# Propionate-producing Veillonella parvula regulates the malignant properties of tumor cells of OSCC

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# Abstract

Oral squamous cell carcinoma(OSCC) remains a global health concern with high mortality and morality around the world. Emerging evidence implicates the abnormal abundance and species of oral microbiota are associated with the development of OSCC. *Veillonella parvula* is an anaerobic Gram-negative coccus and the resident member of the normal oral microbial community. In our study, 16S rDNA (V4) amplicon sequencing of salivary microbiome genome revealed that OSCC patients with a high expression of trop2 showed a dysbacteriosis and characterized with a significant decreased distribution of *veillonella parvula*. In vitro study, *Veillonella parvula* treatment promoted the apoptosis inhibited the proliferation and invasion ability of TROP2-high expressed head and neck sauamous cell carcinema HN6 cells compared with the human oral keratinocyte (HOK) cells through its metabolite products rather than the bacteria cell itself. Further, Propionate the main metabolite of *Veillonella parvula* also plays the similar anti cancer role through inhibiting the activation of TROP2 related PI3K/Akt pathway. These results suggest that propionate, the SCFA metabolite of *Veillonella parvula* induces cell apoptosis, inhibits cell proliferation and invasion through reducing the activation of TROP2/PI3K/AKt pathway in OSCC cells.


# Introduction

Oral squamous cell carcinoma (OSCC) is one of the main head and neck squamous cell carcinomas (HNSCCs), with an roughly 300,000 new cases and 140,000 OSCC-related deaths annually, ranking eighth among human malignancies as the considerable reasons cancer-associated mortality worldwide[1, 2]. Despite recent advances in diagnosis and therapy, the overall 5-year survival rate of OSCC patients remains lower and less than 60% in cases involving early detection and proper treatment[3, 4]. Furthermore, OSCC often causes dysfunctions in chewing and swallowing, as well as speech and esthetic disorders, which can worsen patients' quality of life[5]. Therefore, it is urgent to identify the mechanism and explore a new therapeutic target of OSCC.

The human trophoblast cell surface (TACSTD2/TROP2/M1S1/GA733-1) is a surface glycoprotein with a single transmembrane extracellular domain and a short tail. It is overexpressed in many human cancers, including ovarian[6, 7], gastric[8, 9], colorectal[10, 11], pancreatic[12] and laryngeal cancers[13]. TROP2 contributes to carcinogenesis properties by regulating cancer cell adhesion, invasion, migration and vascularization through multiple pathways[14]. Our previous studies suggested that the overexpression of TROP2 modulates the growth and metastasis of OSCC cells by activating PI3K/Akt signaling[1].

Bacterial cells known as the microbiome at certain sites of the human body have been believed to be involved in disease formation and health maintenance. In recent years, since the development of high-throughput next-generation sequencing (NGS) technology and the completion of the Human Microbiome Project[15], a number of microbiota species related to gastrointestinal diseases[16], cancers[17], neurogenerative diseases[18], and other related health problems have been identified[19]. To date, studies have employed high-throughput sequencing technology to detect the relationship between microbiome dysbiosis and oral cancers. Mager *et al*. reported an elevation of *Capnocytophaga*, Provatella

melaningtoenica and Streptococcus mitis in the saliva of OSCC patients[20]. Recently, Zhao et al. demonstrated that *Fusobacterium, Dialister, Peptostreptococcus, Filifactor, Peptococcus, Catonella*, and *Parvimonas* were enriched in OSCC lesions[21]. Börnigen et al. identified a specific loss of community diversity in oral cancer samples[22]. Large-scale metagenomic analysis from Granato *et al.* demonstrated that the relative abundances of Centipeda, Veillonella, and Gemella are correlated with tumor size among OSCC patients[23]. Although numerous bacterial species have been identified to play crucial roles in the occurrence and development of OSCC, the regulatory mechanism is still unclear[24].

Short-chain fatty acids (SCFAs), principally including acetate, propionate and butyrate, have fewer than six carbons produced from carbohydrates by the microbiome and contribute essential functions to the expression and activation of host proteins. Among them, propionate is metabolized by *Veillonellae* from lactate or succinate, acts as an agonist of G protein-coupled receptor 41 (GPR41) and GPR43, influences insulin-induced glucose uptake[25], and inhibits the proliferation of liver cancer and colon cancer cells[26, 27]. In addition, propionate also suppresses invasion by activating large tumor suppressor kinase 1 and inhibiting extracellular signal-related kinase 1/2 in breast cancer cells[28, 29]. However, the functional repertoire of propionate contributing to human OSCC remains largely unexplored. Since the molecular mechanism of the TROP2/PI3K/Akt pathway was identified in our previous study[1, 30], we herein explore the interaction between the oral microbiota and host TROP2-related pathway activity in the development of OSCC.

## Materials And Methods

## DNA Extraction and 16S rDNA Gene Amplicon Sequencing

No antibiotic drugs were used in participants within one month, 2ml of saliva samples from each participants were collected and placed in a sterile tube and then stored at -80 C for future use. Genomic DNAs were extracted using the Magnetic Swab DNA Extraction kit (WH0117, Apronal Inc.) according to the manufacture's guidelines. The integrity and size of DNA samples were confirmed by 1.0% agarose gel electrophoresis and the concentrations of DNA samples were measured by the NanoDrop spectrophotometer(Nano Drop, Germany). 16S rDNA gene amplicon sequencing libraries of the V4 region were prepared using the primers 319F: 5'-ACTCCTACGGGAGGCAGCAG-3', and 806R: 5'-GGACTACHVGGGTWTCTAAT-3', the amplicons were quantified and the pools were sequenced on the MiSeq PE300 sequencing instrument (Illumina, USA) by 2x300bp chemistry according to the manufacture's guidelines.

## 16S rDNA Sequencing Data Analysis

Reads of end-matched were assigned to each samples based on the unique barcodes and then truncated by cutting off the barcode and primer sequences. Then modified reads were merged by the tool of Fast Length Adjustment of SHort reads(FLASH). Sequences were assigned to the same operational taxonomic units (OTUs) with a similarity threshold ≥ 97% using the CD-HIT online tool(V.4.6.1). Abundance of OTUs

were normalized using the PyNAST and Mothur softwares and α- and β- diversity were evaluated by quantitative insights into microbial ecology(QIIME) and principal coordinate's analysis (PCoA).The Wilcoxon rank-sum test and Welch's t-test were calculated to analyze bacterial abundance and diversity while the heatmaps were drawn based on the nonparametric Wilcox test ($p < 0.05$, $q < 0.1$) at the genus level.

