Effects of Tumor-Associated *E. coli* Metabolites on Migration of Colorectal Cancer Cells

Original Articles

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Abstract

Colorectal tumors have a close connection with the gut microbiome. Correlation between rearrangement in microbiome composition and disease progression has already been shown. However, the questions about the mechanisms underlying microorganisms and cancer cells interaction and the immediate effects of tumor-associated microbiomes on cancer cells remain open. In this work, we investigated the effects of metabolites of tumor-associated *E.coli* strains on migration of human colorectal cancer cell lines (HCT116, SW480 and HT29). We showed differenties in some biochemical enzime activity of *E.coli* strains and the spectrum of synthesized organic acids by tumor-associated and probiotic *E.coli* M-17 strains. Most of the strains associated with colorectal cancer were unable to utilize sucrose. Specifically, tumor-associated *E.coli* produced more fumaric, malic and maleic acids, whereas the *E.coli* M-17 produced more short fatty acids such as propionic, 2-oxobutyric and α-ketoglutaric acids. Upon exposure to metabolites from tumor-associated *E.coli* strains, HCT116 and SW480 cells showed an increased migration activity and HT29 cells - decreased migration activity in 2D and 3D culture models. Immunocytochemistry assay revealed decrease of E-cadherin in HCT116 and SW480 cells and FAK- in HT29, which explain different effects of *E.coli* metabolites on migratory capacity of colorectal cancer cells. Therefore, these results suggest that the effect of tumor-associated *E.coli* strains on cancer cells migration depends on their innate type of migration and enhance FAK-dependent single-cell migration accompanied by the loss of E-cadherin in cancer cells with initially low FAK expression. At the same time, this effect was not observed in cancer cells with collective migration phenotype.

1.Introduction

Colorectal cancer is one of the most common malignancies remains a lethal disease worldwide [1]. In the case of colorectal cancer, microbiota and its metabolites are considered an essential player in the disease progression [2]. It is generally accepted that there are close associations between the gut microbiota composition and its metabolites [3] and the progression of colorectal cancer [4]. The crosstalk between the host cancer cells and gut microbiota mediated by metabolites can favor cancer progression, enhanced invasiveness and cancer cell survivability.

However, the direct effects of microorganisms and their metabolites on cancer cells have been poorly described.

Escherichia coli (E. coli) is the most common representative of colon microbiota. Commensal *E. coli* is known to promote the regeneration of damaged colon epithelial cells [5]. However, an increase in the colonization of colon mucosa by mucosa-associated *E. coli* has been found in patients with colorectal cancer [6].

Our study was designed to understand the role of *E.coli* metabolites isolated from patients' tumor samples in regulation of migratory capacity of colorectal cancer cells. The effects of the metabolites on three colorectal cancer cell lines migration were examined on cell monolayers and 3D tumor spheroids.

2.Methods. ***Bacterial strains and cultivation.*** *E.coli* strains (Col-93, Col-101, Col-102, Col-103) were isolated from colorectal cancer biopsy samples. Human colon samples were collected at Nizhny Novgorod Regional Oncologic Hospital (Russia). The study with the use of patients' samples was approved by the ethics committee of the Privolzhsky Research Medical University (approval № 09 from 30.06.2023). All methods were carried out in accordance with relevant guidelines and regulations.

The bacterial species identity was determined by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI ToF Autoflex speed, Bruker Daltonik GmbH, Germany). Biochemical properties were studied by bacterial biochemical identification kits (RPC Diagnostic Systems, Russia). Routine cultivation was carried out using nutrient agar (24 h, 37 °C). Probiotic strain of *E.coli* M17, which is widely used as a component of probiotic drugs to correct dysbiotic conditions, served as a positive control in all the experiments.

3. Metabolite preparation. The bacterial strains were seeded in DMEM with 4.5 mg/L glucose (PanEco, Russia) in a concentration of 1×10^6 CFU/ml and cultured for 24 h at 37 °C. Then metabolites were obtained by filtration of the growth media through bacterial filter 0.2 μm (Corning, USA). The resulting solution was diluted 1:1.5 with DMEM with 5% fetal bovine serum. The final medium was checked for pH, which was in neutral range (7.0-7.2).

4.Target liquid chromatography – mass spectrometry analysis. Cell-free metabolites were prepared by centrifuging the culture at 20,000 rcf for 20 min. Finally, they were filtered through 0.2 μm filters (Corning, USA). High-performance liquid chromatography with electrospray ionization triple quadrupole tandem mass spectrometry was used for detection of short-chain fatty

acids and organic acids in supernatant liquid. The samples of metabolites were prepared according to the manual (Shimadzu Corporation) and analyzed using a mass spectrometer LCMS-8050 coupled with the Nexera XR liquid chromatography system (Shimadzu, Japan).

