Research Article

A Snapshot of Oral Microbiota in Patients with Colorectal Cancer

Deniz Can Guven,[1] Koray Ergunay,[2] Annika Brinkmann,[3] Alpaslan Alp,[2] Fatma Nur Kittana,[2] Yakut Akyon,[2] Engin Yilmaz,[4] Derya Karakoc,[5] Erhan Hamaloglu,[5] Suayip Yalcin,[1] Andreas Nitsche,[3] Mutlu Hayran,[6] Omer Dizdar[6]

[1]Department of Medical Oncology, Hacettepe University Cancer Institute, Ankara, Turkey
[2]Department of Medical Microbiology, Hacettepe University Faculty of Medicine, Ankara, Turkey
[3]Robert Koch Institute, Centre for Biological Threats and Special Pathogens 1 (ZBS 1), Berlin, Germany
[4]Department of Medical Biology, Acibadem Mehmet Ali Aydinlar University Faculty of Medicine, Istanbul, Turkey
[5]Department of General Surgery, Hacettepe University Faculty of Medicine, Ankara, Turkey
[6]Department of Preventive Oncology, Hacettepe University Cancer Institute, Ankara, Turkey

Abstract

Objectives: Human oral microbiota is evaluated in several cancers; however, data on colorectal cancer (CRC) are very limited. Based on this, we aimed to investigate the composition of oral microbiota in CRC and to compare them with healthy controls.

Methods: Saliva samples from 32 patients who were diagnosed with CRC and from 32 healthy controls in the same age group were evaluated in this study. Bacterial populations were identified through the multiplication of partial 16S rDNA sequences and comparison with databases after next-generation sequencing (Illumina MiSeq).

Results: In family-level examinations, the relative abundance of Pasteurellaceae was decreased and that of Neisseriaceae was increased in patient group (p=0.008 and p=0.036, respectively). The relative abundance of Bacillales showed a negative correlation with disease stage (r=-0.638, p<0.001), while the relative abundance of Proteobacteria showed a positive correlation with the stage of disease (r=0.450, p=0.01).

Conclusion: In our study, there were significant differences between the relative abundances of Pasteurellaceae and Neisseriaceae in the saliva of CRC patients. Additionally, the correlation between disease stage and abundance of Bacillales and Proteobacteria members was a novel finding, which needs validation in larger series. These findings might have implications for screening and prognostication of CRC.

Keywords: Colorectal cancer, neisseriaceae, next-generation sequencing, oral microbiota, pasteurellaceae

The association between colon microbiota and colorectal cancer (CRC) has been widely investigated. Many researchers reported changes in fecal or colonic mucosal microbiota in patients with CRC.[1-3] Common strains in co-lon microbiota including Escherichia coli[4] and Bacteroides fragilis[5] were found to be frequently associated with CRC. Interestingly, pathogenic organisms of the oral cavity such as Fusobacterium nucleatum and Porphyromonas gingi-

valis were also reported to be enriched in GI tract malignancies including CRC[6] as well as pancreatic cancer[7] and esophagus cancer.[8] Furthermore, previous studies showed increased risk of a number of cancers including CRC in patients with periodontal diseases.[9-11]

Given the increased abundance of periodontal pathogenic bacteria in fecal[1,12] and tumor specimens 2 3 of CRC patients, similar anaerobic conditions of CRC tissue and oral mucosa[13], and clinical association between periodontal diseases and CRC, examining the oral microbiome and its possible associations with CRC appears highly relevant. Saliva is generally considered as a good representative of oral microbiota and saliva specimens are convenient for analyzing oral microbiota due to easy sampling and handling and improved stability.[14] There are limited number of previous studies evaluating oral microbiota in CRC, with various patient populations, study designs, samples (saliva vs. oral rinse) or methodologies making the interpretation of results difficult.[3,15,16] We previously showed increased amount of Fusobacterium and Porphyromonas using PCR in patients with colon cancer.[17] In the present study, we aimed to investigate the saliva microbiome composition in patients with CRC in comparison with healthy controls.

Methods

Patient Selection

Newly diagnosed CRC patients and age and sex-matched healthy controls without prior diagnosis of chronic gastrointestinal (GI) disorder were included in the study. All CRC patients were diagnosed with colonoscopy. Patients with inflammatory bowel disease and hereditary CRC syndromes and patients who used antibiotics within the previous month were excluded from both groups. Informed consent was obtained from all the patients that participated in the study. Tumor stages and histologic properties were retrieved from patient files and hospital registries. Five milliliters (mL) of saliva were collected from each patient using non-sterile tubes and stored at -20 ºC until analyses.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local Ethics Committee.

