

The Role of Gut Bacterial Metabolome in Colorectal Cancer

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ABSTRACT: Colorectal cancer (CRC) is one of the most common cancers, affecting around five million people worldwide. Accumulating evidence suggests that gut bacteria are closely associated with CRC, and bacterial metabolites play critical roles in human health and disease. In this study, I hypothesized that gut bacterial metabolites are essential for CRC formation, and changes in the levels of specific bacterial metabolites may play a role in CRC tumorigenesis. To test this hypothesis, bacterial supernatants were prepared from four bacterial species that have previously been reported to be associated with CRC, including *Staphylococcus epidermidis* (SE), *Fusobacterium nucleatum* (FB), *Escherichia coli* (EC) and *Staphylococcus aureus* (SA). The effects of these bacterial supernatants on the viability of COLO205 cells (a representative CRC cell line) were examined. Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics experiments were also performed to determine the levels of different metabolites in these supernatants. Bacterial supernatants prepared from EC and FB were found to inhibit COLO205 cell growth significantly ($p < 0.01$). In addition, the levels of microbial metabolites ciliatine, N-benzylformamide, and aconitic acid were increased dramatically in both supernatants prepared from EC and FB, suggesting potential key roles of these three metabolites in inhibiting COLO205 cell growth as well as CRC tumorigenesis.

KEYWORDS: Microbiology; Applied microbiology; Colorectal cancer; Gut bacteria; Metabolite.

■ Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States.^{1,2} Only in 2021, about 149,500 individuals were diagnosed with CRC, and 52,980 died from this disease based on statistical analysis by the American Cancer Society. A better understanding of CRC tumorigenesis can provide new therapeutics to treat this disease and improve the health of more than five million people suffering from CRC worldwide. Recent evidence suggests that gut bacteria are closely associated with CRC.³⁻⁵ In addition, metabolites from bacteria have been shown to play essential roles in human health and disease.⁶⁻⁸ Based on these previous observations by others, I raised the research question, "Is there a link between gut bacteria and CRC through gut bacterial metabolites?". I hypothesize that the metabolites of gut bacteria play critical roles in the formation of colorectal cancer, so changing the levels of specific bacterial metabolites will affect colorectal tumorigenesis. This study was conducted to test this hypothesis with two goals: 1) to determine if supernatants prepared from bacterial cell culture affect (either inhibit or promote) the growth of COLO205 cells (a representative CRC cell line) and 2) to identify bacterial metabolites in these bacterial supernatants that may lead to the observed effects on COLO205 cell growth. The significance of this research is that it may reveal previously unknown mechanisms for CRC tumor formation and development by linking microbial population/metabolism and CRC pathogenesis. The outcome of this study has the potential to open new doors for CRC prevention and therapeutics.

■ Methods

Four bacterial species that have previously been reported to be associated with CRC were used in this study.^{9,10} The ex-

periments were performed in three steps (Figure 1). Bacterial supernatants were first prepared from these bacterial species (Step 1), and the supernatants were then aliquoted for both CRC cell culture experiments (Step 2) and liquid chromatography-mass spectrometry (LC-MS)-based metabolomic analysis (Step 3). Individuals were diagnosed with CRC, and 52,980 died from this disease based on statistical analysis by the American Cancer S.

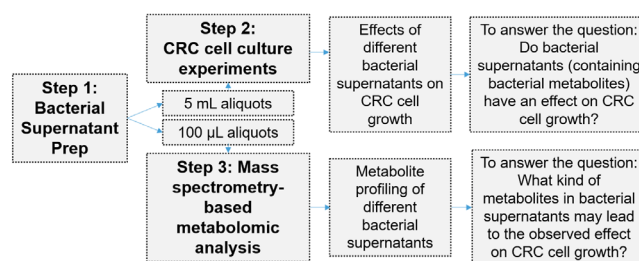


Figure 1: Experimental design.