# Immunohistochemical Staining

Paraffin sections (4-μm ) from participants were soaked in alcohol solutions according to the gradient of 95,85 and 75%; 5 min per solution to realize dewaxing and rehydration. 3% hydrogen peroxide was added to inhibit the activation of endogenous peroxidase. Antigen retrieval was performed by sodium citrate acid buffer at 95˚C, 10 min. Anti-TROP2(1;200, AF650; R&D Systems, Inc.) was incubated with specimens overnight at 4°C. The peroxidase (dako; Agilent Technologies, Inc.) was used to detect the reactions and 3,3'-diaminobenzidine etrahydrochloride with hematoxylin were used as the counterstain. Slides stained with TROP2 were scanned under power magnification (x200) to identify the five areas with the highest TROP2 density and then imaged at a magnification of x400. The total cells and TROP2 positive cells were counted in each field respectively. The experiments were performed separately by two experienced researchers with a double-blind manner. The percentage of positive cells in each field (0-100%) was calculated and compared. All experiments were prepared in accordance with the national guidelines approved by the Medical Ethics committee of children's Hospital of Nanjing Medical University (February 24, 2022) and informed consent was given by all participants.

**Isolation of** Veillonella Parvula **from the Saliva Samples**

10ul of saliva samples were diluted in 10ml sterilized water and inoculated on the *Veillonella* Medium Base (pancreatic digest of casein 5g/L, yeast extract 3.0g/L, sodium thioglycolatem 0.75g/L, basic fuchsin 0.002g/L, agar 15g/L, 1.2% sodium lactate solution, 0.1% vancomycin PH7.5) cultured at 37℃ in a anaerobic bag for 16–18 hours. Colony Forming Unites(CFU) of each plates were counted and 30 colones of each plates were cultured in *Veillonella* liquid medium. The genomic DNA was extracted and the full 16SrRNA sequencing was amplified with using primers 27F:AGAGTTTGATCCTGGCTCAG and 1429R:GGTTACC TTGTTACGACTT, sequences were matched in NCBI database by Basic Local Alignment Search Tool (BLAST)to identified the species of bacteria colones. To confirm, primers of *Veillonella parvula* were designed according to the sequences and real time PCR assay was performed in DNA samples.

# Cell Culture

The OSCC cell lines HN6 and normal oral epithelial cells (HOK)were obtained from Children’s Hospital of Nanjing Medical University. HOK and HN6 cell lines were maintained in DMEM medium(Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% FBS(Invitrogen; Thermo Fisher Scientific, Inc.), penicillin (100 IU/ml) and streptomycin (100 IU/ml) at 37℃ in a humidified,5% CO 2 atmosphere.

# Cell Viability Assay

Cells were seeded into a 96-well plate with a density of $2x10^3$ cells per well, the *Veillonella parvula* cells and heat inactivated *Veillonella parvula* cells (0.05mg/ml) which were washed for 3 times with PBS, and same dilution-culture supernatant were co-cultured with cells for 2hrs then replaced by fresh medium for another 24 hrs. In the propionate treatment group, cells were co cultured with propionate for 24hrs. Cell proliferation assays were performed using a CCK-8 assay (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Generally speaking, 10ul of CCK8 were added into each well after the 4h or 24h culture time, and incubated for 4 hours before absorbance was recorded at 450 nm using an GEN5 Reader(BioTek Instruments, Inc.).

# Invasion Assays

For the transwell Invasion assay, $1x10^4$ HN6 or HOK cells were plated in the top chamber with an matrigel (1:15, 3D cell culture hydrogel kit; Biozellen) coated membrane (8 μm, 24-well plate, BD Biosciences). *Veillonella parvula* NCTC11810 cells, heat inactivated *Veillonella parvula* NCTC11810 cells, culture supernatant and 10mM of sodium propionate solution were used to treat the cells. Cells plated in the top chamber in serum-free media were incubated with *Veillonella parvula* NCTC11810 cells, heat inactivated *Veillonella parvula* NCTC11810 cells and culture supernatant for 2 h and 24 h with sodium propionate respectively. Cells in the upper chamber were removed with a cotton swab slightly while cells on the lower surface of the membrane were stained with 0.1% crystal violet for 30 min at room temperature and counted under the optical microscope.

**Flow cytometry analysis of apoptosis.**

$1x10^6$ /ml cells were harvested with trypsin-EDTA free solution, washed for 2 times with PBS and incubated for 5 min in 500μl binding buffer, 5ul of Annexin V-FITC and Annexin V-PI staining were added according to the setting of tubes( FITC positive tube, PI positive tube, FITC-PI double negative tube and target samples ) respectively, and incubated for 15 min at R.T. in the light avoided environment. Apoptosis analysis was assessed using CytoFLEX system (Beckman Coulter, Inc.).

# Western Blotting Analysis

Antibodies including: Anti-TROP2 (1:1000; AF650; R&D), anti-Akt (1:10,000;ab179463; Abcam), anti-p-Akt(1:1000; AA329; Beyotime), anti-PI3Kp85 (1:1,000; T40115s; CST), anti-P-PI3Kp85 (1:1,000;4228s; CST), anti-cleaved gasderminD(1:1000; 36425;CST),anti-β-tubulin(1:1000;2146;CST) and anti-GAPDH (1:5,000; ab181602; Abcam). Membranes were incubated with the antibodies(diluted in 5% skim milk) over night at 4 ℃. Membranes were Washed with PBST for three times and incubated with secondary antibodies at room temperature for 2 hours. The signals were visualized and quantiated using an enhanced chemiluminescence system (GE Healthcare) and densitometry analysis was performed usingenhanced chemiluminescence system (Tanon, China). Each experiment was repeated at least three times.

# Statistical Analysis

GraphPad (Prism 5.0) was used for statistical analysis. Comparisons between groups were calculated using unpaired t-test and data were reported as the mean ± SEM. $P < 0.05$ was considered to indicate a statistically significant difference.

# Results

1. **The structural composition of saliva microbiota was altered in patients with high TROP2 expression and**

Saliva samples from 11 subjects, including 5 patients diagnosed with OSCC and 6 volunteer subjects, were collected from February to April 2022 from Nanjing Stomatological Hospital. The mean age of the total cohort was 56.4 years and comprised 50% men. Based on the diagnostic results, TROP2 expression in oral tissues of OSCC patients and volunteers was detected by immunohistochemistry staining, and the expression was 56.70% in OSCC tissues, which was higher than that in normal oral tissues (16.45%) (***Fig. 1*** **G,H**).

