium is another pathogenic Fusobacterium Fusobacterium and other pathogens with CRC. studies linking pathogens such as Fusobacterium to survival through peripheral immune modulation Compared to PCR, loop-mediated isothermal amplification (LAMP) is a simple, non-costly and accurate method for pathogens testing that was shown to be more sensitive than PCR for Fn detection [108]. In all these cases applying the word "pathogen" is incorrect. Most fusobacteria, including Fusobacterium nucleatum, are indigenous for healthy mouth microbiota. Although some fusobacteria species can be involved in pathogenesis. Declaring a bacterium to be a pathogen can not be done at will. I recommend to replace**

the word "pathogen" through bacteria in all cited sentences, or exactly the author should define what he means.

Answer:

We agree. We made the appropriate changes to the manuscript by omitting the word pathogen when present, as detailed in the revised version of the manuscript.

3. **The presentation is often to affirmative and misleading at the same time**
   For example:
   "… Fn is the most detected species of Fusobacterium in CRC tissue [40-52]. Furthermore, Fn ssp Animalis is the most abundant subspecies of Fn in CRC tissue in two out of three studies that had more indepth analysis….."
   Using "was" instead of "is", is probably more appropriate.
   "Most abundant" should be "frequent". Otherwise the concentrations of microorganism should be mentioned.
   The above statement is misleading.
   What the authors avoid to say in the above sentence is the fact, that in each person (CRC tissue sample) sequences of multiple different fusobacteria species were detected in different composition with some Fusobacteria predominant (most detected, abundant, frequent…. whatever), which makes the possibility of infection through a single pathogen unlikely and Fusobacteria using some kind of changes in environment occurring around carcinogenesis more probable. The authors should discuss this point and bring their explanation of this fact instead of hiding it behind "most".

Answer:

We clarified our statements, per the reviewer's suggestion, as shown in the revised manuscript and as follows:

"*Fn* was the detected species of *Fusobacterium* in CRC tissue in 13 out of the 15 studies that presented species-level analysis [41–53]. In two out of three studies that presented subspecies-level analysis, *Fn* subspecies *animalis* was the most frequent subspecies of *Fn* in CRC tissue[40, 52, 54]. Other *Fusobacterium* species, such as *F. periodonticum, F. varium, F. ulcerans F. necrophorum,* and *F. gonidiaformans*, were also identified in CRC tissue in the five remaining studies [49, 52, 54–56]. *Fn*, *F. periodonticum, F. varium*, and *F. ulcerans* species can actively invade host cells, independently of mucosal compromise or presence of coinfection with other bacteria [57, 58]. Conversely, *F. necrophorum* and *F. gonidiaformans* are termed passive invaders, and their presence in CRC could be due to the disruption of the mucus layer seen with CRC or to coinfection with other invasive bacteria. In the largest study comparing genes of *Fusobacterium species*, active invaders such as *Fn* were found to harbor larger genomes, encode adhesions, and contain twice as many genes encoding membrane-related proteins compared with other *Fusobacteria*l species termed passive invaders[57]. Thus, the presence of multiple *Fusobacteria*l species could be due to their virulence and/or to

early changes in the colonic environment that facilitate their presence in CRC tissue. Further studies are warranted to answer this question."

We also clarified potential mechanisms for *Fusobacterium/Fn* presence in colonic neoplastic tissue as follows:

"The mechanism by which *Fn* reaches the colonic epithelium are unclear. However, some *Fn* strains display the potential to disrupt the colonic mucosal barrier, suggesting that it can be transmitted from the colonic lumen to the epithelium, potentially causing colorectal disease[58]. Other *Fusobacteria* may take advantage of coinfection with other invasive bacteria or of disruption of the mucosal layer, seen with CRC. Another mechanism by which *Fusobacteria* home and localize to dysplastic colorectal epithelium is the blood-borne route[83]. In a novel study, a host lectin (Gal-GalNAc) was shown to mediate *Fn* attachment to CRC and precursor cells through interaction with an Fn protein, fibroblast activation protein 2 (FAP2)[83]. The expression of Gal-GalNAc is increased in a stepwise fashion in colorectal adenoma and matched surrounding normal tissue to villous adenomas with highest levels seen in CRC[83, 84]."