Microbiota Analyses

Bacterial DNA was extracted according to recommended manufacturer protocol with GeneMATRIX Swab-ExtractDNA Purification Kit (Gdansk, Poland). Bacterial 16S rDNA sequences were amplified using previously described primers targeting the V3-V4 region.[18] Attachment of sequencing adapters to PCR products, amplification, and library preparation were performed using NextEra XT Index and NextEra DNA Library Prep kits (Illumina, San Diego, California, United States), as suggested by the manufacturer. Product clean-up and library quantification and optimization were performed using Agencourt AMPure XP reagent (Beckman Coulter Biosciences, Krefeld, Germany) standard protocol and Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Sequencing runs were performed in Illumina MiSeq system (Illumina Inc.). Sequence data handling was performed using Geneious v11.1 (Biomatters Ltd, Auckland, New Zealand), MALT V0.3.8, and MEGAN v6.11.[19] The trimmed bacterial reads were mapped to NCBI-NT RefSeq 16S database, with hits down to 95% identity. For OTU (operational taxonomic unit) identification and taxonomic binning, LCA-assignment algorithm (with 95% minimum identity) and 16s percent identity filter (species assignment at 99% identity) in MEGAN package were used. Relative bacterial abundances were calculated using the reads numbers of the corresponding OTUs.

Statistical package for social sciences 22 program was used for analysis in the study. P-values below 0.05 were considered statistically significant.

Results

General Characteristics

Thirty-two CRC patients and 32 controls were included in the study. Patient characteristics are shown in Table 1. Median age (57 in patients vs. 56 in controls, p=0.946) and gender distribution (M/F 53.1% and 46.9% in patients vs. 59.4% and 40.6% in controls, p=0.614) were similar between patients and controls. Most of the patients had left-sided (75%) and advanced (59.4% stage III and IV) tumors. Microsatellite instability status was evaluated in 21 patients and was found high in three of them (Table 1).

Table 1. Characteristics of patient group

Tumor localization	n (%)
Left	24 (75)
Right	8 (25)
Stage	
I	2 (6.3)
II	11 (34.4)
III	9 (28.1)
IV	10 (31.3)
MSI status	
High	3 (14.3)
Low	18 (85.7)

Microbiota Analyses

Relative abundances of total bacteria were similar in the patient and control groups. In addition, analyses at the
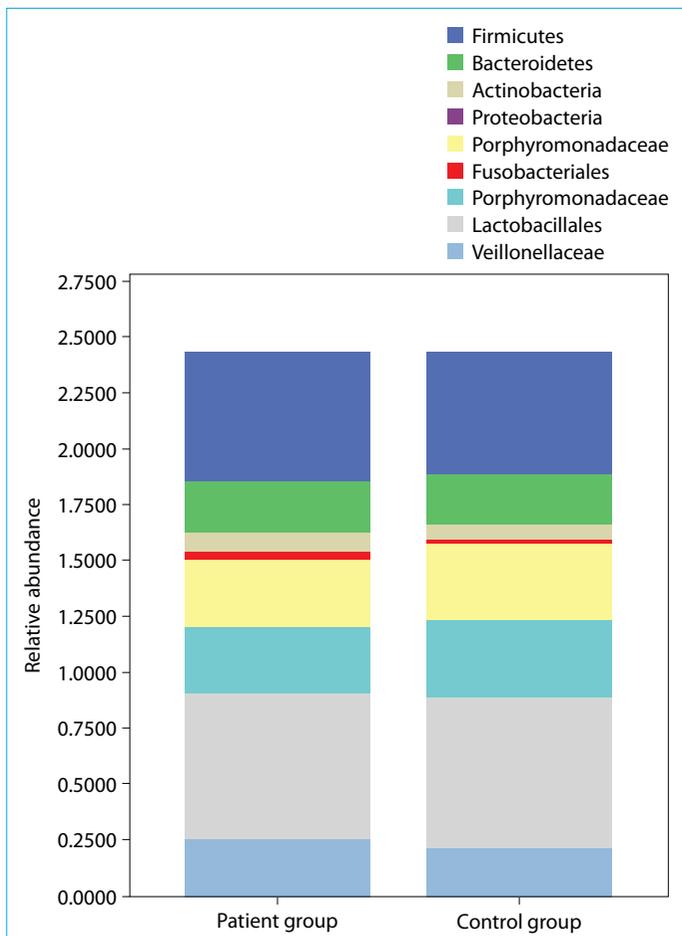

Figure 1. Relative abundances of major phyla in patient and control groups.

phylum level showed no significant differences between the relative abundances of Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Spirochaetes phyla (Fig. 1). In the analyses at the family level, the relative abundance of Pasteurellaceae was decreased and that of Neisseriaceae was increased in the patient group (p=0.008 and p=0.036, respectively) compared with controls (Fig. 2).

