Metabolite Secretions of *Lactobacillus plantarum* YYC-3 May Inhibit Colon Cancer Cell Metastasis by Suppressing the VEGF-MMP2/9 Signaling Pathway

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Abstract

Background Colorectal cancer (CRC) is a major clinical challenge, and the gut microbiome plays important roles in the occurrence and metastasis of CRC. Lactobacillus and their metabolites are thought to be able to suppress the growth of CRC cells. However, the antimetastatic mechanism of Lactobacillus or their metabolites toward CRC cells is not clear. Therefore, the aim of this study was to assess the inhibitory mechanism of cell-free supernatants (CFSs) of L. rhamnosus GG, L. casei M3, and L. plantarum YYC-3 on metastasis of CRC cells.

Results YYC-3 CFS showed the highest inhibitory effect on CRC cell growth, invasion and migration, and inhibited MMP2, MMP9, and VEGFA gene and protein expression, and protein secretion. Furthermore, it suppressed the activities of MMPs by gelatin zymography. Moreover, the effective compounds in these CFSs were analyzed by Q Exactive Focus liquid chromatography-mass spectrometry.

Conclusions Our results showed that metabolite secretions of YYC-3 may inhibited cell metastasis by downregulating the VEGF/MMPs signaling pathway. These data suggest that treatment of CRC cells with metabolites from L. plantarum YYC-3 may reduce colon cancer metastasis.

1. Background

Colorectal cancer (CRC) is a major cause of cancer-related mortality worldwide, and many patients with CRC present at advanced stage with distant metastasis [1-3]. Tumor metastasis involves the invasion of cancer cells through extracellular matrix (ECM); cancer cells use matrix metalloproteinases (MMPs) to degrade ECM during invasion and metastasis [4]. MMPs are calcium-dependent and zinc-containing
endopeptidases that can be induced by an autocrine VEGF/VEGFR signaling pathway loop. The VEGF pathway is involved in multiple cellular processes, including cell proliferation, migration, invasion, and vascular cell permeability. The gut microbiome plays a critical role in maintaining the health of the gastrointestinal tract through host-microbe and microbe-microbe interactions [5–7]. Studies on the impact of gut microbiota and the effects of gut microbiota metabolite secretions on proliferation and invasion of colon cancer cells may inform new strategies for treatment of colon cancer metastasis.

Lactic acid bacteria (LAB), such as Lactobacillus and Bifidobacterium, are members of the gut microbiome [8–10]. Some metabolites secreted by LAB exhibit antibacterial properties, for example, bacteriocin; these metabolites may be potent substitutes for antibiotics [11, 12]. In recent years, the applications of LAB have extended to the field of cancer prevention, especially for CRC. Previous reports have demonstrated that certain LAB, including Lactobacillus casei, L. paracasei, L. plantarum, and L. reuteri, can inhibit the growth of human cancer cells [13–15]. The anticancer mechanisms attributed to these LABs include promoting apoptosis of cancer cells through intrinsic and extrinsic apoptosis pathways, antiproliferative regulation of the cell cycle in cancer cell lines, and sequestering of reactive oxygen species by antioxidative enzymes to prevent carcinogenesis [16]. However, to the best of our knowledge, few studies have explored the relationship between the antibacterial and anticancer properties of LAB.

Fusobacterium nucleatum has a symbiotic relationship with colon cancer that encourages proliferation of colon cancer cells [17]. In our previous work (not published), we identified L. rhamnosus GG, L. casei M3 and L. plantarum YYC-3 from 120 strains of LAB by the high antibacterial activities of their cell free supernatants
(CFSs) toward F. nucleatum. However, the direct effects of the CFSs of these bacteria on colon cancer cells remain unclear.

The aim of this study was to investigate the inhibitory effects of metabolite secretions of L. rhamnosus GG, L. casei M3 and L. plantarum YYC-3 on cell metastasis, and their molecular mechanisms for the human colorectal carcinoma cell lines Caco-2 and HT-29.