To investigate the characteristics of the saliva microbiome, we analyzed the bacterial composition and community structure via 16S rDNA gene sequencing. Based on the microbial genomics samples, an average of 806,197 reads were aligned per sample. Alpha diversity based on Chao1 distance and beta diversity based on an unweighted principal coordinate analysis (PCoA) showed that the bacterial diversity of OSCC indicated an ecological resemblance among samples in each group, revealing a marked decrease and distinguishing it from the HC group (***Fig.1 A,B***), respectively. Although microbial variability at the phylum level cannot explain individual differences, we can roughly distinguish the difference between the two groups from the microbiome relative abundance. The dominant bacterial species of each group were similar and belonged to five main phyla: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*, and *Fusobacteria.* Moreover, levels of *Elusimiceobia, Chloroflexi, Verrucomicrobia, Deferribacteres, and Planctomycetes* increased in the OSCC group, while levels of *Synergistetes, Candidatus-Gracilibacteria* and *Cyanobacteria* decreased slightly (***Fig. 1C***). We further analyzed the taxonomic profile at the genus level, and the relative abundance among the 30 major genera showed that Prevotella, *Treponema, Lautropia, and Lactobacillus* were markedly decreased (Wilcox. test. $p<0.05$) in OSCC patients, while *Aggregatibacter and Rothia* were relatively increased (wilcox. test. $p<0.05$) (***Fig.1D***). Next, we compared wilcox. test. P values for the top 30 species of bacteria between the OSCC group and HC group at the species level. Five species, including *Lactobacillus casei, Neisseria subflava, uncultured Haemophilus sp., Veillonella sp. HGFM67* and *Veillonella sp. oral clone VeillB9* were significantly decreased (Wilcox. test.$p<0.05$) while *Veillonella parvula* was relatively decreased((wilcox. test.$p=0.05$)) in OSCC patients. We also found that two species, *Bacilli unclassified and Rothia mucilaginosa,* were significantly increased in the OSCC group (***Fig.1*** **E**). We further analyzed the bacterial community structure by using linear discriminant effect size (LEfSe), an algorithm for bacterial biomarker discovery that uses linear discriminant analysis (LDA) to estimate the effect size of each taxon that is differently represented in two groups, and found several high-dimensional biomarkers in two groups (***Fig. 1F***).

These results indicated that the composition of the saliva microbiota in patients with high TROP2 expression was significantly altered in OSCC patients.

2. **The screening and identification of *Veillonella parvula* NCTC11810**

*Veillonella* species *are* known as early colonizers of the oral microbiome and normally inhabit the mouth, upper respiratory tract, intestine and vagina[31, 32]. Recent studies illustrate the association of *Veillonella spp.* with mixed infections in children. However, the function and importance of *Veillonella* species in OSCC cancer are uncertain[33].

Since *Veillonella sp. HGFM67, Veillonella sp.* oral clone *VeillB9* and *Veillonella parvula* were decreased in OSCC patients to different degrees. To investigate the difference in *Veillonella* between the two groups in vivo, we collected 3 more samples of control group and analyzed the colony forming units (CFUs) of *the Veillonella* genus on Veillonella agar medium and found that the CFUs of Veillonella bacteria were not different in OSCC patients compared with those in the HC controls, which was consistent with the taxonomy profile results (***Fig. 2 A,B*** and ***Fig. 1,D***). For further exploration, we purified 34 colons from columbia blood plates by dilution separation methods from HC saliva samples and identified one colon of *Veillonella parvula* NCTC11810 with 99.71% sequence similarity **(*Supplementary Fig.S1*)**. Next, we detected the expression level of *Veillonella parvula* NCTC11810 by real-time PCR and found a 10-fold reduction in the OSCC group (***Fig. 2C***).

3. ***Veillonella Parvula* NCTC11810 Cells and Culture SupernatantInhibited the Proliferation and Invasion Ability of HN6 Cells**

As in our previous study, the expression of TROP2 was evaluated in HN6 cells compared with HOK cells (***Fig.3A***). To investigate the biological function of *Veillonella parvula* NCTC11810 in OSCC cells, we treated HOK and HN6 cells with *Veillonella parvula* NCTC11810 cells, heat inactivated *Veillonella parvula* NCTC11810 cells and culture supernatant for 2 hours. The viability of cells was investigated by CCK8 assays, and the results showed that cell proliferation at 24 hours was significantly reduced in HN6 cells treated with *Veillonella parvula* NCTC11810 and culture supernatant fluid compared with heat inactivated bacterial cells. For another cell line, *Veillonella parvula* NCTC11810, heat inactivated *Veillonella parvula* NCTC11810, and the culture supernatant treatment seemed to have no effects on the proliferation ability of HOK cells (***Fig.3 B***). In transwell assays, the number of cells that migrated across the Matrigel was counted, and the results indicated that both culture supernatant and bacterial cells reduced the cell invasion ability 24 hours after treatment in HN6 cells, but heat *inactivated Veillonella parvula* NCTC11810 cells could not inhibit the invasion of HN6 cells; however, the number of HOK cells that migrated across the Matrigel was not different among the treatment groups. (***Fig.3C,D***)

The data above indicated that the viable *Veillonella parvula* NCTC11810 cells and its metabolite products inhibited the proliferation and invasion ability of HN6 cells.

4. ***Veillonella Parvula* NCTC11810 Cells and Culture Supernatant Promoted the Apoptosis of HN6 Cells**

To further confirm the function of *Veillonella parvula* NCTC11810 in OSCC cells. HOK and HN6 cells were treated with the measures above, subjected to PI/Annexin V staining and analyzed by flow cytometry analysis to determine the percentages of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant), and late apoptotic cells (upper right quadrant). The results revealed that HN6 cells in the late apoptotic stage accounted for 9.25% in the *Veillonella parvula* NCTC11810-treated group and 17.48% in the culture supernatant-treated group, which were markedly increased compared with those of the heat inactivated *Veillonella parvula* NCTC11810-treated and control groups. The percentage of late apoptotic HOK cells was not different among the *Veillonella parvula* NCTC11810, heat-inactivated *Veillonella parvula* NCTC11810 and culture supernatant groups compared with those of the control group. (***Figure 4 )***

The data above indicated that apoptosis was significantly induced by the viable *Veillonella parvula* NCTC11810 cells and its metabolite products in HN6 cells.

The cytological phenotype of the *Veillonella parvula* NCTC11810 cell treatment but not the heat-inactivated *Veillonella parvula* NCTC11810 cell treatment inhibited the proliferation and invasion and promoted the apoptosis of HN6 cells might be because *Veillonella parvula* NCTC11810 cells were added to the cell culture plates, and the nutrient substance in the culture medium might promote the metabolism of *Veillonella parvula* NCTC11810 cells to some degree and induce the secretion of SCFAs, which play an antitumor function in HN6 cells.

The results suggested that the metabolite products of *Veillonella parvula* reduced proliferation and invasion and promoted apoptosis in TROP2-overexpressing HN6 cells. In the human oral cavity, the enriched culture of anaerobic microorganisms, for example *Veillonella parvula,* was shown to favor the process of fermenting lactic acid into propionate through the acrylate pathway[34-36]. Propionate has been found to inhibit the proliferation of liver cancer cells and induce the apoptosis of breast cancer cells, indicating a potential anticancer property; however, studies on propionate for the proliferation, invasion and apoptosis of OSCC are lacking. Thus, we investigated the anticancer effect of sodium propionate (SP) on the proliferation, invasion and apoptosis of OSCC cells.

