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 colon 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\textsuperscript{[6]} as well as pancreatic cancer\textsuperscript{[7]} and esophagus cancer.\textsuperscript{[8]} Furthermore, previous studies showed increased risk of a number of cancers including CRC in patients with periodontal diseases.\textsuperscript{[9-11]}

Given the increased abundance of periodontal pathogenic bacteria in fecal\textsuperscript{[1,12]} and tumor specimens\textsuperscript{2 3} of CRC patients, similar anaerobic conditions of CRC tissue and oral mucosa\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[3,15,16]} We previously showed increased amount of Fusobacterium and Porphyromonas using PCR in patients with colon cancer.\textsuperscript{[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 °C 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.\textsuperscript{[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.\textsuperscript{[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>
<thead>
<tr>
<th>Tumor localization</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>24 (75)</td>
</tr>
<tr>
<td>Right</td>
<td>8 (25)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (6.3)</td>
</tr>
<tr>
<td>II</td>
<td>11 (34.4)</td>
</tr>
<tr>
<td>III</td>
<td>9 (28.1)</td>
</tr>
<tr>
<td>IV</td>
<td>10 (31.3)</td>
</tr>
<tr>
<td>MSI status</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Low</td>
<td>18 (85.7)</td>
</tr>
</tbody>
</table>
Microbiota Analyses

Relative abundances of total bacteria were similar in the patient and control groups. In addition, analyses at the 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], Staphylococccae[12] and Escherichia coli.[4] Some of these bacteria such as Fusobacterium, Porphyromonas, and Prevotella are
also periodontopathogenic 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. Subgingival plaque and colorectal tissue have similar anaerobic conditions, which may explain the survival advantage of periodontopathogenic bacteria in CRC tissue. 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. 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. 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. 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. 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. 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. Pasteurellaceae abundance was reportedly increased in the tongue coating microbiome of patients with pancreatic cancer. 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. 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, Flemer et al. showed decreased abundance in oral swabs and Kageyama et al. showed increased abundance levels in saliva samples of patients with CRC compared with healthy controls. 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. Previous studies conducted on oral microbiota of CRC patients did not report any association between microbiota changes and disease stage. 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. Mima and Viljoen 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. 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. 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. 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.

Disclosures
Acknowledgement: We would like to thank Hacettepe Teknokent Technology Transfer Center for their precious support in the advanced editing of this article.

Ethics Committee Approval: The study was approved by the Ethics Committee of Hacettepe University with approval number of GO19/232.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors report no conflicts of interest.

Financial Disclosure: This manuscript did not receive any funding.


References