2. Results

2.1. The Effect of Cytotoxicity and Growth by Cell Free Supernatants from Lactobacillus Toward Colon Cancer Cells

The effect of Lactobacillus CFS on Caco-2 and HT-29 cells’ growth (Fig. 1) and cytotoxicity (Tables 1 and 2) were evaluated. In both Caco-2 and HT-29 cells, an increasing concentration of Lactobacillus CFS resulted in enhanced cytotoxicity. At a concentration of 800 µl/ml, the cell viability was most inhibited (as high as 100%) in Caco-2 cells treated with CFS of M3 (Table 1). Generally, the IC$_{50}$ value of all treatments group (GG, M3, and YYC-3) were 456.7 µl/ml, 381.1 µl/ml and 403.6 µl/ml respectively. Similarly, HT-29 cells were also inhibited in a dose-dependent manner after treatment with CFSs of GG, M3, and YYC-3, the IC$_{50}$ value of them were 417.1 µl/ml, 516.6 µl/ml, and 211.5 µl/ml respectively. Their inhibitory rates were comparable to those of 2.5 µM 5-flourouracil (positive control) which could inhibit about 50% of HT-29 cells. Treatment with MRS medium showed no significant reduction in the viability of Caco-2 and HT-29 cells.
Table 1
The inhibitory effect of the CFSs of LAB on the growth of Caco2 cells.

| Concentration (µl/ml) | GG   | M3   | YYC-3 |
|----------------------|------|------|-------|
| 200                  | 9.7  | 20.1 | 16.6  |
| 300                  | 25.4 | 36.2 | 32.6  |
| 400                  | 46.2 | 53.7 | 48.2  |
| 500                  | 61.6 | 75.6 | 66.4  |
| 600                  | 77.4 | 84.7 | 88.9  |
| 700                  | 80.7 | 95.1 | 92.4  |
| 800                  | 90.9 | 100  | 97.6  |

IC<sub>50</sub> (mM)

| Concentration (µl/ml) | GG   | M3   | YYC-3 |
|----------------------|------|------|-------|
| 200                  | 456.7| 381.1| 403.6 |
| 300                  |      |      |       |
| 400                  |      |      |       |
| 500                  |      |      |       |
| 600                  |      |      |       |
| 700                  |      |      |       |
| 800                  |      |      |       |

Table 2
The inhibitory effect of the CFSs of LAB on the growth of HT-29 cells.

| Concentration (µl/ml) | GG   | M3   | YYC-3 |
|----------------------|------|------|-------|
| 200                  | 11.5 | 5.7  | 33.2  |
| 300                  | 29.6 | 10.3 | 68.5  |
| 400                  | 52.2 | 22.5 | 74.6  |
| 500                  | 65.1 | 46.2 | 83.7  |
| 600                  | 83.5 | 66.9 | 88.5  |
| 700                  | 90.1 | 82.4 | 96.6  |
| 800                  | 96.1 | 87.7 | 100   |

IC<sub>50</sub> (mM)

| Concentration (µl/ml) | GG   | M3   | YYC-3 |
|----------------------|------|------|-------|
| 200                  | 417.1| 516.6| 211.5 |
| 300                  |      |      |       |
| 400                  |      |      |       |
| 500                  |      |      |       |
| 600                  |      |      |       |
| 700                  |      |      |       |
| 800                  |      |      |       |

Figures 1a and 1b showed the inhibitory effect of CFSs on cell growth for a duration of 72 h. All the treatment groups showed no significant inhibitory effect on cell growth before 24 h. However, after treatment for 48 h, the CFS of YYC-3 significantly (p < 0.05) inhibited HT-29 cell growth at a concentration of 80 µl/ml. However, at same concentration, CFSs of GG and M3 showed no significant inhibitory effect at 48 h; significant inhibitory activities were only observed after treatment for 72 h. Nevertheless, all treatments group significantly inhibited the growth of Caco2 cells at 48 h. Although, comparison among treatment groups showed no significant difference between them.