5. **SP inhibited the proliferation and invasion of HN6 cells of OSCC.**

To determine the regulatory mechanism of *Veillonella parvula* in OSCC cells with high TROP2 expression, we treated HN6 and HOK cells with sodium propionate (SP) in diluted concentrations of 10 mM, 20 mM, 50 Mm and 100 mM. Proliferation was investigated by CCK8 assays 24 hours later, and the data suggested that cell viability was affected by the concentration of SP, as a stronger inhibition ability appeared in HN6 and HOK cells treated with higher concentrations of SP. Furthermore, the inhibition ability of SP was more distinct in HN6 and HOK cells at concentrations of 10 mM and 20 mM (***Fig. 5A***). Then, we detected the invasion ability of HN6 cells. The results of the transwell assay 24 hours after 10 mM SP treatment indicated that the invasion ability of HN6 cells was significantly decreased in the SP group, while there was no obvious change in HOK cells (***Fig.5B,C***). Even the low concentration of SP that

our data showed may inhibit the proliferation and invasion ability of OSCC cells with high TROP2 expression compared with that of normal cells.

6. **Exposure to SP induced apoptosis in HN6 cells.**

Since the culture supernatant of *Veillonella parvula* NCTC11810 promoted the apoptosis of HN6 cells, cells can take up propionate and use it as a metabolic substrate, providing energy through mitochondrial oxidation[37]. To further investigate the biological function of propionate in HN6 cells, 10 mM SP was added to HN6 cells for 24 hours, stained with PI/Annexin V and analyzed by flow cytometry . The results demonstrated that the overall apoptosis rate of SP-treated HN6 cells was 33% and significantly increased compared with that of the HOK group (3.36%); in particular, the late apoptotic percentage was 25.56% in HN6 cells, and the early apoptotic percentage was 7.97%, which were higher than those of HOK (3.37% and 0.25%, respectively) ***(Fig.6 and Supplementary Fig.S2)***.

7. **SP Inhibited the TROP2/PI3K/Akt Pathway in HN6 Cells**

Our previous study indicated that TROP2 might induce cell proliferation and invasion by activating the PI3K/Akt signaling pathway in OSCC, osteosarcoma and gallbladder cancer cells[38, 39]. To detect whether SP regulates cell behavior by modulating the Trop2/PI3K/Akt pathway, Western blotting was performed to explore the expression of related proteins in HN6 and HOK cells. As shown in ***Fig.7***, the expression levels of PI3K and Akt were not significantly changed between the SP treatment and control groups in HOK and HN6 cells ***(Fig. 7A,B)***. However, the expression levels of phosphorylated PI3K and phosphorylated Akt were totally inhibited in HOK cells and SP-treated HOK and HN6 cells but relatively activated in HN6 cells ***(Fig. 7C,D)***. Moreover, the expression level of TROP2 in SP-treated HN6 cells was remarkably inhibited compared with that in HN6 control group***(Figure.7E)***.

These results suggest that SP, the main metabolite of *Veillonella parvula* NCTC11810, may inhibit proliferation and invasion ability and promote the apoptosis by affecting the activity of the TROP2-related PI3K/Akt pathway.

# Discussion

OSCC arises in the oral cavity and tends to metastasize to the lymph node[40]. The occurrence and migration of OSCC is a complex and multi-step process consisted of a progression from dysplasia to carcinoma and involving a variety of epigenetic and genetic changes in the genome that lead to dysfunction in cell proliferation, cell death and cell apoptosis. With the advent of new technologies in the field of medicine, a large number of genetic and/or environmental factors, such as the dysregulation of *TP53, TROP2*, cell cycle regulators,smoking, alcohol consumption and chronic oral diseases, have been studied and are available for medical research, however, the survival rate remains poor. Since the widespread use of molecular-targeted therapy, several target proteins have been studied related to the malignant progression of OSCC, among which TROP2 has been implicated as a marker highly enriched in cell membrane and cytoplasm [41] especially when cells become malignant and in some cases of cancer

metastasis and recurrence[42, 43] and provides crucial signals in the MAPK and PI3K/Akt pathways for cell proliferation, survival, self-renewal, and invasion[44]. Chemotherapeutic agents such as cisplatin, 5-fluorouracil, and paclitaxel have now become first-line chemotherapeutic agents for OSCC [45, 46], but most patients acquired drug resistance and leaded to a poor prognosis eventually.

Propionate is the main metabolic product of *Veillonellae* and relay signals from the oral microbiota to the host[47, 48]. Accumulating data have shown that the beneficial effects of propionate differ considerably in potential effects on some kinds of cancers; however, studies on propionate regarding its proliferation, invasion and apoptosis of OSCC are lacking.

Our findings suggested a reduction in *Veillonella parvula* NCTC11810 in the saliva microbiome of OSCC patients. By isolating and identifying the saliva sample, a strain of *Veillonella parvula* NCTC11810 was separated, and its reduced expression level was confirmed in OSCC patients. Furthermore, the proliferation and invasion ability of HN6 cells were inhibited by the culture supernatant of *Veillonella parvula* NCTC11810 and bacterial cells but not by heat inactivated bacterial cells compared with HOK control cells (Fig. 4,5). In addition, *Veillonella parvula* NCTC11810 culture supernatant promoted the apoptosis of HN6 cells compared with that of bacterial cells or heat-inactivated bacterial cells (Fig. 6). Since *Veillonella parvula* NCTC11810 cells were added to the cell culture plates, the nutrient substance in the cell culture medium might promote the metabolism of *Veillonella parvula* NCTC11810 cells and induce the secretion of propionate and/or other SCFAs, which play an antitumor role in HN6 cells.

Since propionate is the main metabolic product of *Veillonella parvula* NCTC11810, to explore the molecular mechanism of propionate in the TROP2-related pathway in OSCC cells, we treated HN6 and HOK cells with different concentrations of sodium propionate and found that sodium propionate inhibited the proliferation and invasion and promoted the apoptosis of HN6 cells even at a low concentration (Fig. S1). Moreover, the suppressive effects of propionate on proliferation and invasion and the induction of apoptosis in cancer cells were mediated through the TROP2/PI3K/Akt pathway, as the protein levels of P-PI3K and P-Akt were almost completely decreased in the sodium propionate-treated HN6 cells. In addition, we explored the expression of PI3K and Akt and their phosphorylation levels in heat-inactivated *Veillonella parvula* NCTC11810-treated cells and found that the stripes completely disappeared in both HOK cells and HN6 cells. Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria. Related studies suggested that LPS elicits a lytic cell death called pyroptosis by the canonical caspase-1 inflammasomes or by activation of caspase-4, -5 and − 11, which cleave gasdermin-D (GSDMD) in its middle linker to release autoinhibition on its gasdermin-N domain and execute pyroptosis via its pore forming activity. In this study, our preliminary data also showed that viable and heat inactivated *Veillonella parvula* NCTC11810 treatment increased the cleavage of GSDMD compared with SP treatment and the control group in HN6 cells (Supplementary Fig. S3), which might suggest the pyroptosis activation of *Veillonella parvula* NCTC11810 cells, but the mechanism still needs to be explored.