5.Antibiotic susceptibility testing. The disk diffusion susceptibility test was used to determine the susceptibility of isolates of *E. coli* to 9 antibiotics - ampicillin, trimethoprim, amoxicillin, norfloxacin, ciprofloxacin, ofloxacin, cefotaxime, ceftriaxone and ceftazidime. The test was performed using the standard protocol. A bacterial inoculum 1–2 × 10^8 CFU/mL was applied to the surface of a nutrient agar plate with a diameter of 60 mm. A commercially prepared, fixed-concentration paper antibiotic disk was placed on the inoculated agar surface. The results were assessed after 18–24 h incubation of the plates at 37 °C. The zones of growth inhibition surrounding the antibiotic disk were measured to the nearest millimeter. The zone diameters of each drug were interpreted according to the manufacturer's instruction (NICF, Russia).

6.Matrix production assay. To analyze matrix production, the biofilms were grown for 48 h in DMEM with 4.5mg/L glucose (PanEco, Russia) in 96-well plates. The bacterial concentration was 1×10^6 CFU/ml. Then the bacterial biofilms were washed three times by phosphate buffered saline (PBS) and stained by Congo red for 15 min. The staining solution containing 1% Congo red and 10% Twin 80 was prepared in PBS. After staining, plates were washed three times by PBS and ethyl alcohol was used for extraction of Congo red from the cells. The optical density was measured at a multichannel spectrophotometer BioTek Synergy Mx (BioTek, USA) at a wavelength of 500 nm.

7.Bacterial biomass growth. The biofilms were washed three times by PBS, fixed with 96% ethyl alcohol for 15 minutes, stained by 0.1% gentian violet solution (3 min). Next, the dye was eluted by 96% ethyl alcohol at constant shaking (10 min) and the optical density was measured using a multichannel spectrophotometer at a wavelength of 570 nm.

8.Colon cancer cell lines. Human colon adenocarcinoma cell lines HCT116, SW480 and HT29 were routinely cultured in DMEM (PanEco, Russia) with 5% fetal bovine serum (HyClone, USA) and passed twice a week. The cells were cultured in a CO_2-incubator, 37 °C, RH 80%, CO_2 5%. The cell lines were obtained from the cell collection of the Ivanovskiy Institute of Virology (Moscow, Russia).

9.Spheroids formation and cell migration assay. To obtain tumor spheroids, HCT116 and HT29 cancer cells were seeded in low attachment 96-well plates (Corning, USA) in the amount of 200

cells in 200 µl /well and cultured in the presence of bacterial metabolites. The bacterial metabolites were obtained after overnight culturing the bacterial strains in DMEM with 4.5 mg/L glucose (PanEco, Russia) in a concentration of 1×10^6 CFU/ml at 37 °C, filtered (0.2 µm, Corning, USA) and diluted 1:1.5(See section 3). Spheroids grown in a pure DMEM medium (200 µl per well) were used as a negative control. The size of spheroids was measured in 3, 5 and 7 days for HCT116 and in 4 and 7 days for HT29, because the latter have lower cell division rate.
For migration assay the spheroids after 5 days of culturing were used. They were gently transferred in a small Petri dish (3.5 mm) and incubated for either 2 (HCT116) or 5 days (HT29). Light microscopy images of the spheroids were acquired after their attachment using DMIL microscope (Leica, Germany) at magnification 100x, and the zones of cell migration were measured in ImageJ (V 1.4.3.67) software.

10.“Wound healing” assay. The study of migration activity was carried out on the model of "wound healing" using cultural inserts Culture-Insert 2 Well (Ibidi, USA). The cell suspension (70 µl) with a concentration of 1×10^5 cells/ml was placed into the wells, incubated for 24 h (37° C, 5% CO_2), and silicone liners were removed after the formation of the monolayer. Then we added medium contaned bacterial metabolites (1:1.5) and cultured until “healing” the monolaer “wound”. We changed medium every 2 days. Light microscopy images (Leica, Germany) of the "wounds" were obtained in 1-5 days after removal of the liners at magnification 100x. Migration zones were measured using ImageJ (V 1.4.3.67) software.

11.Immunostaining assay. For immunocytochemical staining, the cells were cultured in 96-well plates for 24 h after seeding and fixed in 4% formaldehyde for 15 min. The following primary antibodies were used: rabbit antibodies against E-cadherin (ab15148, Abcam, USA), rabbit antibodies against focal adhesion kinase (FAK) (ab131435, Abcam, USA). Subsequently cells were stained with Alexa555-labeled goat anti-rabbit IgG secondary antibody (ab6825, Abcam, USA). Staining was performed in accordance with the manufacturer's protocol. In addition, the cells were stained with DAPI (1:1000) to visualize the cell nuclei and detect cancer cells. Fluorescence images were observed using DMIL fluorescence microscope (Leica, Germany) equipped with the following filters: A4 UV BP 360/40 400 BP 470/40 for DAPI and TX2 green BP 560/40 595 BP 645/75 for Alexa. The intensity values in Fig.4 were obtained when processing photos in the ImageJ (V 1.4.3.67) software. Zones with a non-zero signal were

selected for processing and the average fluorescence intensity normalized per area was calculated.