2.2. Analysis of effective compounds in the CFS

The effective compounds in these three CFSs were analyzed by Q Exactive Focus liquid chromatography-mass spectrometry. Figure 1(C-E) shows identification of four effective compounds, which included oleic acid, cytidine, oleanolic acid, and yohimbine. The molecular weights in the database were 522.36, 243.09, 456.36, and 354.19, respectively. The production of these four compounds was highest in strain YYC-3, followed by GG then M3. Compared with the other three compounds, the output of oleic acid was the highest.
2.3. Analysis of Cell Invasion and Cell Migration

The ability of the Lactobacillus CFS to inhibit the invasion and migration of colon cancer cells was analyzed using Transwell™ assays (Fig. 2). Treatment with CFS limited the ability of Caco-2 and HT-29 cells to invade and traverse through the filter membranes in Fig. 2A, and suppressed cell migration (Fig. 2B, *P < 0.05*). For Caco-2 cells, treatment with YYC-3 CFS resulted in the highest inhibition of cell invasion. Only 40% of the cells (36 ± 2.1 cells) traversed through the filter membranes, significantly fewer than in the untreated group (90 ± 4.3 cells). Caco2 cell migration was also significantly inhibited (*P < 0.001*) when treated with the CFS of strain YYC-3 in Fig. 1B that 34% of cells (56 ± 1.8 cells) had through the filter membranes. Although the other two CFSs also significantly limited cellular invasion and migration, YYC-3-treated cells generally showed the highest inhibition.

Similarly, the highest inhibition of HT-29 cell invasion and migration was observed on treatment with the CFS of strain YYC-3.

2.4. Analysis of Gene Expression

The effects of Lactobacillus CFS on the gene expression of MMP2, MMP9, and VEGFA in colon cancer cells was analyzed using real-time (RT)-PCR (Fig. 3). Compared with negative control, expression of MMP2, MMP9, and VEGFA was significantly decreased in all the CFS-treated groups. In Caco-2 cells, the M3 treatment group had significantly lower MMP2 (~ 0.7-fold) and MMP9 (~ 0.6-fold) gene expression than the negative control group, and other treatment groups had even lower MMP2 and MMP9 gene expression (~ 0.3-fold). In HT-29 cells, the gene expression of MMP2 and MMP9 was significantly lower in all treatment groups compared with the negative control group (~ 0.3 to 0.4-fold). Moreover, treatment with YYC-3 CFS resulted in the
largest reduction in these genes expression, to about 0.3-fold compared with the negative control group.

2.5. Analysis of Protein Expression

Western blotting was used to evaluate the inhibitory ability of Lactobacillus CFS on protein expression of MMP2, MMP9, and VEGFA in colon cancer cells. Treatment with M3 CFS repressed MMP2 and VEGF expression in Caco-2 cells, but did not induce a significant reduction (P > 0.05) in MMP9 protein (Fig. 4a). Treatment of HT-29 cells with M3 CFS, however, did result in a significant decrease in expression of MMP2, MMP9, and VEGFA (Fig. 4b). Treatment with YYC-3 resulted in the greatest inhibition of MMP2, MMP9, and VEGFA expression in both Caco-2 and HT-29 cells.

2.6. Assessment of Protein Secretion

The secretion of MMP2, MMP9, and VEGFA proteins in the YYC-3 treated group were less than those of other groups (Fig. 5A and B). The treatment of Caco2 cell with YYC-3 CFS, showed that MMP2, MMP9, and VEGFA (360.8, 96.9, and 326.4 pg/ml respectively) were significantly lower than the untreated group (P < 0.001). However, in HT-29 cell, YYC-3 CFS treatment resulted in a protein secretion level of 406.1, 109.5, and 367.5 pg/ml in these proteins respectively, also significantly lower than the untreated group (P < 0.001). Furthermore, when YYC-3 treated group was compared with the positive control (5-FU), there was a significant difference (P < 0.05) in MMP2 and VEGFA protein secretion levels; although no significant differences (P > 0.05) in the MMP9 was observed in both Caco2 and HT-29 cells.

