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A new anti-colon cancer tumor pathway of Phenyllactic acid by reducing adhesion of *Fusobacterium nucleatum*

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Abstract

The incidence of colorectal cancer (CRC) is increasing, with 1,931,590 new colorectal cancer patients worldwide in 2020. *Fusobacterium nucleatum* (*Fn*) has been widely reported as a harmful microorganism that promotes the development of CRC. *Fn* was found to enhance proliferation and metastasis of CRC cells by regulating cellular glucose metabolism and amino acid metabolism in trials. In a previous study, phenyl lactic acid (PLA), a natural metabolite of various probiotic bacteria, was found to have the ability to inhibit Fn as an organic acid. Therefore, further in-depth analysis of the mechanism of action of PLA in weakening *Fn* invasion into tumor cells provides a new therapeutic avenue for exacerbating CRC problems caused by intestinal pathogenic bacteria.

Keywords: phenyllactic acid; *Fusobacterium nucleatum*; colorectal cancer cell; metabolomics.

Practical Application: Phenyllactic acid (PLA), an edible metabolite of probiotic bacteria, was used to inhibit colon cancer caused by adherence of *Fusobacterium nucleatum*. It not only achieves a high utilization of probiotics but also provides a new research direction for the prevention and adjuvant treatment of colorectal cancer.

1 Introduction

Colon Cancer (CRC) is the third-recognized cancer in the world (Rawla et al., 2019). Its incidence of disease and the mortality rate are extremely high, which is the world's second-largest mortality cancer. Global colorectal cancer deaths increased by 6.27% from 2018 to 2020 (Ferlay et al., 2019). In a Danish study of early-onset CRC in 2020, the proportion of young and middle-aged, and elderly patients was compared, and CRC patients were gradually getting younger (Frostberg & Rahr, 2020).

In recent years, the relationship between microorganisms and disease occurrence and development of diseases has been exposed (Rosenberg et al., 2007). Intestinal microbiota induces human metabolism and immune function through signaling pathways, and receives signals from symbiotic microorganisms or invading pathogens to the intestinal epithelium, initiating and promoting CRC (Song et al., 2020). A large amount of evidence shows that *Fusobacterium nucleatum* (*Fn*) has a symbiotic relationship with CRC (Haruki et al., 2020). *Fn* promotes the proliferation and metastasis of CRC cells, and CRC cell lines infected with *Fn* formed larger tumors in nude mice than uninfected cells (Chen et al., 2020). *Fn* can not only accelerate the growth of tumor size but also accelerate tumor metastasis in mice (Yang et al., 2017). The biofilm produced by *Fn* covers the surface of cancer cells and provides a physical protective layer to resist the erosion of drugs (Thurnheer et al., 2019). It acts as an "immune escape train" during the metastasis of cancer cells and provides a fast path for the spread of cancer cells. The metabolites of *Fn* are pathogenic (Sun et al., 2019a). Butyric acid in metabolites has a destructive effect on oral cells (Shirasugi et al., 2018), and *Fn* produces adhesins to make bacteria adhere to the surface of CRC cells (Meng et al., 2021), which is also the premise for *Fn* to invade host cells. It can regulate E-cadherin/β-catenin signal transduction and promote the development of CRC. In addition, caspase activation and recruitment domain 3 expression were upregulated to activate the IL-17F-NF-κB signaling pathway to promote ulcerative colitis (Janati et al., 2019). Research findings, Biofilms formed by this bacterium are associated with decreased E-cadherin in colon epithelial cells, increased IL-6 and Stat3 activation in epithelial cells, and up-regulation of crypt epithelial cells in normal colon mucosa, and have been confirmed to promote cancer in clinical (Dejea et al., 2014). Therefore, it is urgent to seek a biological prevention and control method to reduce the incidence and mortality of CRC.

Traditional treatments include aspirin and nitrozolazole (Brennan et al., 2021) which are treated CRC drug-resistant by inhibiting the *Fn*, but aspirin damages the gastrointestinal tract, and nitrozolazole causes diarrhea. Probiotic Treatment are attracting attention for their safe, natural and healthy features (Adiyoga et al., 2022; Al-Megrin et al., 2022). Phenyllactic acid (PLA) as a new organic acid metabolized by probiotic bacteria such as *Lactobacillus plantarum* (Sun et al., 2019b), which is a kind of small-molecule organic bacteriostatic substance, PLA has a stable structure and can withstand high temperatures. PLA inhibits a variety of food-borne pathogenic bacteria, as early

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as 1998 Dieuleveux et al. (1998) found that PLA could inhibit the growth of *listeria*, by destroying the cell membrane and cell wall structure of bacteria, a potential difference is created at the cell surface. Since the hydroxyl group of PLA polar structure can bind to bacterial surface protein structure, it stimulates bacterial protein hydrolysis and pore formation, leading to bacterial cytoplasm loss and ATP and K+ imbalance (Liu et al., 2018). In addition, PLA can enter bacteria and affect the RNA synthesis pathway of cells, hinder protein expression and synthesis, and reduce the biofilm formation of spoilage bacteria (Liu et al., 2020). Adding PLA to food to inhibit contamination caused by foodborne spoilage and pathogenic bacteria and their biofilms can extend the shelf life of food, and selecting strains with high PLA production for food production as well as consuming probiotic supplementation can better regulate intestinal flora, improve intestinal immunity, and reduce intestinal diseases caused by pathogenic bacteria in the intestinal tract.

During our previous study, the bacteriostatic properties of hundreds of *lactobacillus* metabolites to *Fn* were analyzed Yue et al. (2020) found that the metabolites of *Lactobacillus Plantarum* had the strongest effect on *Fn* involved CRC, it can inhibit the proliferation of *Fn* by secreting antibacterial metabolites. PLA is one of the metabolic bacteriostatic substances of *Lactobacillus Plantarum*, and its effect on *Fn* is worth exploring. In this study, the proliferation of CRC cells by *Fn* was characterized by microscopy, and metabolites in the extracellular matrix were analyzed by metabonomics. As an antibacterial substance, the toxicity and damage of PLA on cells and cancer cells were analyzed. The minimum concentration of inhibiting *Fn* and the mechanism of action were determined, and the effect of PLA on reducing the adhesion between *Fn* and CRC was analyzed by SEM and TEM, thereby reducing the risk of colon cancer, and promoting intestinal micro ecological balance. To prove that PLA can be applied in the preventive and regulatory of this kind of CRC.