12.Statistical analysis. Statistical analysis was performed using Statistica 10 (StatSoft. Inc., Tusla, OK, USA). The nonparametric Mann-Whitney U-test was used to compare the data. P-values ≤ 0.05 were considered statistically significant.

13. Results

Four bacteria species were isolated from patients' tumor and, using a combination of biochemical assay and MALDI ToF spectroscopy, identified as *E. coli*. The antibiotic sensitivity and biochemical enzyme activity for the tumor-associated *E. coli* strains were obtained and compared with standard probiotic *E. coli* M17 strain. Unlike M17, all the tumor-associated *E.coli* strains were resistant to at least one of the antibiotics tested. Three out of four strains (Col-101, Col-102, Col-103) showed resistance to ampicillin, and one (Col-93) - to amoxicillin (Fig. 1A).

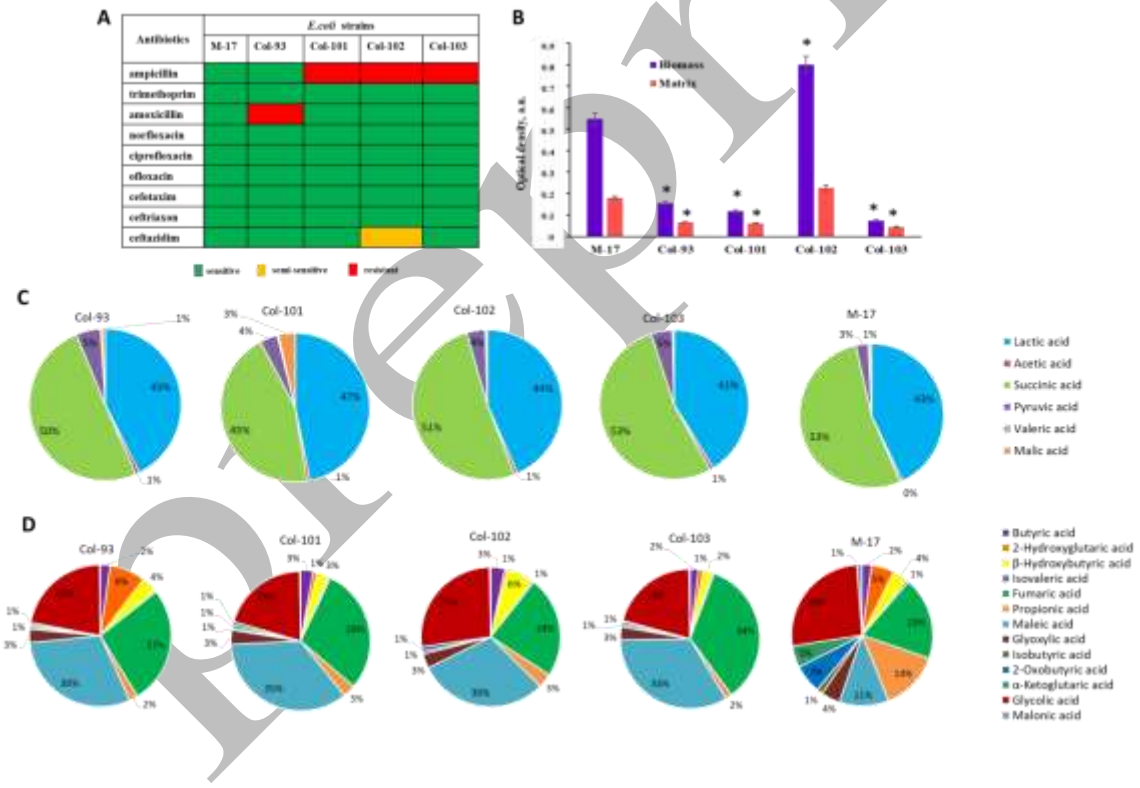

Fig. 1. Characterization of patients' derived E.coli strains. Antibiotic susceptibility (A), biomass and matrix production of different E.coli strains (B), proportion of selected major (C) and minor (D) metabolites produced by E.coli strains (C). Mean±SD, n=10, * $p \leq 0.05$ with M17.

Analysis of the biofilm activity showed that three tumor-associated strains (Col-93, Col-101, Col-103) increased biomass and synthesized a matrix less actively compared to M17 strain (Fig. 1B). One strain (Col-102) had statistically higher reproduction rate and produced the same volume of matrix as M17. Biochemical activity of the strains has fluctuations in some aminoacids and especially in sucrose. Most of the strains associated with colorectal cancer were unable to utilize sucrose, which is typically observed in only a few *E.coli* that can are typically slow growing or pathogenic strains [7]. The differences in the proportion and composition of the synthesized metabolites were found between tumor-associated and the probiotic strains. The proportion of dominant metabolites (mg/ml) was similar in all strains (Fig.1, C), tumor-associated and M17 control.