2.7. MMP2 and MMP9 Activities by Gelatin Zymography

The activities of MMP2 and MMP9 were test by gelatin zymography. The result was shown in Fig. 5 (C and D). All the treatment group showed the significant inhibitory
activity on MMP2 and MMP9 activity in both Caco2 and HT-29 cells (Fig. 5C, P < 0.05). Among them, YYC-3 treated group showed the highest ability to suppress MMP2 and MMP9 activities, which decreased to 19.1 ± 2.9% (MMP2) and 30.3 ± 1.4% (MMP9) of control in Caco2 cells, respectively. Moreover, for HT-29 cells, YYC-3 downregulated MMP2 and MMP9 activity to 14.2 ± 4.7% (MMP2) and 11.5 ± 3.1% (MMP9) of control, respectively.

3. Discussion

LAB are probiotic microorganisms that have been implicated as anticancer agents, and may inhibit colon cancer cells through direct interactions and/or via the secretion of bacteriocin and other bioactive components [18–20]. However, only few documents had focused on the effect of LAB and their metabolites on colon cancer cell metastasis. In this study, we used the metabolites of three LAB (L. rhamnosus GG, L. casei M3 and L. plantarum YYC-3) to explore the anticancer metastasis and their molecular mechanisms on colon cancer cell lines (Caco-2 and HT-29). We found that the metabolites of these LABs could suppress and metastasis of colon cancer cell by inhibiting the VEGF/MMPs signaling pathway.

Faghfoori et al demonstrated that L. plantarum had cytotoxic effect to HT-29 cells [21] Our results showed that the CFS of YYC-3 had the highest inhibitory activity toward HT-29 cell, with an IC50 value of 211.5 µl/ml. Similarly to the result of Chen et al. [22] who also found that L. plantarum PM153 exhibited an anticancer activity towards HT-29 cell (IC50 value was 299.3 µl/ml) under similar conditions. However, Vemuri et al. [23] found that L. plantarum UALp-05 could not significant influence on HT-29 cells. These results inferred that the inhibitory activity by L. plantarum may be strain-specific.
Cancer-associated mortality is mainly associated with metastasis from the primary tumor to distant organs, such as lung, liver, and brain [24]. In this study, we also demonstrated the inhibitory effect of CFSs by L. casei and L. rhamnosus GG on colon cancer cell invasion and migration using Caco-2 and HT-29 cells. Escamilla et al. [25] reported that the CFSs of L. casei and L. rhamnosus GG posed inhibitory effects on the invasion of HCT-116 colon cancer cells. They also reported that the CFS of L. casei showed higher inhibitory effect on the cell invasion than that of L. rhamnosus GG. Contrary to the report of Escamilla et al., we found that CFS of L. casei M3 reportedly had a lower inhibitory effect on colon cancer cell metastasis when compared with that of L. rhamnosus GG. The variations of our report with that of Escamilla et al could be strain-dependent or cell lines differentiation.

In this study, all treatment groups had significant inhibitory effects on Caco2 and HT-29 cells’ invasion and migration. However, YYC-3-treated group showed the highest ability to inhibit the invasion and migration of the cells, when compared with the other treatment groups. Therefore, it could be inferred that the CFS of YYC-3 may exists the highest effective compounds than the other two stains.