In conclusion, the propionate metabolite of *Veillonella parvula* inhibited proliferation and invasion and promoted apoptosis in an OSCC cell line by inhibiting the activation of the TROP2/PI3K/Akt pathway. Therefore, our study suggested the potential antitumor effects of propionate achieved by regulating the intracellular signaling pathway in OSCC, which might provide insights into a novel therapeutic strategy for OSCC.

# Declarations


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### Conflict of interests

The authors declared no conflict of interests.

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# Figures

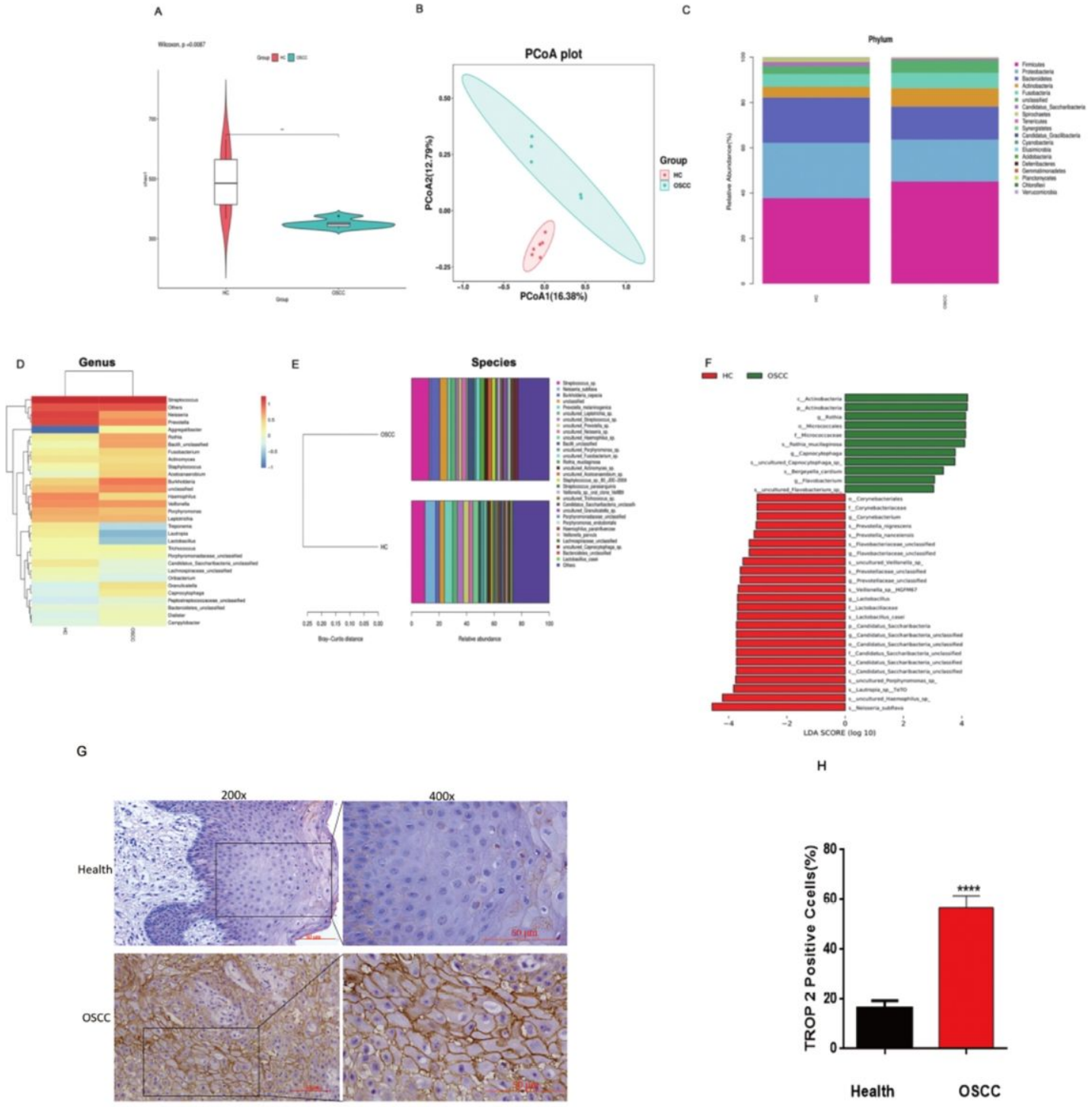



Figure 1

**Microbiota composition and abundance in saliva samples of OSCC analyzed by Illumina MiSeq sequencing.** (A) Alpha diversity of OSCC group and HC group analyzed by Wilcoxon analysis; (B) Beta diversity of saliva microbiome analyzed by PCoA analysis of OSCC group and HC group; (C) Compositional change at the phylum level; (D) Heatmap of community composition at genus level; (E) Cluster analysis results of the groups of saliva microbiota at the species level, p value of *Veillonella parvula was 0.05* ; (F) Association of specific microbiota taxa with the groups by linear discriminant analysis(LDA) effect size(LEfSe); (G) Representative immunohistochemical images of TROP2 protein expression in and healthy controls and tumor tissues of OSCC patients left 200x, right 400x . n=5, **p<0.01.