Table. Daily production of the selected metabolites by *E.coli* strains, Mean±SD (* p≤0.05 vs M17)

Analyte	**M17**	**Col-093**	**Col-101**	**Col-102**	**Col-103**
Lactic acid, mg/l	138.6±13.3	102.2±39.4 *	123.8±23.9	124.6±8.1	112.5±3.7 *
Acetic acid, mg/l	1.7±0.1	2.0±0.8	1.9±0.2	2.1±0.1 *	2.3±0.0 *
Succinic acid, mg/l	170.8±18.5	120.5±40.7 *	118.2±11.4 *	147.4±2.1 *	144.6±4.1 *
Pyruvic acid, mg/l	8.9±0.9	12.6±4.4	10.1±1.2	11.6±0.6 *	12.7±0.2 *
Malic acid, mg/l	1.8±0.3	1.8±0.6	9.7±1.3 *	0.5±0.03 *	0.3±0.02 *
Valeric acid, mg/l	0.60±0.09	0.6±0.2	0.8±0.09 *	0.8±0.01 *	0.8±0.04 *
β-Hydroxybutyric acid, µg/l	31.4±2.9	43.6±19.0	35.8±6.1	70.0±8.3 *	30.2±5.3
ropionic acid, µg/l	122.7±23.0	24.2±8.5 *	37.3±4.3 *	37.0±2.9 *	21.0±2.6 *
Isobutyric acid, µg/l	12.7±2.4	6.1±0.1 *	6.1±0.6 *	6.9±2.0 *	8.5±1.6 *
Butyric acid, µg/l	19.1±3.8	27.3±10.3	36.1±4.1 *	36.4±4.0 *	27.3±1.4 *
2-Hydroxyglutaric acid, µg/l	47.0±6.1	98.7±43.0 *	11.9±0.8 *	6.9±2.8 *	15.2±1.8 *
Isovaleric acid, µg/l	5.2±1.0	4.2±1.9	5.9±1.8	5.1±1.3	6.5±0.5
Fumaric acid, µg/l	170.1±16.1	325.6±114.8 *	372.3±53.2 *	263.2±27.2 *	476.8±15.5 *
Maleic acid, µg/l	101.8±6.1	366.3±229.3 *	446.1±59.6 *	337.4±15.9 *	465.1±63.5 *
Glyoxylic acid, µg/l	38.9±3.3	36.9±11.9	42.1±5.2	37.8±8.4	41.6±7.4
2-Oxobutyric acid, µg/l	59.0±5.3	6.0±2.1 *	9.1±3.6 *	11.5±0.6 *	8.8±0.7 *
α-Ketoglutaric	45.2±16.5	6.3±5.7 *	9.9±1.9 *	0.5±0.2 *	3.4±1.0 *

acid, µg/l					
Glycolic acid, µg/l	235.8±35.3	264.7±72.9	257.9±34.0 *	301.5±2.5 *	286.4±10.1 *
Malonic acid, µg/l	11.2±2.8	2.0±1.3 *	2.9±0.4 *	3.0±1.0 *	3.3±1.5 *

While the balance of minor components (µg/ml) was different for the tumor-associated *E.coli* strains compared to M17 control (Fig.1, D).

We found higher levels ($p \leq 0.05$) of butyric, fumaric, maleic, and glycolic acids in tumor-associated strains' metabolites compared to probiotic strain M17 (Table 1). The amount of malic acid was increased in Col-101 metabolites more than 5 times compared to other strains and M17. Pyruvic acid and valeric acid level was higher in tumor-associated strains, especially for Col-101, Col-102 and Col-103($p \leq 0.05$). Daily production of short-chain fatty acids (SCFAs) such as 2-oxybutyrate, propionate, malonic acid, 2-hydroxyglutaric, 2-oxobutyric, isobutyric acid, α-Ketoglutaric acid (AKG) and isobutyric acid by tumor-associated strains was lower (2-5 times) compared to probiotic strain M17 (Table). Succinic acid was decreased in all patients' strain metabolites. No changes were observed for isovaleric acid and glyoxylic acid.

Metabolites of the probiotic strain M17 did not affect migration of HCT116 cells from the spheroids and slightly ($p=0.035$) inhibited migration of HT29 cells compared to control without any metabolites (Fig. 2).