There are many reports on the inhibitory effect of natural products in tumor metastasis. To evaluate the specific compounds implicated in the inhibitory activity, the CFSs of the three strains were analyzed using Q Exactive Focus liquid chromatography-mass spectrometry, and oleic acid, oleanolic acid, yohimbine and cytidine were identified in all the three CFSs. The concentrations of these substances were positively correlated with the anticancer activity demonstrated by the strains. Interestingly, several reports had also proven that oleic acid, oleanolic acid, yohimbine and cytidine posed anticancer activity by the suppression of tumor metastasis [26–29]. Furthermore, natural products containing these compounds had
been reported to inhibit the expression of VEGF. It is an angiogenic factor, and is closely related to organ development, endothelial cell growth, and blood vessel permeability [30]. VEGFA also facilitates the invasion of cancer cells through the VEGFA/VEGFAR pathway acting via p38 mitogen-activated protein kinase MAPK and phosphatidylkinase B (AKT) in order to induce MMP expression, which are widely used to indicate the metastatic capability of colon cancer cells [31]. MMP2 and MMP9 are gelatinases that are able to degrade and remodel ECM [32]; for these reasons MMP2 and MMP9 play important roles in promoting metastasis of tumor cells.

The inhibition of MMP2 and MMP9 could suppress the metastasis of cancer cells. The CFSs of L. rhamnosus GG and L. crispatus had been reported to downregulate MMP2 and MMP9 in HT-29 and HeLa cells, thus indicated anti-metastatsis [33]. Moreover, Norouzi et al. [34] reported that nisin as the product of Streptococcus Lactate could attenuates expression of metastatic genes of MMP2 and CEA to inhibited colon cancer cell metastasis. In this study, we demonstrated that the metabolite secretions of GG, M3 and YYC-3 suppressed VEGFA expression and secretion, and limited the expression and secretion levels of MMP2 and MMP9; thereby provided the evidence that the inhibition of VEGF-MMP signaling pathway could suppress cancer cell metastasis. Similarly, to the result that YYC-3 treatment group exhibited the highest inhibitory activity on the target gene protein expressions and secretions in Caco-2 and HT-29 cells. This result further validated its highest inhibitory activity demonstrated on cell invasion and migration of Caco-2 and HT-29 cells. However, Escamilla et al. [25] showed that the CFS of B. thetaiotaomicron did not exist the inhibitory effect on colon cancer cell invasion.

The pathway at which the CFSs of YYC-3, M3 and GG inhibit colon cancer cell
metastasis are modeled in Fig. 6. The pathway indicated that these CFSs inhibited the expression of VEGFA, thereby limited the VEGFA/VEGFR pathway. It also showed that the expression of MMP2 and MMP9 were suppressed, and resulted in the inhibition of colon cancer metastasis.

4. Conclusions

In this paper, we demonstrate the inhibition of cell growth and antimetastatic activities of the metabolite secretions of L. rhamnosus GG, L. casei M3, and L. plantarum YYC-3 toward colon cancer cell lines. The inhibitory activities of these CFSs were as effective as that of a conventional chemotherapy drug (5-FU). Among these Lactobacillus strains, L. plantarum YYC-3 showed the best inhibitory activities against metastatic of colon cancer cells. These CFSs suppress VEGFA expression, and may exert their anticancer activities through repression of MMP2 and MMP9 downstream of the VEGFA/VEGFR pathway. This study demonstrates that these three Lactobacillus strains may have potential as alternatives to conventional drugs for the treatment of colon cancer. Further research is required to explore the effects of these probiotic bacteria on colon cancer cells, as well as to assess the inhibitory activities of these bacterial cells and their supernatants in vivo.

5. Methods

5.1. Cell Lines and Culture Conditions

Human colon cancer cell lines (HT-29 and Caco-2) were purchased from Procell Life Science & Technology Co., Ltd., China. HT-29 cell was cultured in Dulbecco’s modified Eagle’s medium (DMEM)/high glucose medium (Procell Life Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS) and Caco2
cell was cultured in minimum Eagle’s medium (MEM) with 20% FBS, both of them contains 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml) in sterile conditions at 37 °C in a humidified atmosphere with 5% CO₂. The culture medium was replaced every 2 days.