In the subgroup analyses of patient group according to disease stage, relative abundance of Bacillales showed a negative correlation with the disease stage (r=−0.638, p≤0.001), while the abundance of Proteobacteria showed a positive correlation with disease stage (r=0.450, p=0.01) (Fig. 3).

Discussion

In this study, we found decreased abundance of Pasteurellaceae and an increased abundance of Neisseriaceae in CRC patients compared with healthy controls. Moreover, we presented for the first time the correlation between some oral flora bacteria and disease stage in CRC.

Gastrointestinal microbiota is a dynamic structure playing an important role in GI homeostasis and protection of the host from invasion of pathogens. Deviations from the normal GI microbiota, so-called dysbiosis, can be related to systemic disorders including cancer.[20] Colorectal cancer is among the most important disease states in which microbiota changes are prominent.[12,21,22] There are multitude of microbiota studies on the feces and CRC tissue reporting an increased abundance of Fusobacterium nucleatum[1,21], Prevotella[22], Porphyromonas[12], Streptococcus gallolyticus subspecies gallolyticus[2], Eubacteriaceae[12], Staphylococcaceae[12] and Escherichia coli.[4] Some of these bacteria such as Fusobacterium, Porphyromonas, and Prevotella are
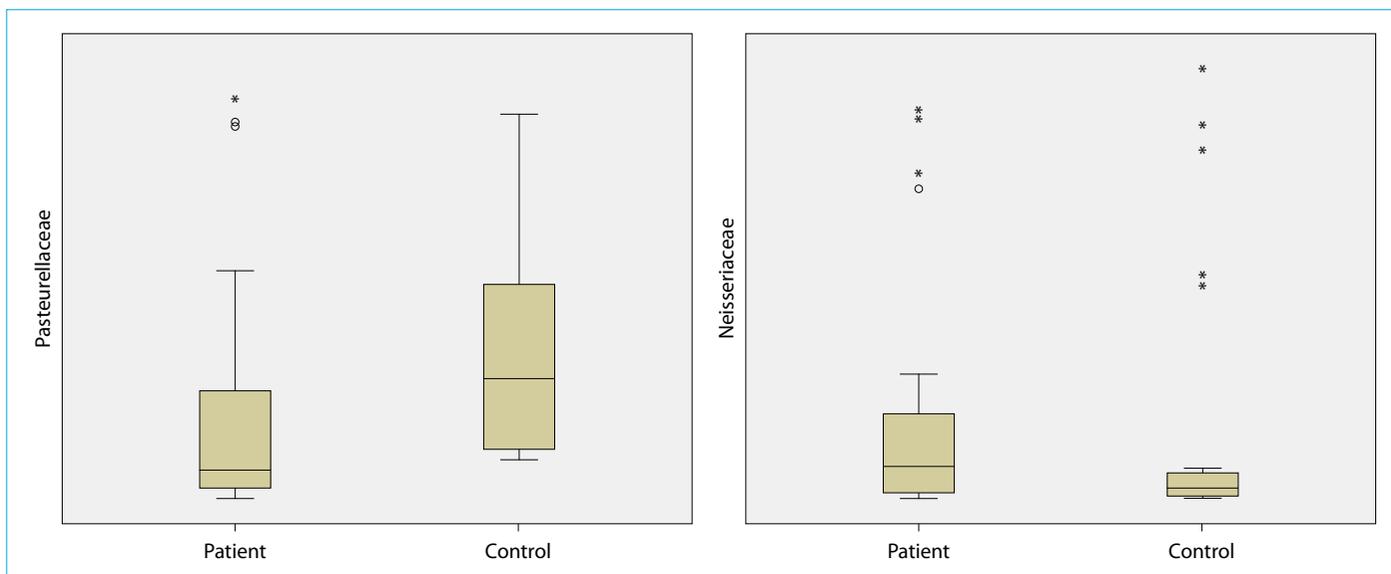