2 Methods

2.1 Bacterial strain and cell culture

Fusobacterium nucleatum ATCC 25586 (ATCC, Manassas,USA) freeze-dried powder, at 37 °C, 80% N_2, 20% CO_2, *Fn* was grown in thioglycolate medium (Hope-bio, Tsingtao, China).

Human colorectal cancer (HT29, HCT116) cells purchased in (Shanghai Cell Bank, China), using Dulbecco's Modified Eagle Medium (Gibco-DMEM) to add 12% Fetal Bovine Serum (FBS) and 50 U/mL penicillin/streptomycin (Gibco), the human normal colon epithelial cells (NCM460) using glutamine-free Gibco RPMI 1640 medium were supplemented plus 10% FBS and 50 U/mL penicillin/Streptomycin/gentamicin (Gibco), at 37 °C, 5% CO_2, 20% O_2, 75% N_2, and seeded in fresh media at 0.5×10^6 cells/mL every 48 hours.

2.2 Co-culture

1×10^7 CRC cells (HT29, HCT116) were inoculated in 48 well plates, each hole was added 0.5 mL DMEM, and cultured in a cell incubator for 24 h to make the adherent firm and grow. 1×10^8 *Fn* was added into CRC, adapted to the growth environment for 4 h, and adhered to the cell surface by inverted microscope.

2.3 Metabolomics analysis

1×10^7 cell culture at DMEM without FBS 24 h, and take the DMEM as control; the 1×10^8 CFU *Fn* adding to the DMEM culture 4 h, 2000 *g* centrifugal 5 min, remove the liquid which is the sample.

The samples were separated by UHPLC (Agilent 1290) chromatography column; The related chromatographic and mass spectrum (MS) conditions were performed similarly to the established method (Chen et al., 2022). The raw metabolomics data were processed by R package (ropls), Chemical Taxonomy, Univariate statistical analysis, and PCA, partial least squares discriminant analysis (PLS-DA), OPLS-DA and Bioinformatics analysis of differential metabolites were conducted for searching the marker compounds between different samples.

2.4 Cell proliferation assay

1×10^4 cells per well in a 96-well plate and grow overnight at 37 °C, add 10% PLA at different mass concentrations, after 48 h, the effects of PLA on HCT116, HT29, and NCM 460 were determined using a Cell Counting-8 Kit (ab228554, Abcam, Shanghai, China) measure absorbance of $OD_{450\ nm}$ (Bio-Rad, Hercules, CA, USA) after 40 min.

2.5 Total superoxide dismutase viability (T-SOD) assay

The CRC cells were cultured in a 6-well plate, added 0, 0.2, 0.4, 0.8, 1.6 mg/mL PLA to the well for 24 h, protein concentration was determined by BCA kit (P0011, Beyotime Biotechnology, Shanghai, China) (Vashist et al., 2011), the Enzyme activity of the cell using Superoxide Dismutase Activity Assay Kit (BC0175, Beijing Solaibao Technology Co., LTD, Beijing, China) according to the manufacturer s protocol. absorbance was determined at 550 nm.

2.6 Observation cell by fluorescent microscope

Cells cultured in 96-well plates were added to chilled 1 mL PBS washed 2 times and treated with 100 mL PBS 2 h, with mass concentrations of 0, 0.5, 1.0 mg/mL PLA 2 h. The damaged cell membranes of PLA were measured by Propidium iodide (PI) (ST511, Beyotime Biotechnology, Shanghai, China) (Gueroui et al., 2002).

2.7 Extracellular exopolysaccharide assay

1×10^4 cells per well in 96-well plates (100 μL volume DMEM), adding 0, 0.2, 0.4, 0.8, 1.6 mg/mL PLA, and crystal violet assay (Schirtzinger et al., 2022) was used to determine extracellular polysaccharide content.

2.8 Minimum inhibitory concentration assay

200 μL *Fn* solution with a concentration of 1×10^6 CFU/mL, the mass concentration of PLA was kept at 0, 0.1, 0.2, 0.3, 0.4, 0.5,

0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL. The absorbance was measured every 2 h. After 24 h by Microplate Reader (HBS-1096A, DeTie, Nanjing, China) (Equation 1):

$$\text{Inhibition ratio} = \frac{OD_X}{OD_0} \quad (1)$$

OD_X: Absorbance at that point in time; OD_0: The absorbance of the 0 groups at this time point.

2.9 Fn membrane was examined by scanning electron microscopy

200 μL *Fn* solution with a concentration of 1×10^6 CFU/mL, the concentration of PLA was kept at 0, 0.5 and 1.0 mg/mL. Using 0.1 mol PBS (pH = 7.2), wash 2 times (15 min), 2000 *g* with 3 min, centrifuge and 1% fixed cells with 80 min, completely black, and washed 2 times with PBS. Dehydration treatment: The treatment time is shown Table 1 below.

After mixing acetone and embedded liquid 1:1, drop into the cell sample, flow slowly along the sidewall, gently stir, and put into the oven for 4 h, to make the cells completely saturated. Add 2 drops of embedded liquid and put it in the oven overnight, after repair and slice for observation by Transmission Electron Microscope (TEM) (Matias et al., 2003).

2.10 Cell count

After digestion, centrifugation, and collection of cells, a fresh complete medium was added to blow cells to single-cell suspension and 0.4% trypan blue solution was added (Barretti et al., 2009). The cell counting plate, and the cover slide were taken, 10 μL cell suspension was slowly added from the side of the counting plate, and the gap between the cover slide and count plate was filled with the cell suspension.