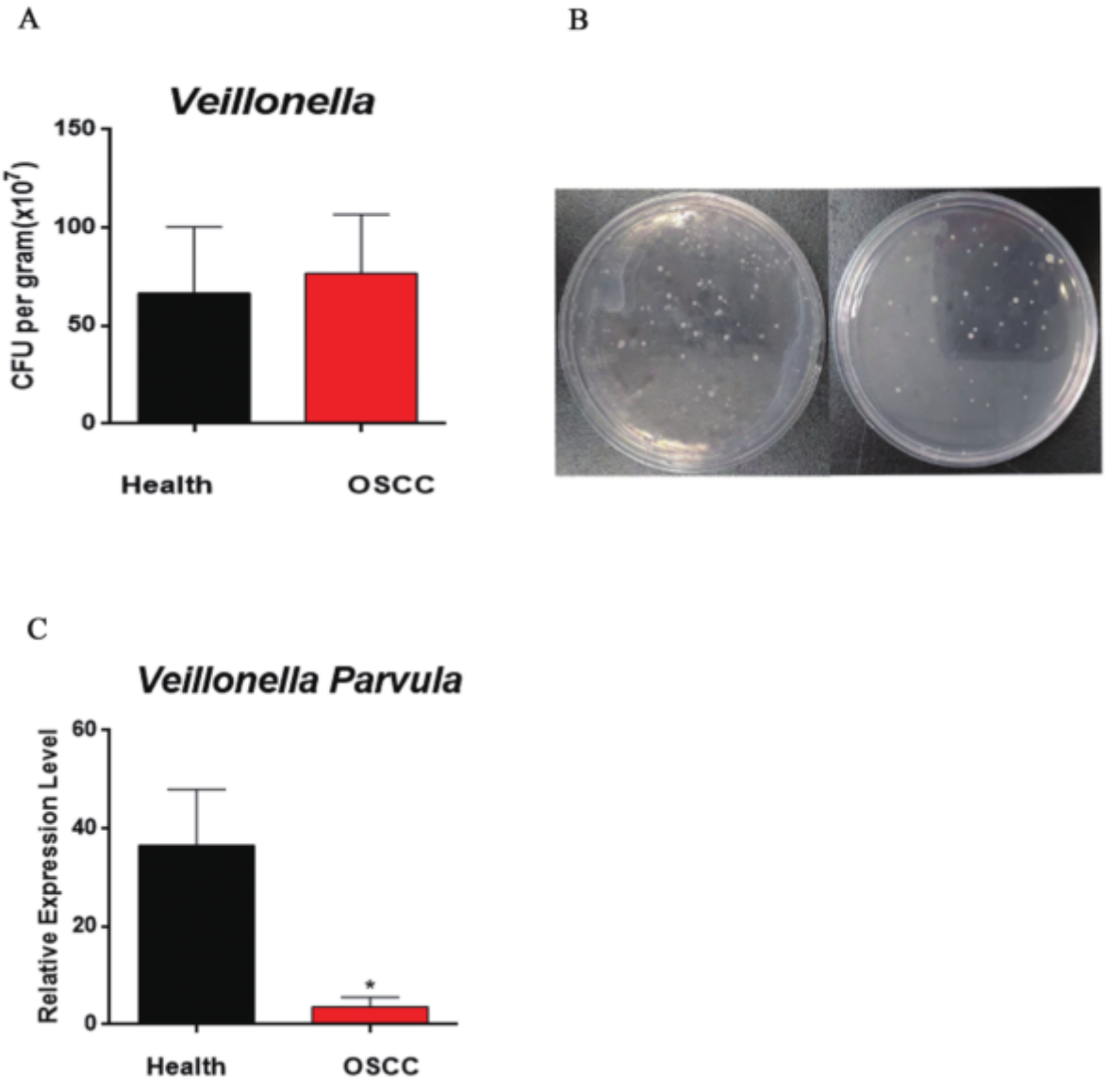
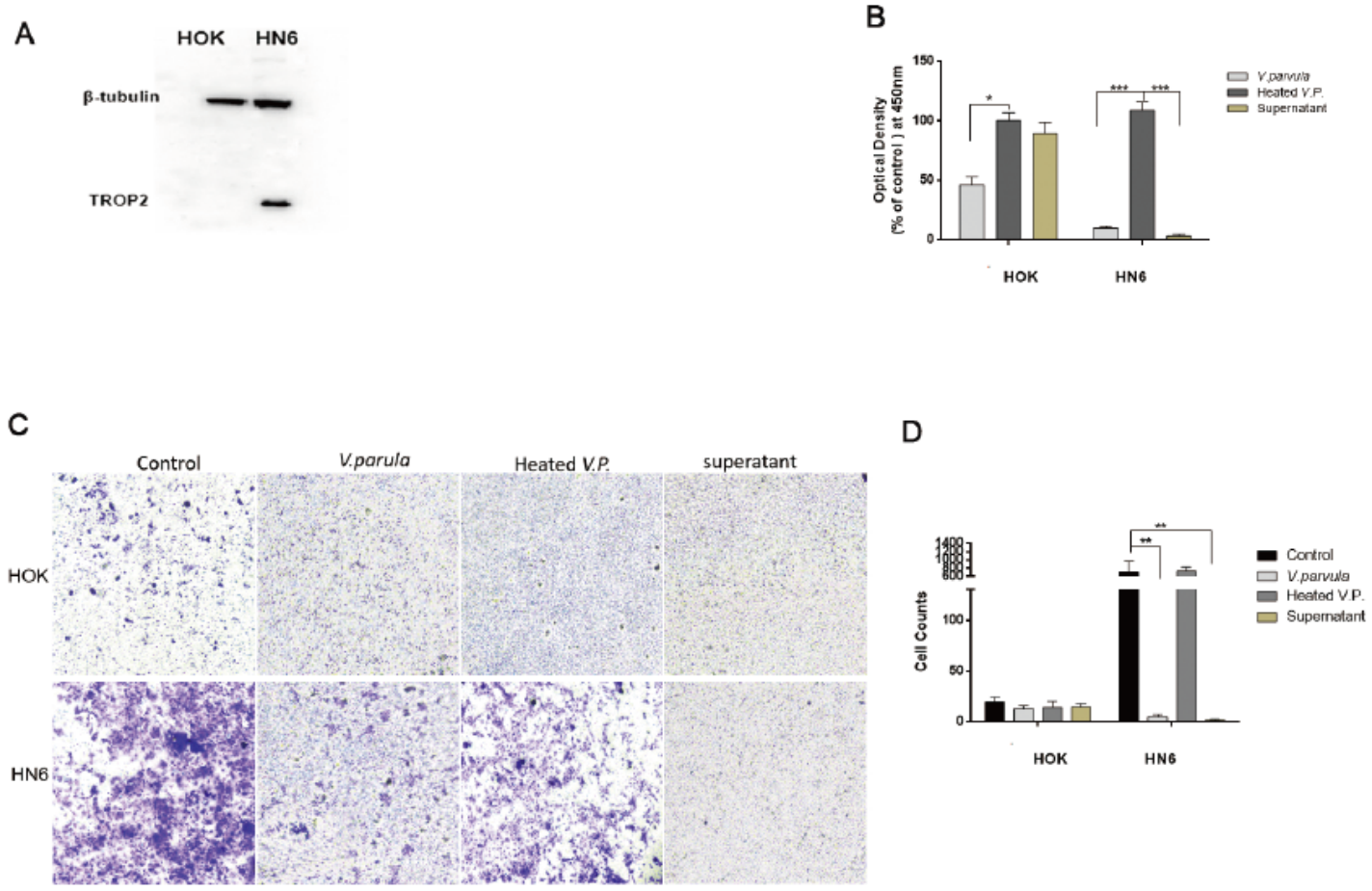