Figure 2. Relative abundances of Pasteurellaceae (left) and Neisseriaceae (right) in patient and control groups.
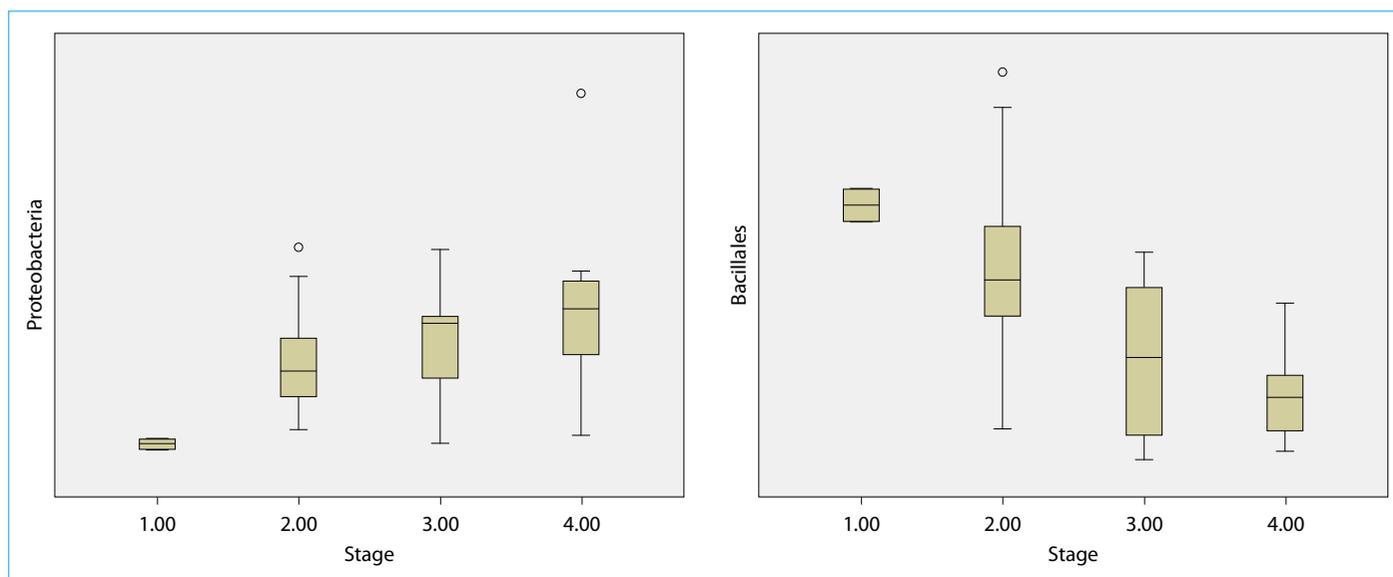
Figure 3. Correlation of Bacillales and Proteobacteria relative abundances according to stages in patient group.

also periodontopathogenic[23] hinting a link between oral flora dysbiosis and CRC.

The concept of "salivaomics" is gaining a rapid momentum with saliva microbiota analyses being used for diagnostic and prognostic purposes in local and systemic diseases. [14] Subgingival plaque and colorectal tissue have similar anaerobic conditions, which may explain the survival advantage of periodontopathogenic bacteria in CRC tissue.[13] We found a similar amount of total bacteria in CRC patients and controls. The total bacteria amounts was also similar in colorectal cancer patients and controls in a previous feces study by Sohbani.[22] This finding reflects that composition changes in microbiota are more prominent rather than total amount bacteria. In our study, relative abundances of periodontopathogenic bacteria were similar between the two groups. Previously, Russo et al. reported a similar abundance of Fusobacterium nucleatum and other oral pathogens in the saliva of CRC patients and controls.[16] Kato et al also showed similar levels of Fusobacterium nucleatum and Porphyromonas spp. in the oral rinse of CRC patients compared to healthy controls, while increased Lactobacillus and increased relative abundance of Rothia was seen in CRC patients.[15] Flemer et al. profiled the oral, fecal, and colonic microbiota of CRC patients and healthy controls and found a decreased abundance of periodontopathogenic bacteria such as Porphyromonas, Parvimonas, and Fusobacterium in the oral swab of CRC patients compared to controls.[3] In contrast to these findings, Kageyama recently reported an increased abundance of Actinomyces odontolyticus and Porphyromonas gingivalis in the saliva of CRC patients. However, a more severe periodontitis, as inferred from the more bleeding teeth with probing, was present

in CRC patients, which may have confounded the findings. [24] Conflicting results may be secondary to the lack of periodontal examination, differences in sampling methods, other potential confounding factors like diet, smoking etc. or due to lack of a real pathophysiology-based association.