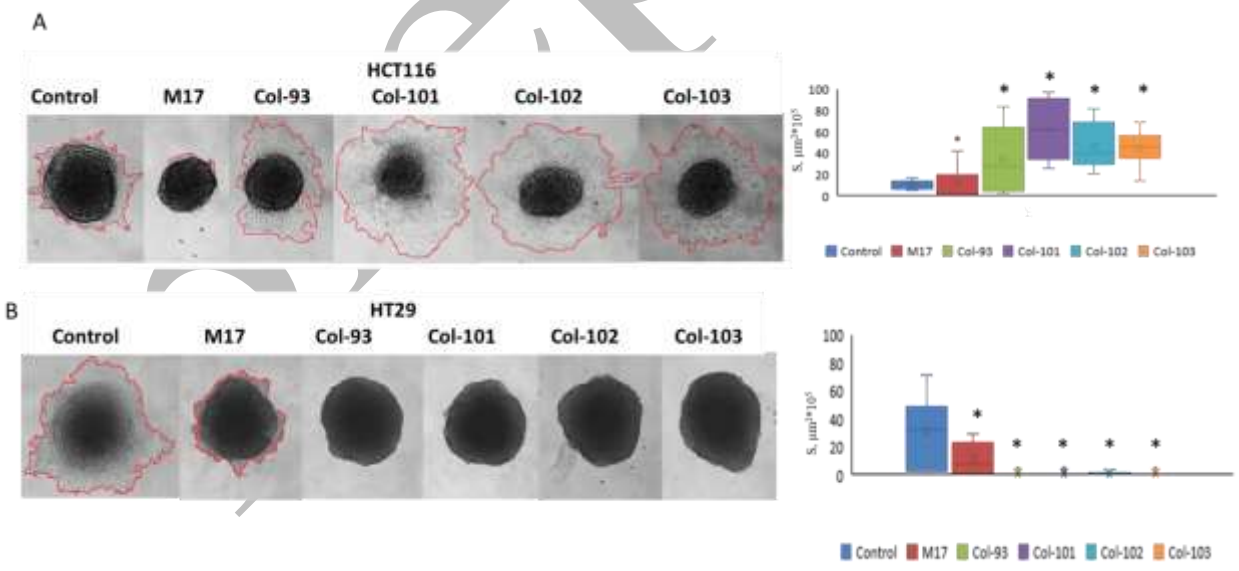

Fig.2 Migration of colorectal cancer cells from the spheroids in the presence of bacterial metabolites. Light microscopy images and the migration area (square) of HCT116 (A) and HT29 (B) spheroids. Mean±SD, n=7, * $p \leq 0.05$ with control without metabolites.

Tumor-associated *E.coli* strains metabolites had different effects on migration of HCT116 and HT29 cells. HCT116 cell line showed higher migration activity in the presence of metabolites from all tumor-associated strains compared to control without any metabolites and M17 strain (Fig.2, A). The largest migration area was observed upon exposure to the Col-101 metabolites.

The opposite effects were observed for HT29 cells, which actively migrated in control without metabolites but inhibited migration in the presence of *E.coli* metabolites (Fig.2, B).

Analysis of cell migration in the model of monolayer "wound healing" was performed for the three cell lines HCT116, SW480 and HT29. Two *E.coli* strains, Col-101 and Col-102, were selected for this test as they demonstrated the most notable effects among other strains obtained from the patients.

Similar to migration from the spheroids, patient-derived *E.coli* metabolites stimulated migration of HCT116 and SW480 cells and inhibited migration of HT29 cells in the "wound healing" model (Fig.3).