2.11 Minimum inhibitory adhesion concentration assay

The absorbance of the culture medium at 600 nm wavelength was determined, inoculating 1×10^4 cells in a 96-well plate, adding 1×10^5 CFU *Fn*, and the absorbance of the culture medium was determined every 2 h under culture conditions. Time to determine minimum value, add PLA with different concentrations at minimum time points and the absorbance change of culture medium was determined.

Table 1. Control table for dehydration treatment of transmission radio microscope.

Add the substance and their concentration	Time
50% ethanol	8 min
70% ethanol	8 min
90% ethanol	8 min
100% ethanol	10 min
100% ethanol	10 min
100% ethanol + 100% acetone	10 min
100% acetone	6 min

2.12 Sample preparation for Scanning Electron Microscope (SEM)

1×10^7 cells were cultured in the T25 cell culture flasks, adding 1×10^8 CFU *Fn* to the culture for 4 h, and 10% PLA (5 mg/mL) was added to the culture for 2 h. Remove medium solution, adding paraformaldehyde fixator to orifice plate, Hiroki's (Miyazaki et al., 2012) pretreatment method was used to treat the bacteria and observed by SEM and computer.

2.13 Sample preparation for TEM

1×10^7 cells were cultured in the T25 cell culture flasks, with 1×10^8 *Fn* co-culture for 4 h, PLA (0.5 mg/mL) was treated for 2 h, pancreatic enzyme digested into cell suspension, centrifugal (1800 *g*) 5 min, and fixed with polyglutaraldehyde (4 °C). Using 0.1 mol PBS (pH = 7.2), wash 2 times (15 min), centrifuge (2000 *g*) 3 min, and 1% fixed cells with 100 min, The rest are the same as method 2.4.

2.14 Data extraction, synthesis, and statistical analysis

All the tests were performed in triplicates, and the data are expressed as mean ± standard deviation (SD). All data were estimated by single-factor variance analysis of one-way (ANOVA) or Duncan's multiple-range tests by using SPSS 26.0, considered to be statistically significant with $P < 0.05$, and Origin 2018 software to draw a graph.

3 Results

3.1 Observation of co-culture of CRC cells with Fusobacterium nucleatum

Many reports have confirmed that *Fn* exists near and in CRC cells (Su et al., 1992), *Fn* adheres to the cell surface (Engevik et al., 2021), this bacterium can reduce the body's immune regulation (Zundler et al., 2022), and stimulate the proliferation and metastasis of cancer cells (Kostic et al., 2013). *Fn* was added to the culture medium of CRC and observed by light microscopy as in Figure 1a, compared to the control group (A), the HT29 and HCT116 cells grew faster which co-culture with *Fn*, and had more cells in the same area, along with a more pronounced darker shade around the cells. The pro-proliferative effect of *Fn* on NCM460 [Figure 1a(C)] was not significant compared to that of HCT116 and HT29, and the cell proliferation effect on HCT116 was significant. This proves that the addition of *Fn* not only improves the proliferation capacity of CRC but also *Fn* sticks to the cell surface to form a special bacterial layer. Therefore, further exploration of the metabolites of HCT 116 by LC-MS analysis to look for specific substances or metabolic pathways of *Fn* that promote proliferation. Metabolomics has had its biggest clinical impact in the field of oncology, and this has already generated several reviews (Robertson et al., 2011), for the toxicologist, the important point is that a metabolomics approach has the potential to reveal novel biochemical sequelae of toxicant administration that can lead to mechanistic insights and identification of biomarkers of cause and effect.