We found a decreased abundance of Pasteurellaceae in the saliva of CRC patients. Pasteurellaceae are found in the biofilm microbiota of subgingival plaques and may have procarcinogenic properties.[25] Pasteurellaceae abundance was reportedly increased in the tongue coating microbiome of patients with pancreatic cancer.[26] The mechanism underlying the reverse direction of oral Pasteurellaceae abundance in the two different GI cancers is difficult to interpret, as a similar trend was observed in the abundance of oral Fusobacterium abundance in CRC and pancreatic cancer.[3,17,27]

Neisseriaceae abundance was increased in the saliva of CRC patients in our study. Three previous studies showed conflicting outcomes; Kato et al. showed similar levels of abundance in oral rinses[15], Flemer et al. showed decreased abundance in oral swabs[3] and Kageyama et al. showed increased abundance levels in saliva samples of patients with CRC compared with healthy controls.[24] Better representation of the gingival plaque using the saliva rather than oral rinse or oral swab, considering the predilection of bacteria in the periodontal pocket, might explain differences in the results.[28]

Previous studies conducted on oral microbiota of CRC patients did not report any association between microbiota changes and disease stage.[3,15,16] In a study by Dai et al. on feces samples, the levels of Streptococcus sp., Shewanella woodyi, and Mycoplasma penetrans were decreased with increased stage of CRC.[29] Mima[21] and Viljoen[5] reported

the association of Fusobacterium nucleatum with tumor stage with increased levels associated with later stages, while Bonnet et al. reported an increased abundance of pathogenic cyclomodulin positive E. coli in stage III and IV cancers.[4] These studies were conducted in CRC tumor tissue. We found increased relative levels of Proteobacteria and decreased levels of Bacillales with increased stages, with a clear and statistically significant trend observed in the analyses. Proteobacteria has previously been demonstrated to have higher abundance in intestinal inflammation and tumors.[20,22] Proteobacteria might have a cofactor role in disease progression in CRC. On the other hand secretome of Bacillales species are known to have protective effects on gastrointestinal mucosa.[30] These findings might have implications in prognostication of patients and determining those at high risk of advanced disease.

Main limitation of our study is the lack of adjustment for confounding factors including nutritional factors, smoking and alcohol consumption, and periodontal diseases. If our findings are replicated, targeted interventions against specific pathogens may result in prevention and treatment of CRC. Larger studies with comprehensive dietary surveys, dental/periodontal examination and concomitant fecal microbiota assessment as part of the protocol are needed to elucidate potential role of oral microbiota in prevention, diagnosis, and determination of prognosis in patients with CRC.

References

1. Amitay EL, Werner S, Vital M, Pieper DH, Höfler D, Gierse IJ, et al. Fusobacterium and colorectal cancer: causal factor or passenger? Results from a large colorectal cancer screening study. Carcinogenesis 2017;38:781–8.

2. Abdulamir AS, Hafidh RR, Bakar FA. Molecular detection, quantification, and isolation of Streptococcus gallolyticus bacteria colonizing colorectal tumors: inflammation-driven potential of carcinogenesis via IL-1, COX-2, and IL-8. Mol Cancer 2010;9:249.

3. Flemer B, Warren RD, Barrett MP, Cisek K, Das A, Jeffery IB, et al. The oral microbiota in colorectal cancer is distinctive and predictive. Gut 2018;67:1454–63.

4. Bonnet M, Buc E, Sauvanet P, Darcha C, Dubois D, Pereira B, et al. Colonization of the human gut by E. coli and colorectal cancer risk. Clin Cancer Res 2014;20:859–67.

5. Viljoen KS, Dakshinamurthy A, Goldberg P, Blackburn JM. Quantitative profiling of colorectal cancer-associated bacteria reveals associations between fusobacterium spp., enterotoxigenic Bacteroides fragilis (ETBF) and clinicopathological features of colorectal cancer. PLoS One 2015;10:e0119462.

6. Komiya Y, Shimomura Y, Higurashi T, Sugi Y, Arimoto J, Umezawa S, et al. Patients with colorectal cancer have identical strains of Fusobacterium nucleatum in their colorectal cancer and oral cavity. Gut 2019;68:1335–7.

7. Torres PJ, Fletcher EM, Gibbons SM, Bouvet M, Doran KS, Kelley ST. Characterization of the salivary microbiome in patients with pancreatic cancer. PeerJ 2015;3:e1373.