5.2. Bacterial Culture and Preparation of Cell-Free Supernatant

L. rhamnosus GG and L. casei M3 were obtained from our lab. L. plantarum YYC-3 was isolated from fermented rose juice. All the strains obtained were cultured using 5% inoculum size in 100 ml MRS broth, and then incubated anaerobically at 37 °C without shaking for 24 h. Thereafter, 1 ml of these cultures, containing about 10⁹ colony-forming units/mL of LAB, was centrifuged at 10000 rpm for 10 min and the supernatants were retained. The pH of the supernatants was adjusted to 7.2 using 1 M NaOH, and then the supernatants were passed through 0.22-µm filters (Sartorius Stedim Biotech). The filtrates obtained were used for analysis. 2.5 µM (Selleck, USA) of 5-FU was used as positive control in this manuscript.

5.3. Analysis of Cytotoxicity

Cell Counting Kit-8 (CCK-8) (Solarbio, China) assays were used to evaluate the cytotoxic impact of Lactobacillus CFS on CRC cells. Briefly, 5 × 10⁴ CRC cells were seeded into each well of a 96-well plate in 100 µl medium without FBS. Cells were incubated overnight in normal cell growth conditions to enable cells to adhere to the wells. Then, the medium in each well was replaced, and different concentrations of Lactobacillus CFS (20, 30, 40, 50, 60, 70, 80, and 100% CFS, diluted with complete medium) were added and cultured for 24 h to test cytotoxicity effect. 100 µl complete medium were used as negative controls; 2.5 µM 5-FU was used as a positive control for cytotoxicity. After 24-h incubation, 10 µl of CCK-8 solution were
added to each well and the plates were incubated at 37 °C for an additional 4 h. Cell viability was measured using a spectrophotometer (TECAN, Spark 20M, Switzerland) at 450 nm. Inhibition ratio (%) = [(OD_{untreated} - OD_{treated})/(OD_{untreated})] × 100%.

The analysis was performed in triplicate. The IC_{50} value was estimated by inhibitory rates.

5.4. Analysis of Cell Growth

To analysis the inhibited effect of these CFSs on colon cancer cell growth, CCK-8 was used to carried out. 8 × 10^3 cells were seeded as described above, 10% (v/v) CFSs were added in the treated groups, and cultured for 0, 24, 48, 72 h, respectively, for analysis cell growth. The steps were followed by the manufacturer’s instruction.

5.5. Assay of Cell Invasion and Cell Migration Ability

To evaluate the effect of CFS from Lactobacillus on CRC cell invasion and migration, Transwell™ chamber assays (Corning Incorporated Costar, USA) were used. Briefly, 3 × 10^5 CRC cells were added into the upper chamber of each Transwell insert. For the analysis of cell invasion, the upper chambers had been coated with 200 µl of Matrigel (200 µg/ml) diluted with serum-free DMEM (cell migration didn’t need the step of coat with Matrigel). The lower chamber contained 600 µl DMEM with 10% FBS (HT-29) or MEM with 20% FBS (Caco2). Cells were allowed to invade for 24 h. After that, the upper chamber was washed with phosphate-buffered saline (PBS) and carefully wiped with a cotton swab to remove excess PBS and residual cells from the upper chamber. Cells that had invaded through the Matrigel onto the bottom side of the Transwell insert were soaked in 95% ethanol and stained with crystal violet. Finally, an inverted microscope was used to observe cells that had invaded and
ToupView 3.7 was used as digital camera to take the images. Then, five views’ cell of each well were observed to determine the main values [35]. The analysis was performed in triplicate.