4. **I do understand the enthusiasm of the authors, however the overstatements and blurred terminology devalue the manuscript. This is especially noticeable in conclusions starting with the first and very strong sentence:**
   "Fn enrichment in colorectal mucosa seems to be an early event occurring during colon carcinogenesis, even prior to the formation of adenoma or serrated lesions but not sufficient on its own to initiate CRC."
   What do the authors mean with enrichment? Adherence? Invasion? Adsorption?

   What do the authors mean with colorectal mucosa: epithelial cells, mucus, stroma?

Answer:

Thank you. We made changes to the manuscript to clarify this statement:

"The histopathology of these findings is ambiguous, but some data suggest that *Fusobacteria* have been observed within the colonic bacterial biofilms, in the colonic mucus layer, within colonic crypts, and invading the colonic epithelium [33, 46, 60, 77]."

What do they want to express with even in "early event of carcinogenesis", even prior to carcinogenesis? Do the authors mean that Fn is primary to cancer? As what? Infection? Colonization? Phagocytosis? How multiple individually composed Fusobacteria sequences found in each sample can be primarily?

Answer:

We agree that this was a strong statement, and we revised it in the body of manuscript:

“Quantity of *Fn* rDNA copies and inflammatory markers were both higher in visually normal rectal mucosa of adenoma patients compared with healthy controls[77, 89]. Fluorescence in situ Hybridization (FISH) confirmed the presence of Fn in the mucus layer and within colonic crypts of normal appearing colonic mucosa in that study[77]. Bacterial biofilms were also found to cover normal appearing colorectal mucosa adjacent to CRC; and this was associated with an increase in colonic epithelial proliferation, *IL6* and *STAT3* activity as well as decreased E-cadherin in the normal appearing colonic epithelium[33]. All this suggests that *Fn* is associated with increased colorectal inflammation in CRC tissue. There is also an association between of presence of *Fn* rDNA and inflammation in visually normal appearing colorectal epithelium. The presence of inflammation in normal appearing colonic epithelium could potentially be due to presence of bacterial biofilms. These findings are interesting since inflammation is considered to be a marker of carcinogenesis which suggest a potential early role for *Fn* in carcinogenesis even prior to adenoma formation [93].

We also revised our conclusion:

“Further, researchers have observed increased detection and quantity of *Fn* rDNA in the visually normal mucosa of colorectal neoplasia patients when compared with healthy controls. The pathophysiology and significance of this finding is unclear, as is its relation to cancer progression.”

I would start the conclusions with:
Sequences of Fusobacteria and especially Fusobacterium nucleatum are significantly more often detected in colorectal tissues and stools of patients with CRC than in healthy controls. The histopathology of this findings is unclear: no relevant data exist to whether the bacteria are adherent, invasive, taken up by macrophages, polluting the crypts, or simply stick to vulnerable and not protected mucosal areas. The lack of this information makes the interpretation of the data difficult.
Fusobacteria are indigenous for healthy mouth microbiota, highly adherent to teeth and oro- pharyngeal epithelium in the presence of low viscous saliva environment, but unspecialized for viscous environment and therefor normally only transient in the intestine, the mucosa is covered with a mucus layer, which is impenetrable for bacteria.
In case of a disturbed mucus barrier (locally over carcinoma or generally in the colon due to conditions preceding carcinoma formation like ulcerative colitis and other), oral Fusobacteria may get advantages in attaching to the denuded regions of colonic epithelium, leading to all described peculiarities in occurrence of Fusobacteria sequences in colonic cancer and making Fusobacteria to an interesting indicator of condition
Although the mechanism and sequels of this attachment (invasion, colonization, phagocytosis) are presently unclear, a strong correlation

between colonic mucosa associated Fusobacteria and cancer, make the possibility of Fusobacteria coinvolvement in colonic carcinogenesis to an intriguing concept, which still has to be unraveled in details.

Answer:

We appreciate this important comment. We made major revisions to the conclusion. The changes are highlighted in the revised version of the manuscript.

5. **English is not my native language. For my opinion many of the sentences are grammatically wrong or at least difficult to understand:**
   For example:
   “Fn is demonstrated to have cancer promoting properties in several rodent models support a role in the human colon cancer cascade.”

   Either the thoughts are jumping or something is lacking.
   I recommend an English native editor to go through the text.

Answer:

Thank you for pointing that out. My coauthors are native English speakers and have reviewed the manuscript one more time and made major edits to assure its clarity. We also sent the manuscript to an editing service and made the appropriate revisions.