8. Chen X, Winckler B, Lu M, Cheng H, Yuan Z, Yang Y, et al. Oral microbiota and risk for esophageal squamous cell carcinoma in a high-risk area of China. PLoS One. 2015;10:e0143603.

9. Kim GW, Kim Y-S, Lee SH, Park SG, Kim DH, Cho JY, et al. Periodontitis is associated with an increased risk for proximal colorectal neoplasms. Sci Rep 2019;9:7528–28.

10. Güven DC, Dizdar Ö, Akman AC, Berker E, Yekedüz E, Ceylan F, et al. Evaluation of cancer risk in patients with periodontal diseases. Turk J Med Sci 2019;49:826–31.

11. Dizdar O, Hayran M, Guven DC, Yılmaz TB, Taheri S, Akman AC, et al. Increased cancer risk in patients with periodontitis. Curr Med Res Opin 2017;33:2195–200.

12. Wu N, Yang X, Zhang R, Li J, Xiao X, Hu Y, et al. Dysbiosis signature of fecal microbiota in colorectal cancer patients. Microb Ecol 2013;66:462–70.

13. Flynn KJ, Baxter NT, Schloss PD. Metabolic and community synergy of oral bacteria in colorectal cancer. mSphere 2016;1:e00102–16.

14. Yoshizawa JM, Schafer CA, Schafer JJ, Farrell JJ, Paster BJ, Wong DT. Salivary biomarkers: toward future clinical and diagnostic utilities. Clin Microbiol Rev 2013;26:781–91.

15. Kato I, Vasquez AA, Moyerbrailean G, Land S, Sun J, Lin HS, et al. Oral microbiome and history of smoking and colorectal cancer. J Epidemiol Res 2016;2:92–101.

16. Russo E, Bacci G, Chiellini C, Fagorzi C, Niccolai E, Taddei A, et al. Preliminary comparison of oral and intestinal human microbiota in patients with colorectal cancer: a pilot study. Front

Microbiol 2018;8:2699.

17. Guven DC, Dizdar O, Alp A, Akdoğan Kittana FN, Karakoc D, Hamaloglu E, et al. Analysis of Fusobacterium nucleatum and Streptococcus gallolyticus in saliva of colorectal cancer patients. Biomark Med 2019;13:725–35.

18. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41:e1.

19. Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, et al. MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Comput Biol 2016;12:e1004957.

20. Schwabe RF, Jobin C. The microbiome and cancer. Nat Rev Cancer 2013;13:800–12.

21. Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, et al. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. Gut 2016;65:1973–80.

22. Sobhani I, Tap J, Roudot-Thoraval F, Roperch JP, Letulle S, Langella P, et al. Microbial dysbiosis in colorectal cancer (CRC) patients. PLoS One 2011;6:e16393.

23. Popova C, Dosseva-Panova V, Panov V. Microbiology of periodontal diseases. a review. Biotechnol Biotechnol Equip 2013;27:3754–59.

24. Kageyama S, Takeshita T, Takeuchi K, Asakawa M, Matsumi R, Furuta M, et al. Characteristics of the salivary microbiota in patients with various digestive tract cancers. Front Microbiol 2019;10:1780.

25. Lax A. The Pasteurella multocida toxin: a new paradigm for the link between bacterial infection and cancer. Curr Top Microbiol Immunol 2012;361:131–44.

26. Lu H, Ren Z, Li A, Li J, Xu S, Zhang H, et al. Tongue coating microbiome data distinguish patients with pancreatic head cancer from healthy controls. J Oral Microbiol 2019;11:1563409.

27. Fan X, Alekseyenko AV, Wu J, Peters BA, Jacobs EJ, Gapstur SM, et al. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. Gut 2018;67:120–7.

28. Vieira Colombo AP, Magalhães CB, Hartenbach FARR, do Souto RM, da Silva-Boghossian CM. Periodontal-disease-associated biofilm: A reservoir for pathogens of medical importance. Microb Pathog 2016;94:27–34.

29. Dai Z, Coker OO, Nakatsu G, Wu WKK, Zhao L, Chen Z, et al. Multi-cohort analysis of colorectal cancer metagenome identified altered bacteria across populations and universal bacterial markers. Microbiome 2018;6:70.

30. Ilinskaya ON, Ulyanova VV, Yarullina DR, Gataullin IG. Secretome of Intestinal Bacilli: A Natural Guard against Pathologies. Front Microbiol 2017;8:1666.