Bacterial supernatant preparation:

Four bacterial species were used in this study, including *Staphylococcus epidermidis* (SE) (ATCC 14990TM), *Fusobacterium nucleatum* (FB) (ATCC 25586TM), *Escherichia coli* (EC) (ATCC 11775TM) and *Staphylococcus aureus* (SA) (ATCC 12600TM). Four representative strains of these species were purchased from ATCC. The bacterial cells were first cultured on Gifu Anaerobic Medium (GAM)-Agar plates containing antibiotics ampicillin overnight. A single colony was then picked for each bacterial species to culture a 5 mL cell culture with GAM containing 50 µL/mL ampicillin. The cells were cultured at 37 °C overnight with continuous shaking at 250 rpm and then used to culture a 50 mL cell culture using the same conditions overnight. OD600 value for the bacterial cell culture solution was measured using a NanoDrop Microvolume

Spectrophotometer. The bacterial solution was then harvested by centrifugation at 4,000 x g (centrifugal acceleration 4,000 times the gravity on earth) for 10 minutes in a benchtop centrifuge to separate the bacterial cells from the supernatant. Then the bacterial supernatant was filtered using a 0.2 µm filter to remove residue cells in the supernatant. The supernatant was finally split into 5 mL aliquots in 15 mL conical tubes for CRC cell culture experiments and 100 µL aliquots in 1.5 mL microcentrifuge tubes for metabolomics experiments. The experiments were repeated three times.

CRC cell culture experiments:

CRC cell line COLO205 cells were cultured in a Roswell Park Memorial Institute medium (RPMI)-1640 medium using a T75 flask in a CO₂ incubator.¹¹ Cells were then plated onto a 96-well cell culture plate with 10,000 cells seeded per well. 100 µL of RPMI-1640 medium was used in each well. The 96-well plate was cultured for one day until cells were ~40-50% confluency. The bacterial supernatants prepared above were diluted with GAM to make three Spent Sup. solutions: 1) Spent Sup. were the undiluted bacterial supernatants; 2) Spent Sup. (0.5) were the diluted supernatant solutions equivalent to OD₆₀₀ = 0.5, and 3) Spent Sup. (0.25) were the diluted supernatant solutions equivalent to OD₆₀₀ = 0.25. 100 µL of Spent Sup., Spent Sup. (0.5) or Spent Sup. (0.25) were added to each individual well. The same volume (100 µL) of RPMI-1640 or GAM was added to control wells. The 96-well plate was returned to the CO₂ incubator at 37 °C and cultured for two days. Cells in each well were then suspended using a Trypsin-EDTA solution. 10 µL of suspended cells were mixed with 10 µL of Trypan Blue (0.4%) dye in a 1.5 mL microcentrifuge tube. 10 µL of the mixed solution was transferred to a cell counter slide. COLO205 cell viabilities were measured by counting viable and nonviable cells using an Invitrogen COUNTESS™ automated cell counter.

LC-MS-based metabolomic analysis:

100 µL of bacterial supernatant from each bacterial species was used for the metabolomics experiment. 250 µL of methanol (HPLC grade) and 50 µL of ¹³C¹⁵N labeled internal standards were added to 100 µL of bacterial supernatant. The ¹³C¹⁵N labeled internal standards were prepared to measure 230 metabolites using stable isotope labeled metabolite standards purchased from Sigma-Aldrich, IROA Technologies, and Cambridge Isotope Laboratories. The mixtures were vortexed for 2 minutes before being stored at -20 °C for 20 minutes. The samples were centrifuged at 14,000 rpm for 20 minutes. 150 µL of supernatant were then transferred to a new 2 mL Eppendorf vial and dried at 30 °C for 1.5 hours in a speed vacuum. Samples were then reconstituted with 250 µL 50% acetonitrile (ACN) and transferred into LC vials for MS analysis using a Thermo Vanquish UPLC system coupled with a Q-Exactive Orbitrap mass spectrometer.¹²⁻¹⁴ An ACQUITY UPLC CSH C18 1.7µm 2.1×100mm column (Waters Corp, Milford, MA, USA) was applied for metabolite separation in both negative and positive ionization modes with separate injections. Mobile phase A was 100% water, containing 5 mM ammonium acetate and 0.1% formic acid, and mobile phase B was acetonitrile and H₂O at a proportion of 95/5 (v/v), containing

5 mM ammonium acetate and 0.1% formic acid. A linear gradient elution program was set as 35% B from 0–1 min, increasing to 95% B over 20 min; after another 10 min at 95% B, the mobile phase composition was then returned to 35% B. The total run time was 35 min. The flow rate was 0.4 mL/min, and the column temperature was 40 °C. The MS/MS data were acquired at m/z 100–1500. The resolution for data collection in full scan and fragment spectra were 140,000 and 17,500, respectively. AGC target was 3e⁶, and the maximum IT was 50ms for full scan mode. While the MS/MS mode parameters were set as automatic gain control (AGC) target at 1e⁵, maximum IT of 50ms, loop count at 10, isolation window of 1.2 m/z, and normalized collision energy (NCE) of 20, 50, and 80 eV. Metabolomic data were analyzed using a streamlined metabolomics data analysis tool, MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca/home.xhtml>).¹⁵