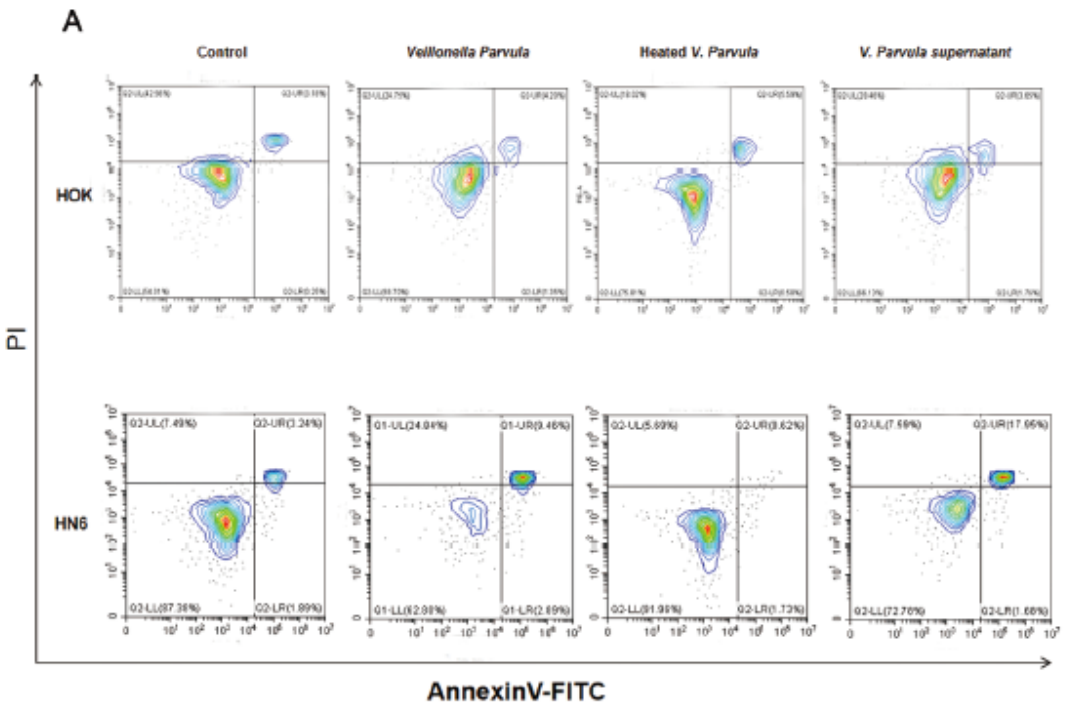

Figure 2

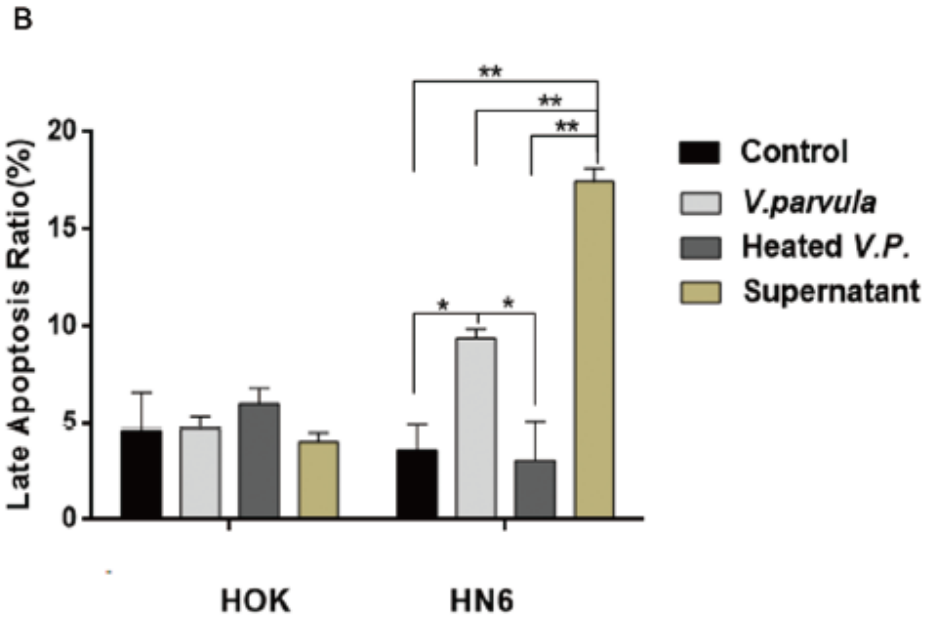
**The identification and expression of *Veillonella parvula* NCTC11810.**(A) CFU analysis of Veillonella genus in saliva microbiota of health controls and OSCC patients; (B) Agar plating of Veillonella genus in saliva microbiota of health controls and OSCC patients; (C) Relative expression level analysis of *Veillonella parvula* NCTC11810 by real time quantitative PCR. OSCC group n=8, Health group n=9*p<0.05.




## Figure 3

**Effects of *Veillonella parvula* NCTC11810 on the proliferation and invasion of OSCC cells.** (A) Expression level of TROP2 in HOK and HN6 cells by Western blotting; (B) Effects of *Veillonella parvula* NCTC11810 on the proliferation of HN6 and HOK cells, cells treated with bacteria cell, heat inactivated bacteria cells and culture supernatant were analyzed by CCK-8 assay. n=3, *p<0.05, ***p<0.001;(C,D) Effects of *Veillonella parvula*NCTC11810 on the invasion of HN6 and HOK cells by Transwell assay,cells treated with *Veillonella parvula* NCTC11810 bacteria cell(*Veillonella parvula*), heat inactivated bacteria cells(Heated *V. Parvula* ) and culture supernatant of *Veillonella parvula* NCTC11810 (*V.Parvula*supernatant) were counted, n=3,**p<0.01.