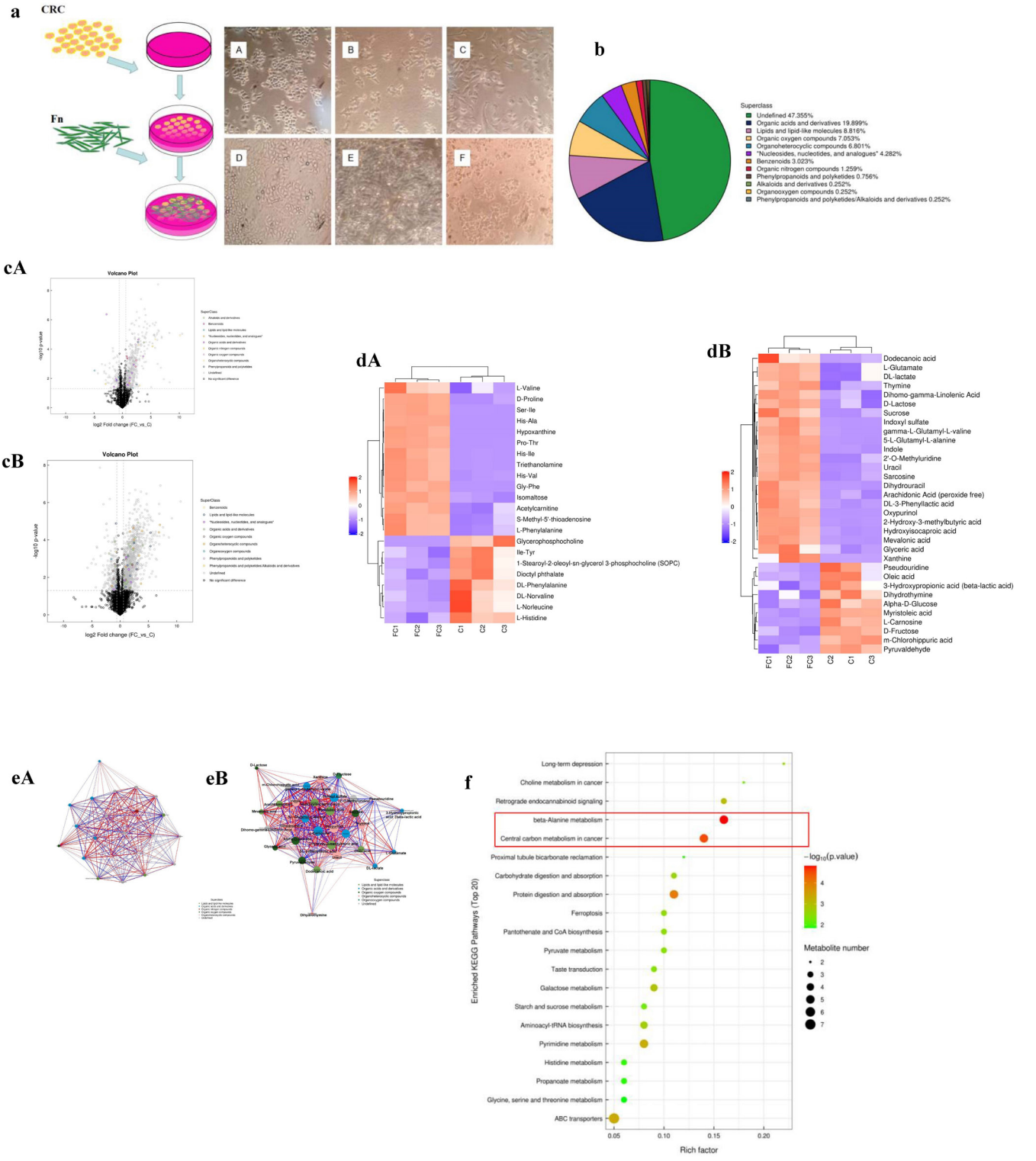

Figure 1. The proliferation of CRC cells can be promoted by Fn adhering to the cell surface. a, The results of Fn and cell co-culture were observed by optical microscope, A HCT116; B HT29; C NCM460; D HCT116+Fn; E HT29+Fn; F NCM460+Fn. b, The proportion of the identified metabolites. c, volcano plot of relative metabolites of HCT116 cells treated by Fn for 24h, compared with that without Fn (n = 3 per group), A: FC vs C Positive ion pattern volcano map; B Negative ion pattern volcano map. d, Hierarchical clustering heat map of metabolites with significant differences in ion patterns, A: Positive ion pattern, B Negative ion pattern. e, Ion-pattern network diagram, A: Positive ion pattern, B Negative ion pattern. f, Enriched KEGG Pathways (the first 20 most significant choices according to P-value), the horizontal coordinate and bubble size indicates the influence factor size of the path, the larger; the color of the bubble indicates the P-value, the darker the color, the more significant the concentration.

3.2 Analysis of Fusobacterium nucleatum on the metabonomics of HCT116 cells

To determine the specific effect of *Fn* on the metabolic pathway to CRC cells, the separated metabolites were classified and analyzed. The quantities of differential metabolites were obtained as shown in Table 2.

The metabolites of *Fn*-added CRC showed significantly different changes in the levels of 55 substances compared to the metabolites of CRC cells. The proportion of various metabolites is as shown in Figure 1b. Organic acids accounted for 19.899% of the total substances and were the most varied metabolites in CRC. The detected types of cell metabolites added with *Fn* become abundant compared to the metabolites of the only cell, and the content of most substances increases, the change of Hypoxanthine increased by about 4 times, and it can inhibit CD8+ of external immune function cells and reduce their immune capacity, thus effectively protecting tumor cells (Tang et al., 2020), Oxypurinol inhibits the conversion of Hypoxanthine into Xanthine by inhibiting the activity of Xanthine oxidase, Hypoxanthine can be phosphorylated by nucleoside phosphorylase or produced by adenine through adenine deaminase or nitrite (Spector, 1988). And low levels of xanthine dehydrogenase trigger an inflammatory response (Pritsos & Gustafson, 1994), weakened Hypoxanthine catabolism, and enhanced anabolism, which are positively associated with cancer cell proliferation.

The results of the metabolic pathway enrichment analysis are shown in the bubble diagram (Figure 1d-1f), the first 20 metabolic pathways, including two pathways to high quantity fit and greater influence, β-Alanine metabolism and Central Carbon metabolism in cancer. By differential analysis that the cell supernatant had 1.888-fold higher Lactate with *Fn* compared with extracellular metabolites without *Fn*, the L-Glutamate was improved by 2.208-fold, the cell metabolism is accelerated, cancer cells consume glucose, produces a large amount of lactic acid (Miro et al., 2021). Similarly, to maintain a high-speed cell operation, the increase in glutamine utilization, in the metabolic process due to the limited number of coenzymes, leads to the accumulation of raw materials in the process, thus higher L-Glutamate content. But overall verified that the proliferation and metabolism of cancer cells is accelerated affected by *Fn*.

3.3 Detection of phenyllactate acid on cell activity

PLA has bacteriostatic properties (Mu et al., 2012) and is expected to be used as a preventive and adjuvant treatment substance for CRC. It must be guaranteed to be non-toxic to the human body. Figure 2a(A)-2a(B) shows that PLA inhibits cell growth of the range of bacteriostasis, PLA at 0.5 mg/mL, HCT 116 and HT 29 cell virulence was (23.42 ± 3.81) % and (24.26 ± 0.71) %, therefore the addition of PLA can't achieve inhibitory the effect of treatment CRC. Similarly, we added PLA to colon epithelial cells NCM460 to analyze the effect of the substance on normal cells. From Figure 2a(C), the effect of PLA on NCM460 cells was not significant ($p > 0.05$). Although the proliferation effect of NCM 460 was weakened with the increase of PLA concentration, the concentration of PLA far exceeded the minimum inhibitory concentration. To reflects the antioxidant capacity and characterize the physiological activity values of cells by determining the effect of PLA on cell SOD viability. Show in Figure 2b, PLA had no significant effect on the free radical scavenging capacity of cells ($p > 0.05$).

Table 2. Number of metabolic variations.

Contrast the group	Different number of metabolites
Total metabolic identification quantity	397
(FC vs Fn) *Fusobacterium nucleatum* invasion cell vs *Fusobacterium nucleatum*	97
(Fn vs C) *Fusobacterium nucleatum* vs cells	88
(FC vs C) *Fusobacterium nucleatum* infecting cell vs cells	55

From Figure 2c *Fn* adhering to the cells was affected by PLA and its membrane permeability was not sufficient to resist the PI dye, producing red fluorescence, the fluorescent blobs reveal the bacterial form, while the cell membrane of the CRC cells could block the entry of the PI dye, and no fluorescence was produced in the colorectal cells. This suggests that PLA can disrupt the membrane structure of *Fn* and alter permeability, but does not affect the integrity of the cell membrane of colon cancer cells. Compared with untreated cells, PLA also did not affect the extracellular polysaccharide of HCT116 and HT29 cells (Figure 2d) and showed a slightly increasing trend for HT29 cells, but the difference was not significant. In conclusion, PLA is safe and reliable, has no effect on the activity of colon epithelial cells, and cannot inhibit CRC cells.