■ Results and Discussion

Effects of bacterial supernatants on the viability of COLO205 cell growth:

In the CRC cell culture experiments, the COLO205 cells were incubated with different amounts of bacterial supernatants. An automated cell counter examined the effects of bacterial supernatants on the viability of COLO205 cell growth. After staining with Trypan Blue, the live cells appeared as halos with bright centers and dark edges, whereas the dead cells had uniform blue color throughout the cells without bright centers in the cell counting analysis. The viabilities were calculated as the percentages of live cells in total cells (live plus dead cells) in each well of the 96-well cell culture plate. After two days of incubation, the viabilities of COLO205 cells with the SE and SA bacterial supernatants did not change significantly compared to cells incubated with RPMI and GAM control media (Figure 2). However, the original SE bacterial supernatant (Spent Sup.) led to a COLO205 cell viability decrease (p<0.05), the diluted SE bacterial supernatant solutions (Spent Sup. (0.5) and Spent Sup. (0.25)) did not demonstrate a similar effect. However, all three different bacterial supernatant solutions (Spent Sup., Spent Sup. (0.5) and Spent Sup. (0.25)) prepared from FB and EC led to a dramatic decrease (p<0.01) in COLO205 cell viabilities (Figure 2).

The observation of COLO205 cell viability decrease in the presence of original and diluted FB and EC supernatants suggested that these bacterial supernatant solutions had an inhibitory effect on cancer cell growth. This inhibitory effect of bacterial supernatants prepared from FB and EC may result from the presence or an increase in one or more bacterial metabolites secreted into the supernatant solution by FB.

and EC bacterial cells. These metabolites are missing or at normal concentrations in the bacterial supernatants prepared from SE and SA bacterial cells.

LC-MS-based metabolomics analysis revealed distinct metabolite profiles and top metabolites:

In the LC-MS-based metabolomics analysis, a stable isotope labeled internal standard mixture was spiked into the samples. Of 230 targeted metabolites, 173 were detected in bacterial supernatants in this study. These detected metabolites were subjected to statistical analyses among different experimental

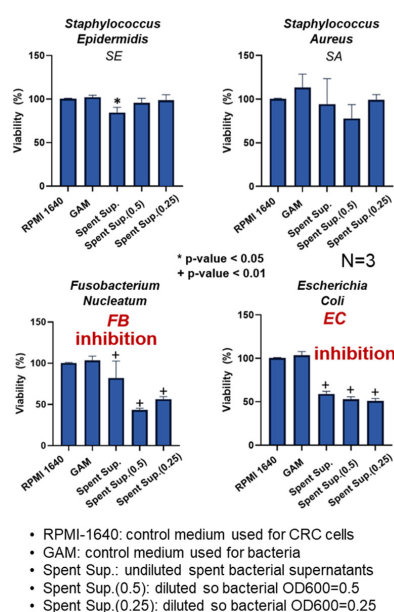


Figure 2: Effects of bacterial supernatants on the viability of COLO205 cell growth. The X-axis shows the different solutions added to the COLO205 cells. They are independent variables. The Y-axis shows the viability of the cells as a percentage. RPMI-1640 medium and GAM are both controls. RPMI-1640 is a medium for CRC cells, and GAM is a medium for bacteria. Spent Sup. is the undiluted spent bacterial supernatants; Spent Sup. (0.5) is the diluted spent bacterial supernatants corresponding to a bacterial optical density (OD)600 of 0.5; Spent Sup. (0.25) is the diluted bacterial supernatant corresponding to bacterial OD600 of 0.25. The p-value is the statistical significance value. The lower the p-value, the better. The p-values were measured using a pair-wise student T-test. P-values < 0.05 are considered statistically significant.

groups to identify the metabolites that may cause the observed COLO205 cell inhibitory effect induced by

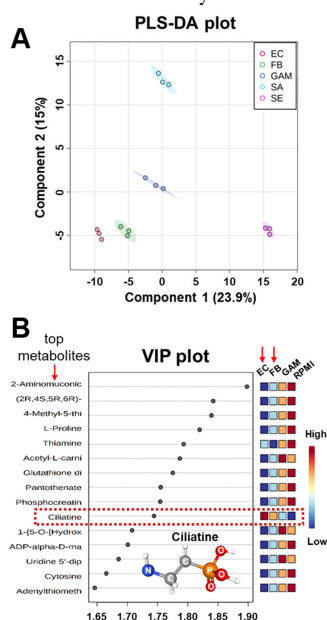


Figure 3: LC-MS-based metabolomics analysis revealed distinct metabolite profiling and top metabolites. A) This PLS-DA plot on the left generated by MetaboAnalyst5.0 shows unique clustering of metabolites, indicating that the metabolomes of the bacteria differ. B) The VIP plot on the right shows the top metabolites analyzed in this study. The X-axis shows the VIP scores. The molecular structure of ciliatine was inserted in the VIP plot. Both PLS-DA and VIP plots were generated by MetaboAnalyst5.0.