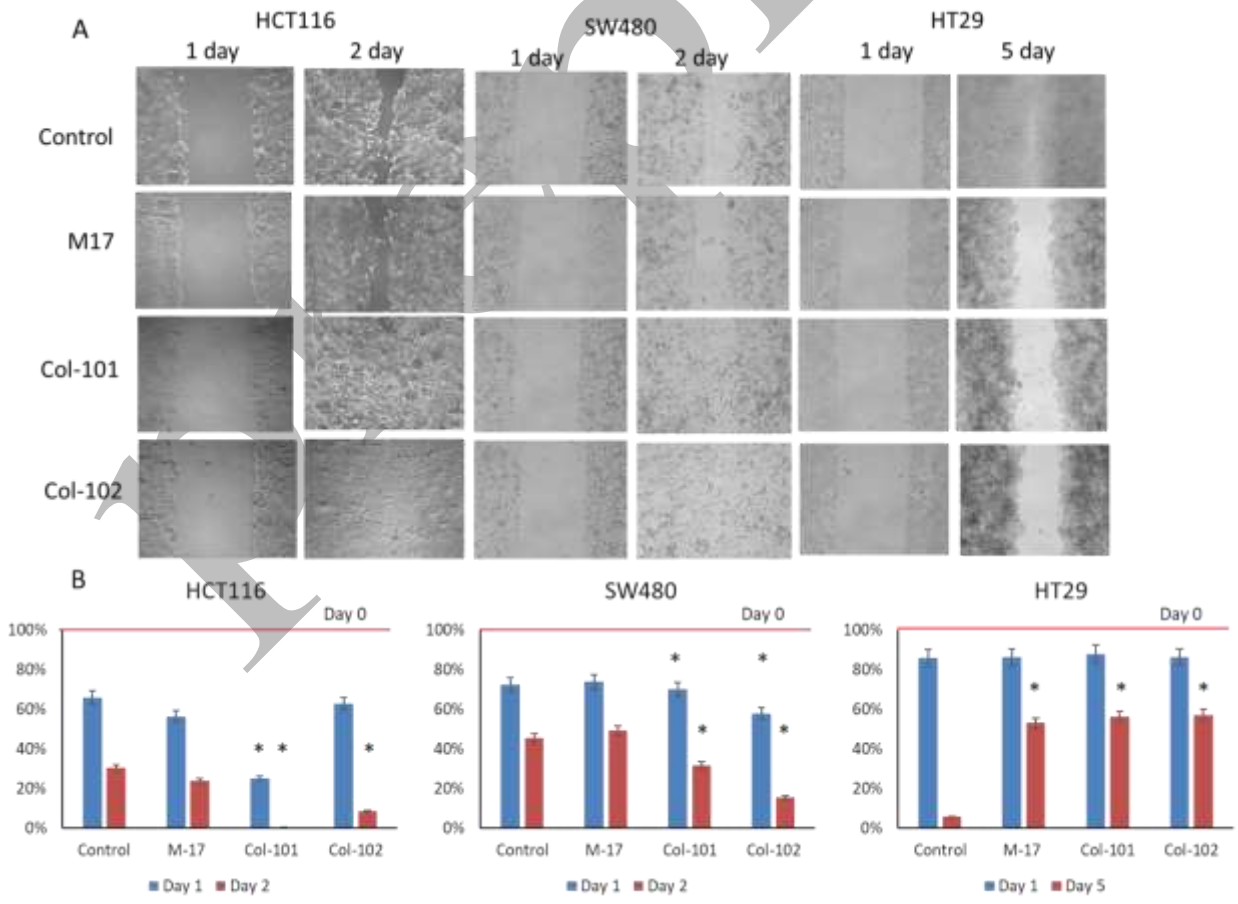

Fig.3. The “wound healing” assay in the presence of *E.coli* metabolites. (A) Light microscopy images of HCT116, SW480 and HT29 cells; (B) The relative square of the “wound” in the cell monolayer. Mean±SD, n=5, * p≤0.05 with control without metabolites at the same day.

The M17 metabolites did not change migration of HCT116 and SW480 cells and inhibited migration of HT29 cells. Therefore, the experiments on the cell monolayers and tumor spheroids revealed that tumor-associated *E.coli* metabolites affected migratory capacity of colorectal cancer cells and could either increase or decrease it depending on the specifics of cancer cells.

To identify the molecular mechanisms through which *E.coli* metabolites had different effects on colorectal cancer cell lines, the expression levels of E-cadherin and the focal adhesion kinase (FAK) were analyzed using immunofluorescence.

E-cadherin expression decreased (p≤0.05) in HCT116 and SW480 cell lines upon exposure to metabolites of the Col-101 strain (Fig.4).