3.4 Minimum inhibitory concentration and mechanism of PLA to Fn

PLA inhibits *Fn* growth in culture. We adjusted the pH by $NaHCO_3$ after PLA addition to matching the control medium to avoid conflating pH-based responses. Determine the logarithmic growth period of *Fn*, under the culture conditions in 0 mg/mL PLA (Figure 3a), 0.5 mg/mL PLA make *Fn* growth mildly reduced, as determined by maximum optical density (optical density at 600 nm [OD600]) because OD_{600} is a proxy for bacterial abundance (Beal et al., 2020). However, higher levels of PLA slowed or entirely inhibited *Fn.* PLA more than 0.5 mg/mL, we observed decreased growth yields, these data demonstrate that PLA can inhibit *Fn* growth in culture.

The *Fn* internal structure by transmission electron microscope (TEM), the bacterial edges of normal *Fn* are relatively clear [Figure 3b(A)], the cell wall is more complete, the center is a uniform solid body, and there is a relatively light extracellular protein layer around it, the mucin composition almost no impurities. After treatment PLA with a concentration of 0.5 mg/mL [Figure 3b(B)], the extracellular protein layer with *Fn* was destroyed, there are white gaps or damage in the bacteria and more surrounding impurities. While treated with 1.0 mg/mL PLA [Figure 3b(C)], the cell walls and membranes were damaged, there are obvious vesicular structures on the surface of the thallus, severe bacterial contents are completely detached from the shell, which shows that PLA does affect the cell membranes and walls of *Fn*, these results are consistent with those under the fluorescence microscope.

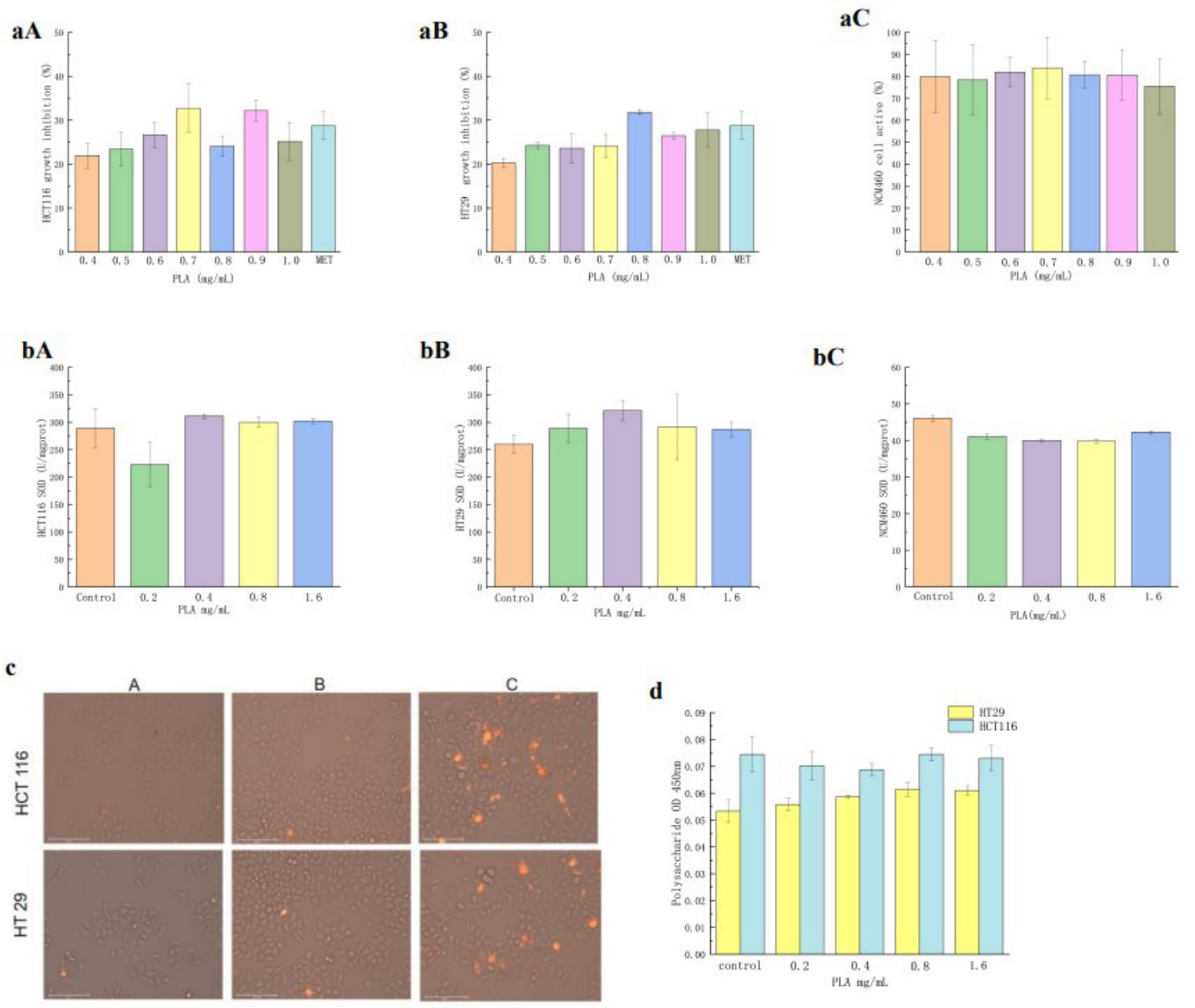

Figure 2. PLA had little effect on cell activity. a, Determination of cell proliferation by PLA, A: HCT116, B: HT29, C: NCM460 (n = 6); b, Effect of PLA on cell SOD activity, A: HCT116, B: HT29, C: NCM460 (n = 6); c, Fluorescent image of CRC cells with Fn, A: 0 mg/mL PLA, B: 0.5 mg/mL PLA, C: 1.0 mg/mL PLA (n = 3); d, Effect of PLA on the extracellular polysaccharide content (n = 6), *p < 0.05.