bacterial supernatants prepared from EC and FB. A partial least squares-discriminant analysis (PLS-DA) plot suggested that the metabolic profiles of different bacterial species are distinct, as evidenced by distinct clustering of the experimental groups (Figure 3A). In addition, the variable importance (VIP) plot shows that ciliatine, a phosphonate, is significantly increased in bacterial supernatants prepared from EC and FB species (Figure 3B). Further heat map analysis of bacterial supernatant metabolomic data among EC, FB, and controls (GAM and RPMI-1640) revealed that in addition to ciliatine, the levels of N-benzylformamide and aconitic acid also significantly increased in bacterial supernatants prepared from EC and FB species (Figure 4, left panel).

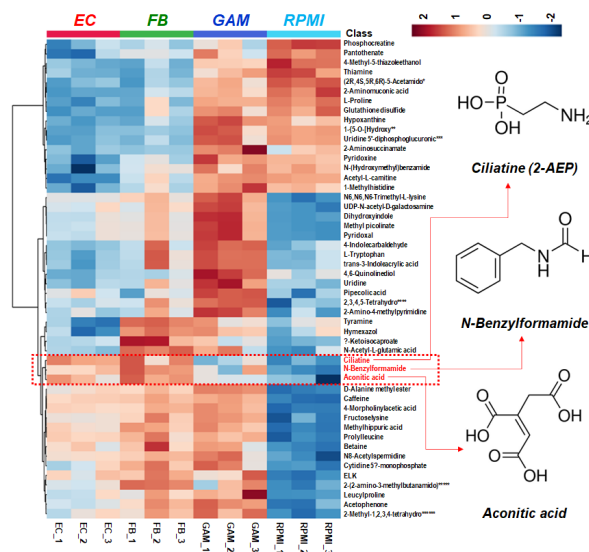


Figure 4: Heatmap analysis revealed that three metabolites significantly increased in bacterial supernatants prepared from EC and FB species.

Left panel, the heat map analysis showed that the levels of ciliatine, N-benzylformamide, and aconitic acid significantly increased in bacterial supernatants prepared from EC and FB species compared to controls (GAM and RPMI-1640). Right panel, the chemical structures of ciliatine, N-benzylformamide, and aconitic acid. The heat map was generated by MetaboAnalyst5.0.

The PLS-DA plot showed unique clustering of metabolites, indicating that the metabolomes of the bacterial supernatants prepared from the four bacterial strains used in this study are distinct from each other as well as the control. In the VIP and heat map analyses, three metabolites, ciliatine, N-benzylformamide and aconitic acid were found to increase significantly in both FB and EC bacterial supernatants that inhibit COLO205 cell growth, suggesting that these three metabolites may play a key role in inhibiting CRC cell growth. Ciliatine is a phosphonate (Figure 4, right panel, top). N-benzylformamide belongs to the class of aromatic compounds known as benzene and substituted derivatives, containing one monocyclic ring system consisting of benzene (Figure 4, right panel, middle). Aconitic acid is an organic acid (Figure 4, right panel, bottom). Further study of the effects of these three metabolites and their related derivatives on the cell growth of COLO205 and CRC animal models may reveal novel mechanisms of CRC tumorigenesis and shed light on CRC therapeutics.

■ Conclusion

In summary, this research proved my hypothesis and suggested that bacterial metabolites could modulate gut bacteria and CRC interactions. Bacterial supernatant prepared from EC and FB demonstrated inhibitory effects on CRC cell line COLO205 cell growth. Three bacterial metabolites in these supernatants, ciliatine, N-benzylformamide, and aconitic acid, may participate in the observed inhibitory effect. To confirm this finding, further studies will be needed to investigate the direct impact of these metabolites and their derivatives on cultured COLO205 cells and CRC mouse models. In addition, computational docking and chemoproteomics will help to identify potential protein targets of these metabolites and their derivatives to reveal molecular mechanisms of their possible therapeutic effects toward CRC.

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