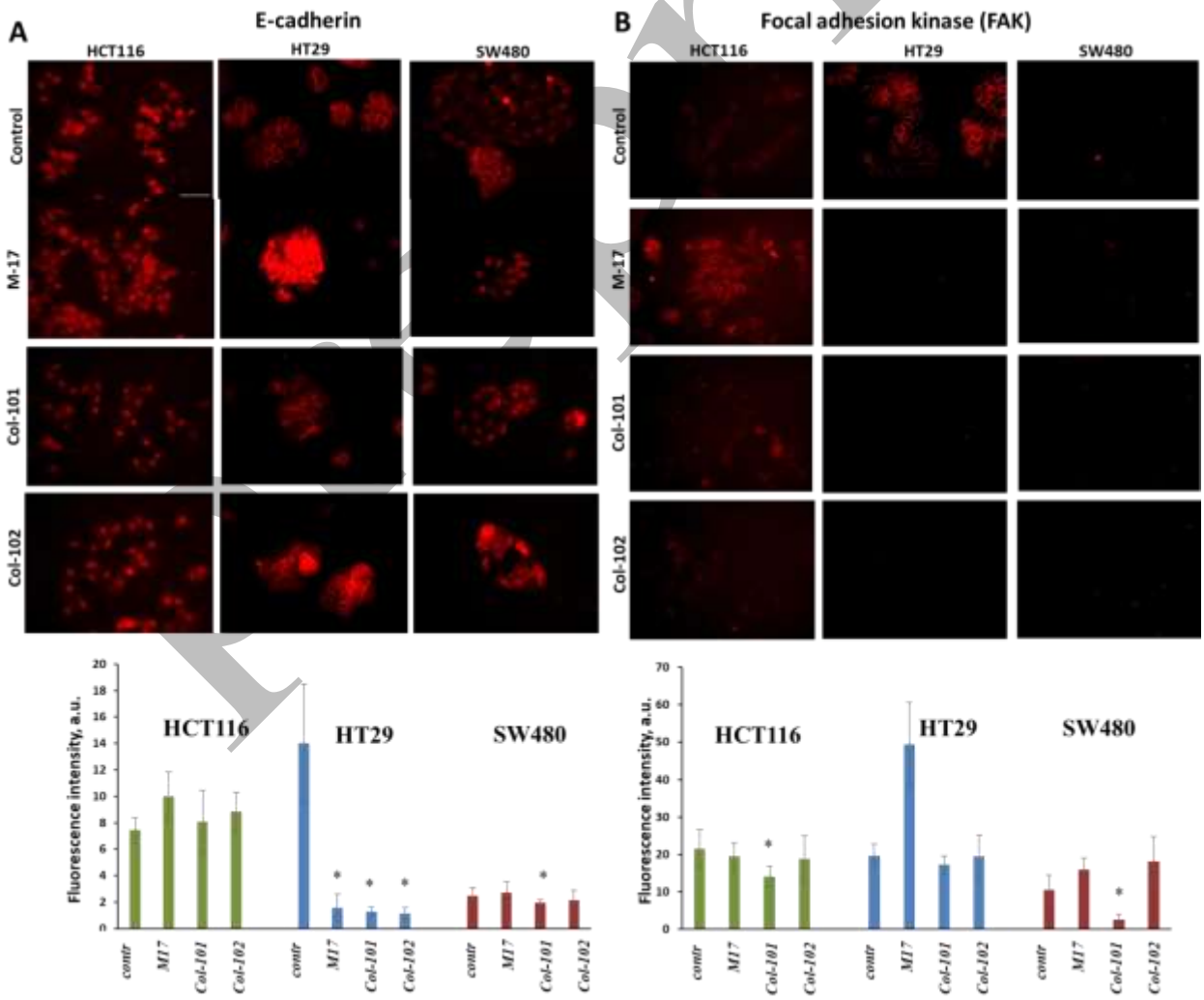

Fig.4. E-cadherin and FAK expression under exposure of cancer cells to *E.coli* metabolites. (A) Fluorescence microscopy images of E-cadherin expression and quantification of fluorescence intensity. (B) Fluorescence microscopy images of FAK expression and quantification of fluorescence intensity. Scale bar 50 μm for all images, Mean±SD, n=25, * $p \leq 0.05$ with control without metabolites.

The probiotic M17 strain induced a marked increase ($p \leq 0.05$) in E-cadherin level only in HT29 cells, and had no effect on two other cell lines. Since down-regulation of E-cadherin, a major component of adherens junctions, facilitates cell motility and migration, its lower level in HCT116 and SW480 cells correlated with their highest migratory activity upon incubation with Col-101 metabolites.

FAK expression decreasing ($p \leq 0.05$) was noted in HT29 cells under the bacterial metabolites of all tested strains, which explains a decrease of migratory capacity of this cell line. Inhibition of FAK activity is known to decrease cell motility due to suppression of cell-matrix attachment. Of note, the initial FAK activity in HT29 cells was higher compared with other cell lines, suggesting their higher migratory potential. In HCT116 and SW480 cell lines FAK level did not change after incubation with bacterial metabolites (Fig.4, B). These results suggest that changes in migratory capacity of colorectal cancer cells under the exposure to tumor-associated *E.coli* metabolites can be mediated by both the loss of cadherin-based cell−cell adhesions and attenuation of the FAK signaling.

14. Discussion

It is known that tumor-associated microorganisms play a critical role in the progression of colorectal cancer [8]. However, the mechanisms underlying the impact of microbiome on tumor progression remain poorly elucidated. Here, we analyzed the effect of probiotic and tumor-associated *E. coli* strains on migratory capacity of colorectal cancer cells in vitro.

First, we compared general characteristics of *E.coli* strains obtained from colorectal cancer patients with the standard probiotic strain M17 and found numerous differences between them. Specifically, the tumor-associated strains showed resistance to at least one antibiotic tested, primarily β-lactam antibiotics (ampicillin, amoxicillin), which can be associated with their ability to intracellularly survive during antibiotic treatment [9]. In addition, their biomass growth and biofilm matrix synthesis were reduced compared to M17, indicating that growth on the enterocytes surface is not a dominant type of growth for tumor-associated strains. Also, it was noticed that tumor-associated strains were adapted to utilize glucose, rather than complex

disaccharides due to decreased sucrose-related saccharolytic activity, which can be explained by the competition for the intracellular glucose with cancer cells. Previously, it has been shown that intracellular localization provides numerous advantages to the invading microbes, including the immune escape and a favorable nutritional environment. A low microbial biomass is consistently present in colorectal tumors, and they play an important role in cancer development [9].