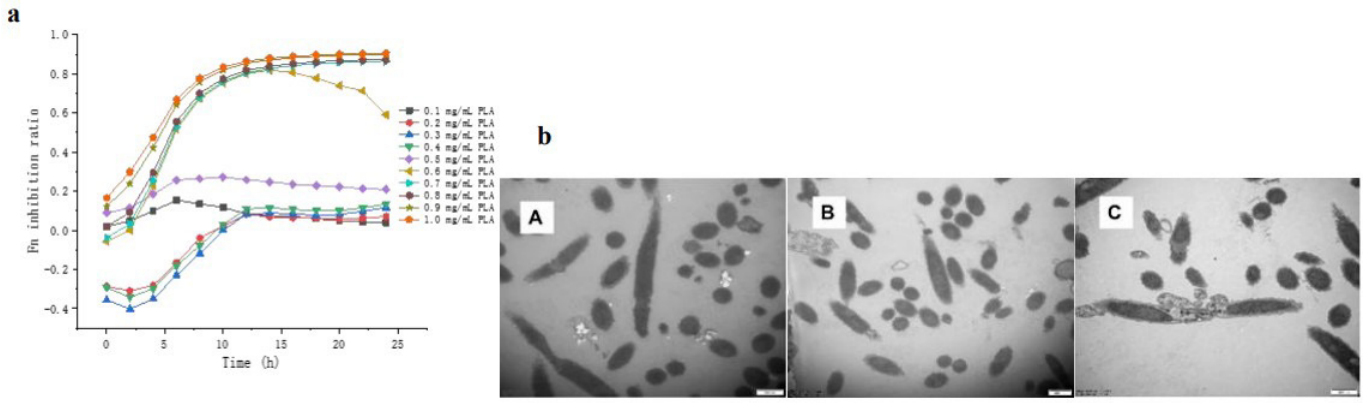

Figure 3. PLA destroys the membrane structure of Fn. a, Effects of phenyllactic acid on Fn inhibition ration (n = 6); b, Effects of phenyllactic acid on Fn membrane by TEM, A: 0 mg/mL PLA, B: 0.5 mg/mL PLA, C: 1.0 mg/mL PLA (n = 3).

3.5 Effects of phenyllactic acid and Fn on cell number

To further assessed the role of Fn and PLA invasion and colonization on cells, and measured the exact number of cells, and analyzed the changes in the number of living cells after exposure to these two (Figure 4b). We count colon cancer cells after 24 h and 48 h. The number of HCT116 cells (48 h) is $(3.9 \pm 0.09) \times 10^4$/mL in normal culture as shown in Figure 4b(A), after the addition of *Fn*, the number of cells increased significantly ($p < 0.05$) and reach $(5.6 \pm 0.12) \times 10^4$/ mL, however, there was no significant ($P > 0.05$) difference between the number of PLA groups and Control groups, even less than Control groups an average of 1×10^3 cells/ mL. The number of HT29 cells (48 h) was $(4.8 \pm 0.148) \times 10^4$ cells/mL [Figure 4b(B)], the number of *Fn* groups reached $(7.2 \pm 0.095) \times 10^4$ cells/mL. As with HCT 116 manifestation. The results showed that adding PLA could control the *Fn* promotion of CRC.

3.6 Minimum concentration determination of PLA against Fn adhesion CRC

From Figure 4c(A), in HCT 116 cells, when the mass concentration of PLA was 0 mg/mL, the absorbance increased, which was due to the *Fn* still growing, so the absorbance was slightly higher. When the concentration of PLA was 0.4 mg/mL, PLA inhibited the growth of *Fn*, the absorbance is minimal. when the concentration of PLA was 0.5 mg/mL, destroyed the condition of adhesion of *Fn* to the cell surface, and it appeared that *Fn* fell off from the cell surface. In HT29 cells [Figure 4c(B)], at concentrations of PLA greater than 0.4 mg/mL, the OD value gradually increased with increasing concentrations, and the *Fn* adhered to the CRC surface was shed, and mass concentration of PLA greater than 0.6 mg/mL resulted in significant shedding of surface-adherent *Fn*. In conclusion, 0.5 mg/mL PLA can detach *Fn* from the cell surface.

3.7 PLA on adhesion of Fn to the cell surface by SEM

A single optical microscope can directly observe the shape of *Fn* and CRC cells (Figure 1a), but the shedding of surface *Fn* could not be directly seen. For this reason, *Fn* adhesion on the cell surface was observed by SEM (Figure 4d). The observation was magnified according to SEM 5 K, and HCT116 cells had abundant cilia and uniform cell morphology. HCT116 cells in Figure 4d(B) were minimally affected by PLA. After co-culture of HCT116 and *Fn*, a large number of *Fn* adhered to the cell cilia, and some even penetrated the cell [Figure 4d(C)]. Figure 4d(D) shows HCT116 cells treat result 0.5 mg/mL PLA after cultured with *Fn*, *Fn* not only dropped off but also reduced surface cilia and increased pores. Figure 4d(d) shows HT29 cells with slightly finer cell morphology. Figure 4d(c) shows the co-culture of HT29 and *Fn*, *Fn* adhered to the cell surface and completely covered HT29 cells. In Figure 4d(d), *Fn* was visibly abscised. Therefore, according to SEM, we know that PLA can reduce the adhesion of *Fn* on the cell surface to help to inhibit the growth of cancer cells.