5.6. Analysis of the Chemical Composition of CFS

CFSs were centrifuged using ultrafiltration devices (1-kDa cutoff, PALL Corporation) at 6500 rpm for 50 min at 4 °C. Then, the compounds of the CFSs that < 1 kDa were analyzed by ultra-performance liquid chromatography (UPLC)-Q Exactive Focus-MS/MS (Thermo Scientific Co. Ltd). A C18 column (2.1 × 100 mm, 1.8 µm) was used in UPLC with 0.1% HCOOH (A) and acetonitrile (B) as mobile phase. The flow rate was as 0.3 ml/min, and the gradient was: A/B 95/5 (0 min), 10/90 (15 min), 10/90 (20 min), 95/5 (20.1 min), 95/5 (30 min). Mass spectrometry with an electrospray ionization source was used for metabolite identification. The heated capillary was maintained at 320 °C. The spray voltage was set to 3.8 kV in positive mode and 3.4 kV in negative mode. The sheath and auxiliary gases were at 25 arbs and 8 arbs, respectively. m/z 100 to 1500 was scanned and the three most intense peaks were collided. The compounds were identified using the Compound Discoverer 2.1.0.401 database. Peak area of an identified compound was used for relative quantification.

5.7. Analysis of Gene Expression

The impact of Lactobacillus CFS on the expression of MMP2, MMP9, and VEGF in CRC cells was analyzed using SYBR Green Real-Time PCR. Briefly, CRC cells were plated on a 6-well tissue culture plate and incubated for 4 h with 3 ml CFS from 24-h cultures of strains GG, M3, or YYC-3 (pH adjusted to 7.2 using 1 M NaOH).

Afterwards, cells were washed twice with PBS (pH 7.2). For cell harvest, 1 ml of PBS
was added into each well and the wells were scraped with a cell scraper (Corning Incorporated Costar). Cells were collected into sterile 1.5-ml tubes, and centrifuged at 1800 rpm for 2 min. The tubes and cell pellets were immediately put into liquid nitrogen for quick-freezing. RNA was extracted from each sample according to a previously described method [36]. The quality and quantity of the extracted RNA was evaluated using a NanoDrop® ND-2000 spectrophotometer (Thermos, USA) and 1.5% modified agarose gel electrophoresis. For cDNA synthesis, PrimeScript™ RT reagent kit with gDNA Eraser was used to synthesize cDNA from the mRNA, 20 µl reverse transcription system contains 1 µg RNA. Agarose gel electrophoresis was used to confirm the synthesis of cDNA. The relative gene expression was analyzed by RT-PCR, using GAPDH as a reference gene (primers are listed in Table 3).

According to the manufacturer’s instructions, 2 µl of cDNA was added into an 18-µl reaction mixture containing 10 µM primers and 2 × Master Mix. An ABI 7500 instrument (Applied Biosystems, USA) was used to perform the RT-PCR. The reaction condition was set as 30 seconds at 95 °C for 40 cycles for amplification reaction. After that 10 seconds at 95 °C, 60 °C for 60 seconds, and 95 °C for 15 seconds. Then heating the temperature slowly from 60 °C to 99 °C. Each sample was evaluated in triplicate. Data were analyzed using the $2^{-\Delta\Delta CT}$ method by ABI7500 SD2.3 software.

| Gene name | Forward primer (5’-3’) | Reverse primer (5’-3’) | Product size (bp) |
|-----------|------------------------|------------------------|------------------|
| MMP2      | AATGCCATCCCCCGATAA CCT | CACGCTCTTCAGACTTT GGTTC | 114              |
| MMP9      | GAGCACGGAGACGGGT ATC | ACTCGTCATCGTCGAAA TGG  | 106              |
| VEGF-A    | CAGATTATGCCGATCAA ACCTCACCC | CACAGGGGACGCTCCAG GACTTAT | 190              |
| GAPDH     | CACCCACTCCTCCACCT TTGA | TCTCTTTCCTCTTTGTGC TCTTGC | 188              |