Characterization of the spectrum of organic acids synthesized by different *E. coli* strains during their metabolism showed that the proportion and the level of synthesized substances were different for the probiotic and tumor-associated strains. The positive impact of microbiota is mostly connected with the production of SCFAs as a result of fermentation of dietary fibers, which are commonly indigestible by the human enzymes. SCFAs are thought to serve as anti-inflammatory substances in the gut, improving the intestinal epithelium barrier and can induce apoptosis of colorectal cancer cells [10, 11].

An excess of malonic acid and AKG was produced by the probiotic strain. Malonic acid inhibites succinate dehydrogenase. The succinate and succinate dehydrogenase complex are the central link of the Krebs cycle and regulates the mitochondrial respiratory chain and provides antioxidant defense by binding excess iron ions [12]. Antitumor effect of AKG was demonstrated on human lung carcinoma H460 and colon adenocarcinoma cell lines HCT116 [13]. Multiple studies have shown that metabolites such as butyrate, propionate, acetate, and niacin contribute to protection of the host against malignation and represent an energy source for the colon epithelial cells [14]. However, the oncometabolites like the lactate, glutamate, fumarate, and succinate are involved in tumor survival and progression [15,16]. Oncometabolites make the tumor microenvironment more favorable for cell migration [17]. In the study by Ternes et al. it was shown that gut microbial metabolite formate, produced by *F. nucleatum*, enhanced migration potential of HCT116 cells through the formation of focal adhesion points [18]. Sciacovelli et al. showed on renal cancer patients that fumaric acid inhibits Tet-mediated demethylation of a regulatory region of the antimetastatic miRNA cluster miR-200ba429, leading to the expression of EMT (epithelial-mesenchymal transition)-related transcription factors, which, in its turn, results in enhanced migratory properties and poor clinical outcome [19]. Malic acid was increased in malignant prostatic hyperplasia, suggesting that this metabolite can be used as a biomarker of prostate cancer [20].

The results of our study showed that metabolites of tumor-associated *E.coli* strains had different effects of migration capacity of colorectal cancer cells depending on their original properties.

Here, we observed more active migration of HCT116 and SW480 cells and inhibition of migration of HT29 cells in the monolayer "healing" model under *E.coli* metabolites. Similar results were obtained using the model of 3D tumor spheroids. We assume that these differences could be associated with different expressions of proteins involved in the processes of intercellular adhesion and adhesion to the substrate, such as E-cadherin and FAK. According to "The human Protein atlas" HT29 cell line initially has high expression of genes, associated with collective migration, such as *CDH1* (E-cadherin) and *PDK2* (FAK) and low expression of single-cell migration-associated markers (*ROCK1*, *EZR* and *TALIN).* While HCT116 and SW480 have higher single-cell migration markers, indicating different types of cell migration specific to these cells. In HCT116 and SW480 cells, for which a single-cell migration is typical, the loss of E-cadherin was the main effect of *E.coli* metabolites. Also, it is worth noting that the most pronounced effect was shown for *E.coli* strain Col-101. It was isolated from the tumor with the most invasive phenotype with the presence of the distant metastases to the liver. Loss of E-cadherin expression results in loss of contact inhibition, increase of cell motility and subsequent single-cell migration [21]. In the work by Tarashi et al. similar effect of *B. fragilis* toxin that is associated with colorectal cancer on cleavage of E-cadherin and formation of invasive phenotype was demonstrated [22]. Thirunavukkarasan et al. showed that SCFAs increased the level of expression of E-cadherin, and therefore, prevented the formation of the invasive phenotype of cancer cells [23].

In collective cell migration, E-cadherin mediates epithelial cell–cell adhesion and its expression is required to maintain intercellular junctions [21]. In HT29 cells, characterized by collective migration, the main effect of bacterial metabolites was the loss of FAK, while the expression of E-cadherin did not change. It is known that FAK, when it becomes constitutively active due to mutations or elevated activity of alternative signaling pathways, exerts oncogenic properties and allows cancer cells growth and survival without anchorage to the ECM, which is an important step during metastatic process.

Conclusion

Our research showed that metabolites from tumor-associated *E.coli* strains enhanced FAK-dependent single-cell migration accompanied by the loss of E-cadherin in cancer cells with

initially low FAK expression. At the same time, this effect was not observed in cancer cells with collective migration phenotype. Further studies of the effects of tumor-associated strains on migratory potential of different cancer types are important for development of microbiome correction strategies to improve cancer prognosis.

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Author's Contribution

Conceptualization and supervision: I.N. and D.I.;

Methodology and Investigation: P.M., A.A., S.A., D.I and I.N.;

Writing and draft preparation: I.N., P.M. and D.I;

Writing review and editing: S.M., G.S.;

All authors have read and agreed to publish version of the manuscript.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Competing interests

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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