3.8 PLA on adhesion of Fn to the cell surface by TEM

The cell morphology and damage degree were observed by TEM. As shown in Figure 4e(A), HCT116 cells had complete structure and a clear nuclear membrane. With the addition of Fn [Figure 4e(C)], the different shapes of vesicles exist in CRC, the number of mitochondria increases, and it can also clearly distinguish the morphology of bacteria or cells, half of the bacteria in the packaged state of the cells. After CRC was co-culture with 0.5 mg/mL PLA, *Fn* was present, but it was not attached to the cell, and *Fn* remained in bacterial form. In addition, a large number of cytoplasmic vacuoles are produced in CRC cells and similar mitochondrial vacuolar damage. In other words, *Fn* can embed CRC and affect cell metabolism, and PLA can eliminate this effect, under the synergistic effect of *Fn* and PLA, the degree of cell damage was increased. Similarly, *Fn* added into HT29 cell culture medium [Figure 4e(A-d)], PLA prevents *Fn* from adhering to the CRC surface or removing binding bacteria on the cell surface. The above results are consistent with SEM.

4 Discussion

Many reports have confirmed that *Fn* exists near and in CRC cells, and confirmed *Fn*'s effect on CRC proliferation (Repass et al., 2016; Zhang et al., 2022). They only explain the correlation between the two but do not explain the binding state of bacteria and cells. In this experiment, the promoting effect was verified and adhesion was observed, it was found that *Fn* was not simply attached to the surface of tumor cells, but adhesion to the cells, making *Fn* more firmly attached to the cell surface.

Fn promotes the proliferation of the CRC cells and changes the metabolism. The analysis of the metabolic pathway changes provides strong evidence for exploring the mechanism of *Fn* pro-cancer. Studies have shown that *Fn* contributes to the occurrence, development, and metastasis of colorectal cancer by accelerating host glucose metabolism. From the perspective of the glucose metabolism pathway, SIRT5 protein can regulate the metabolic process through erythrocyte glucose-6-phosphate dehydrogenase, and then affect the cell proliferation rate (Liu & Zhang, 2021). This study found that *Fn* can affect nearly one hundred metabolites of colorectal cancer cells, in the process of purine metabolism, 1-pyrophosphate-5-phosphate and glutamine, produce hypoxanthine nucleotide, can be used as raw material for AMP, and when hypoxanthine nucleotide decomposition, namely hypoxanthine, xanthine and uric acid, the oxopurinol by inhibiting the activity of xanthine oxidase, prevent hypoxanthine to xanthine, on the one hand, the substances improve reduces the immunity, on the other, in the division of cancer cells, need a lot of energy and nucleic acid, make the content of cancer hypoxanthine urine, through this experiment found that *Fn* can improve CRC cells synthesis nucleic acid speed, and speed up the cancer cell proliferation rate. In addition, increasing levels of uracil in metabolic substances, However, the increased content of uracil in colorectal cancer cells is beneficial to improve the pro-cancer effect of *Fn* thus affecting the progression of DNA synthesis in colorectal cancer (Xie et al., 2017). It is confirmed that the intervention of the substance metabolic process is an effective way for the treatment of bacterial cancer.