5.8. Analysis of Protein Expression
The impact of Lactobacillus CFS on protein expression of MMP2, MMP9, and VEGF was explored using western blot analysis. CRC cells were treated and collected according to method described in Sect. 4.6. After sample collection, cells were lysed for 20 min in RIPA buffer on ice. Then the lysed cells were centrifuged at 13,000 rpm for 20 min at 4 °C, and the protein supernatants were collected. Protein supernatants were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (Millipore, USA) by a wet transfer method at 300 mA for 120 min. After protein transfer, the membranes were blocked in 5% skim milk-TBST, and then shaken gently at room temperature for 30 min. Primary antibodies (rabbit anti-human IgG, obtained from Abcam, Cambridge, UK), including MMP2 (1:2000 dilution), MMP9 (1:2000), and VEGFA (1:2000) were added to the membranes, then retained for 10 min at room temperature, then at 4 °C. After 24 h, membranes were washed five times using TBST (3 min for each wash). Secondary antibody (goat anti-rabbit IgG, horseradish peroxidase-conjugated) obtained from TDY Biotech Co., Ltd., China was diluted 10000 times with skim milk/TBST. Membranes were incubated with secondary antibody at room temperature for 40 min with gentle shaking. Electrochemical luminescence reagents were added to the membrane, reacted for 3-5 min, and autoradiography film was exposed to the membrane for 10 s to 5 min. Finally, the film was developed for 2 min, and fixed. Protein band intensities were analyzed using Image-J software and the housekeeping protein was β-actin.

5.9. Analysis of Secretion Level by ELISA kit

EliKine™ Human MMP2 and MMP9 ELISA kit (Abbkine), and Human VEGFA ELISA kit (Abcam) were used to analyze the inhibitory ability of Lactobacillus CFSs on the
secretion level of MMP2, MMP9, and VEGFA. In brief, $3 \times 10^5$ cells were cultured in six-well plates and different treatments were added and incubated for 24 h. After that, the cell supernatants were collected from each group, and the contents of these proteins were analyzed according to the manufacturer’s instruction.

5.10. Assessment of MMP-2 and MMP-9 Activities by gelatin zymography

MMP gelatin-zymography electrophoresis analysis kit (Genmed Scientifc Inc, USA) was carried out to assess the inhibitory effect of CFSs on MMP2 and MMP9 activities. The sample was prepared as described by Escamilla et al. [25]. BCA protein assay kit (BioDee BioTech, China) was used to analyze the concentration of the protein. Then, extracellular MMP2 and MMP9 activities were tested according to the manufacturer’s instruction. Their activities were assessed by the quantification of the bands. The bands images were viewed using Clinx Genosens Capture program, and then quantified by ImageJ system.

5.11. Statistical Analysis

All data in this experiment are expressed as mean ± standard deviation of triplicate values. Data were analyzed by Turkey test of One-way analysis of variance (ANOVA) in SPSS 19.0 software. $P$-value less than 0.05 was considered statistically significant.

Declarations

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**Conflicts of Interest:** The authors declare no conflicts of interest, financial or otherwise.

**Data Availability:** The supplement data are availability from the corresponding author if required.

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Figures
Figure 1

Inhibitory effect of CFSs on cell growth and effective compound analysis of CFSs.
Lactobacillus CFSs inhibit Transwell™ invasion and migration of colon cancer cell.
Figure 3

Relative gene expression of metastasis-related genes in colon cancer cells treated with Lactobacillus CFS.
Figure 4

Relative protein expression from metastasis-related genes in colon cancer cells treated with Lactobacillus CF... SD of three independent experiments; different letters mean P < 0.05 by One-way ANOVA in the same group.
Figure 5

Protein secretion of MMP2, MMP9, and VEGFA and activity of MMPs by colon cancer cells treated with Lactobacillus reuteri. Data are expressed as mean ± SD of three independent experiments; different letters mean P < 0.05 by One-way ANOVA in the same group.
Figure 6

Putative mechanism by which metabolite secretions of *L. plantarum* YYC-3 inhibit