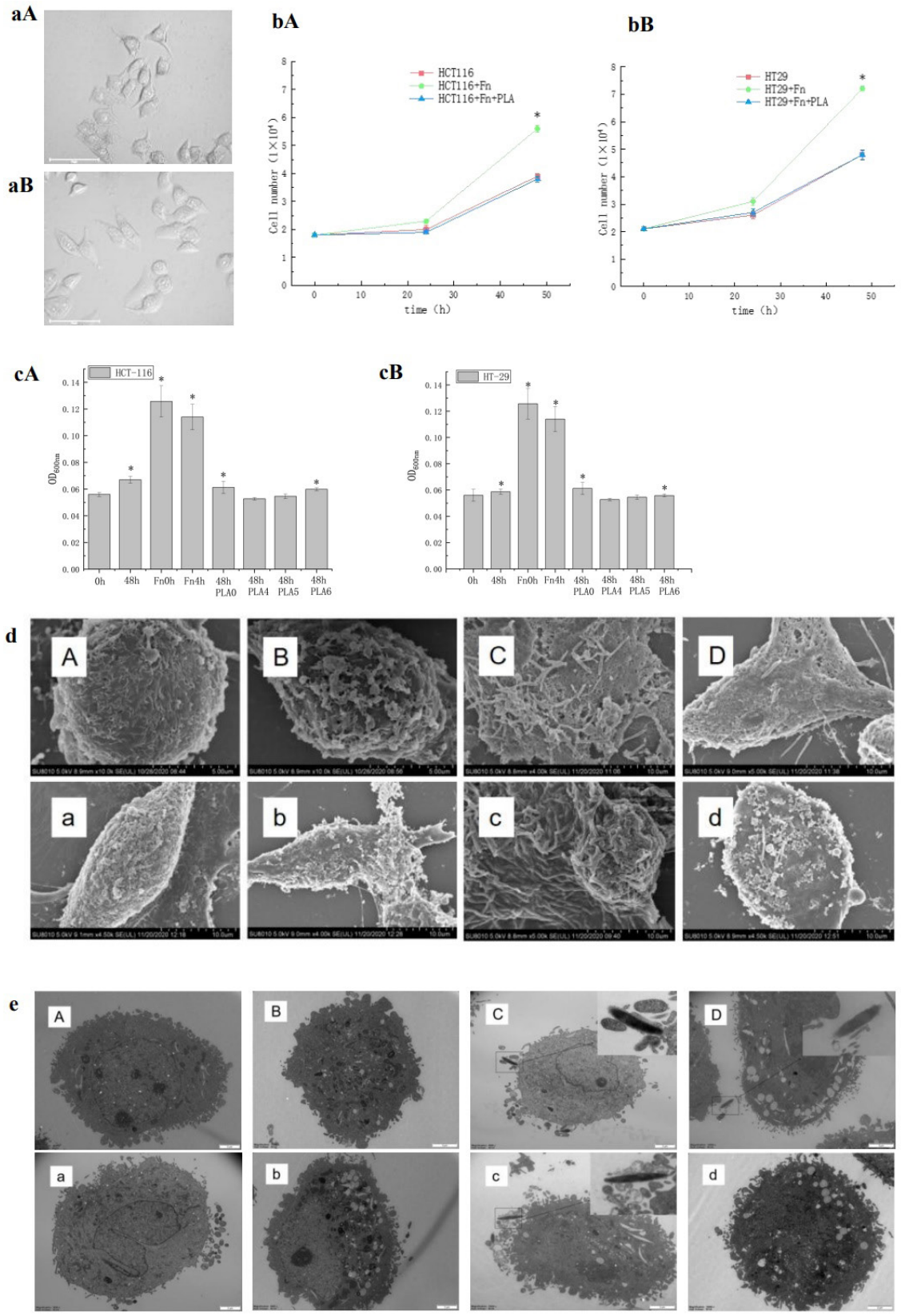

Figure 4. PLA reduced the proliferative ability of Fn against CRC. a, PLA, Fn, and CRC cells under an optical microscope, A: The HCT116, B: The HT29 (n = 3); b, PLA reduced the Fn promoting CRC co-incubated cells number, (n = 6) *p < 0.05; c, Effect of PLA on the adhesion of Fn on the surface of CRC, absorbance value of supernatant at each time point (OD600 nm, n = 6); d, The PLA removes the Fn from the CRC surface by SEM; e, The PLA removes the Fn fromthe CRC surface by TEM, A: HCT116, B: HCT116 + PLA, C: HCT116 + Fn, D: HCT116 + Fn + PLA, a: HT29, b: HT29 + PLA, c: HT29 + Fn, d: HT29 + Fn + PLA (n = 3).

Antibacterial substances of biological sources that inhibit harmful bacteria are good methods (Mao et al., 2020). PLA is a natural organic acid, which has the advantages of being stable, non-toxic and harmless to human body, with anti-bacterial and antioxidant effects (Ren et al., 2021). 《Nature》 points out that breast milk contains a certain amount of aromatic lactic acid, probiotics in the infant's intestines, especially the aromatic lactic acid dehydrogenase (ALDH) of *Bifidobacterium* can produce aromatic lactic acid such PLA, improve the immune capacity of infants (Laursen et al., 2021). It not only inhibits food spoilage and pathogenicity caused by foodborne spoilage bacteria, including Staphylococcus aureus and Listeria monocytogenes, but also inhibits intestinal pathogenic bacteria Such as Enterococcus faecalis, Clostridium perfringens and other pathogenic bacteria form biofilms (Liu et al., 2020). In this study, the effects of PLA on cell proliferation, SOD enzyme activity, and extracellular polysaccharide were measured respectively, and it was confirmed that PLA is not toxic to healthy cells and that it inhibits the development of colon cancer not by killing CRC cells, but by inhibiting the proliferation and metastasis of colon cancer tumors caused by pathogenic bacteria in the intestine. Therefore, determined the minimum bacteriostatic concentration of the PLA to *Fn*, and explored the way that the PLA inhibits the *Fn*. The high-quality concentration of the PLA can destroy the cell membrane structure of *Fn*, which can prevent the flow of material in the cells, and inhibit the growth of *Fn*.

Intestinal microbiota contributes to the tumor progression in various tumor systems, the biological significance of intratumor microbiota remains largely unknown. Many reports have confirmed that Fn can exist near and inside colorectal cancer cells (Su et al., 1992), There is a lack of proper tools to distinguish the function of tumor-resident microbiota (Fu et al., 2022). Despite studies showing that drinking water can efficiently eliminate tumor microbiota 10^2-fold (Iida et al., 2013), but is not the solution. Dejea et al. (2014) found that the biofilm formed by this bacterium was related to the decrease of E-cadherin in colon epithelial cells, the enhancement of IL-6 and Stat3 activation in epithelial cells, and the up-regulation of crypt epithelial cell proliferation in colon mucosa (Tomkovich et al., 2019). Fn's Fap2 can recognize Gal-Gal NAc, which can be used as signal transduction to resist foreign invasion. The O-type sugar chain of CRC can improve the adhesion ability of bacteria and promote the adhesion of Fn. Fn can up-regulate Angiopoietin Like 4, thereby promoting sugar uptake in CRC cells and regulating the effect of colonization (Zheng et al., 2021). The above authors only explain the correlation between the two but do not explain the binding state of bacteria and cells. the effect of phenyllactic acid on Fn was observed by SEM. It was found that PLA could destroy the cell wall and membrane of Fn, resulting in the leakage of the contents inside, and the morphology of the bacteria was changed, with irregular protrusions on the surface. After section treatment, the protrusion structure was generated by osmotic pressure on the membrane, PLA will make Fn dissociate from the biofilm, resulting in the reduction of Fn quantity per unit area of the biofilm. We found that after the treatment of PLA, the *Fn* that enters the cells shed. Under the condition of minimal inhibitory concentration, PLA could make *Fn* lose its proliferation activity, and then reduce its ability to adhere to cells, the results showed that PLA inhibits the physiological effects of *Fn*, passivation has the "adhesion" effect of *Fn*, and reduce the proliferation effect of CRC induced by *Fn*. Further studies by inhibiting the mechanism of bacterial-host cell interactions will help provide new ideas for drug targeting actions. In recent years, more and more studies have confirmed that edible probiotics and their metabolites are widely used for food preservation and regulation of human health and immunity (Pato et al., 2022; Shi et al., 2022). Therefore, in the food industry, it is more highlighted that the selection and breeding of high-yielding PLA strains, metabolic regulation, and the mechanism of their inhibition of spoilage and pathogenic bacteria still need more in-depth research, so that the application of PLA is more extensive.

5 Conclusion

Based on the concept of "beneficial bacteria and inhibitory bacteria", phenyllactic acid, a metabolite of probiotics, was used to inhibit *Fn* and regulate colorectal cancer. It was found for the first time that *Fn* could embed (penetrate) colorectal cancer cells. PLA suppresses the further proliferation of colorectal cancer by *Fn*, and also reduced the *Fn* originally "tied" into the colorectal cell surface. To explore the prevention and adjuvant treatment of colorectal cancer to provide a new direction of research.

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