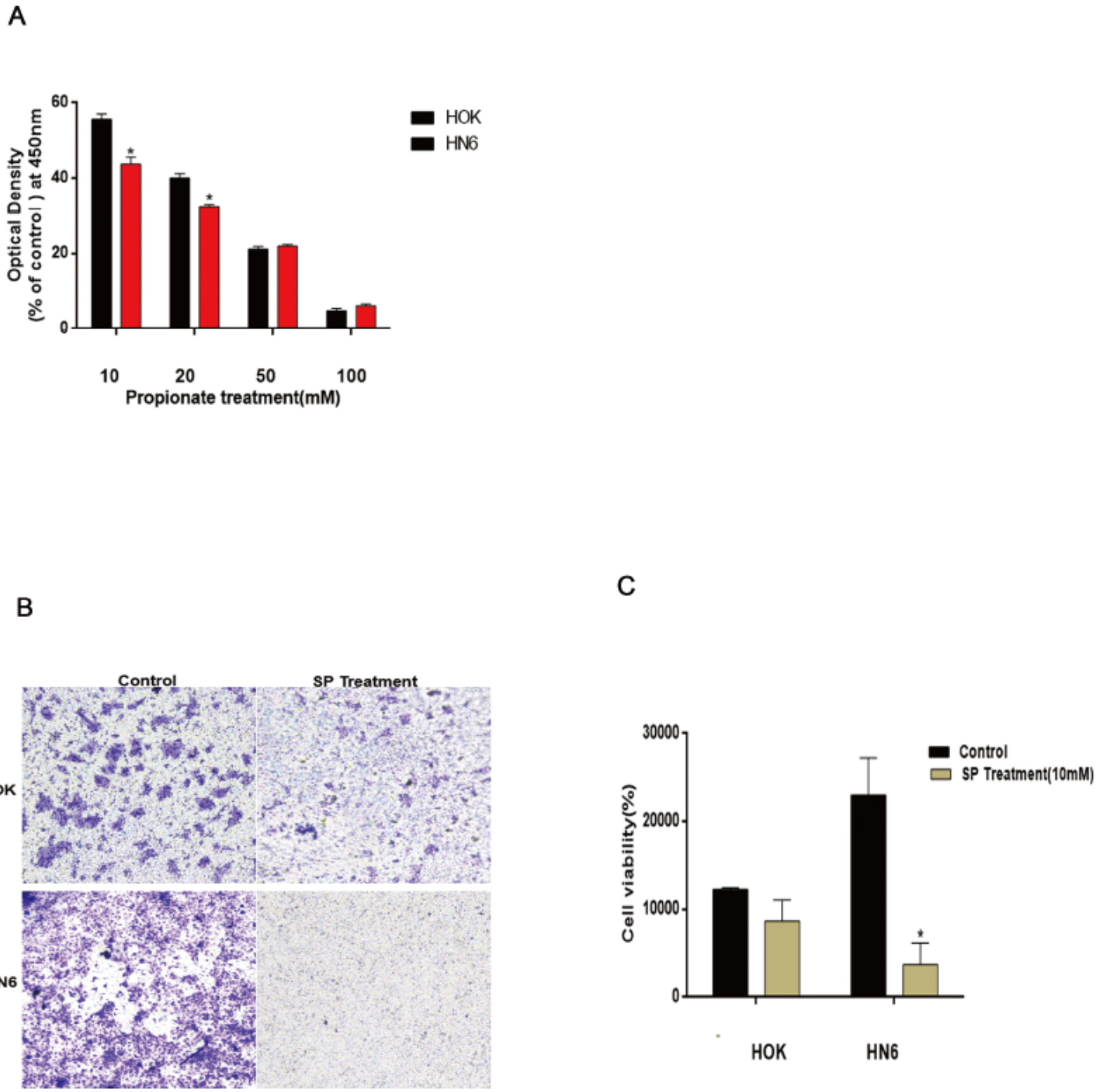




## Figure 4

**Effects of *Veillonella parvula* NCTC11810 on the apoptosis of OSCC cells.**

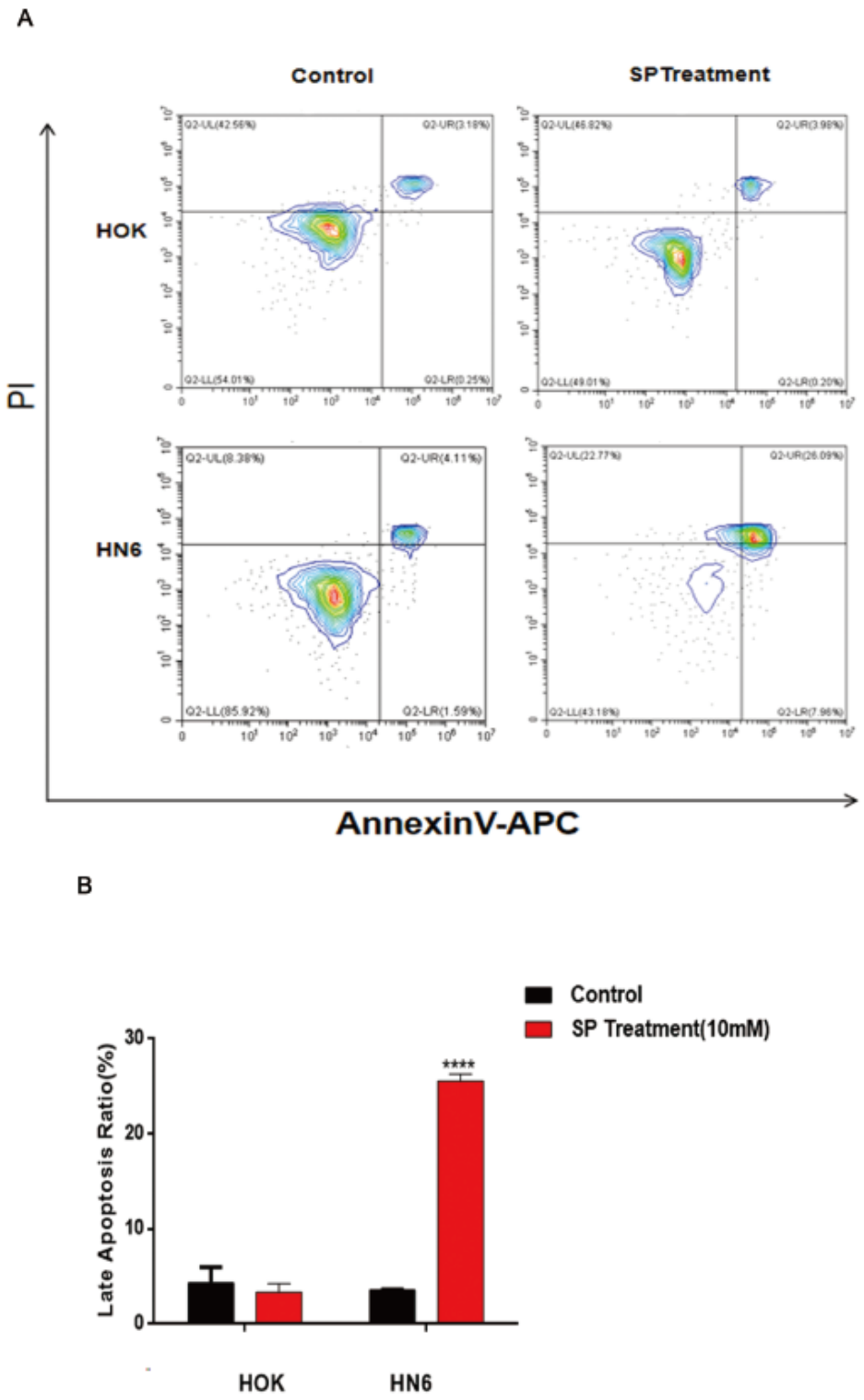
(A) The apoptosis rate of OSCC cells stained with PI/Annexin V staining and analyzed by flow cytometry analysis; (B) Late apoptosis ratio of cells treated with *Veillonella parvula* NCTC11810 bacteria

cells(*Veillonella parvula*), heat inactivated bacteria cells(Heated *V. Parvula*) and culture supernatant of *Veillonella parvula*NCTC11810 (*V.Parvula* supernatant) were compared with control. n=3,**p<0.01.




## Figure 5

**Effects of sodium propionate (SP) on the proliferation and invasion of OSCC cells.** (A) Effects of SP on the proliferation of cells at different concentration by CCK-8 assay.(B) Effects of SP on the invasion of cells at 10mM by transwell assay; n=3, *p<0.05.




## Figure 6

**Effects of sodium propionate (SP) on the apoptosis of OSCC cells.** (A) The apoptosis rate of OSCC cells stained with PI/Annexin V staining and analyzed by flow cytometry analysis; (B) Late apoptosis ratio of cells treated with SP were compared with control groups. n=3,***p<0.001.

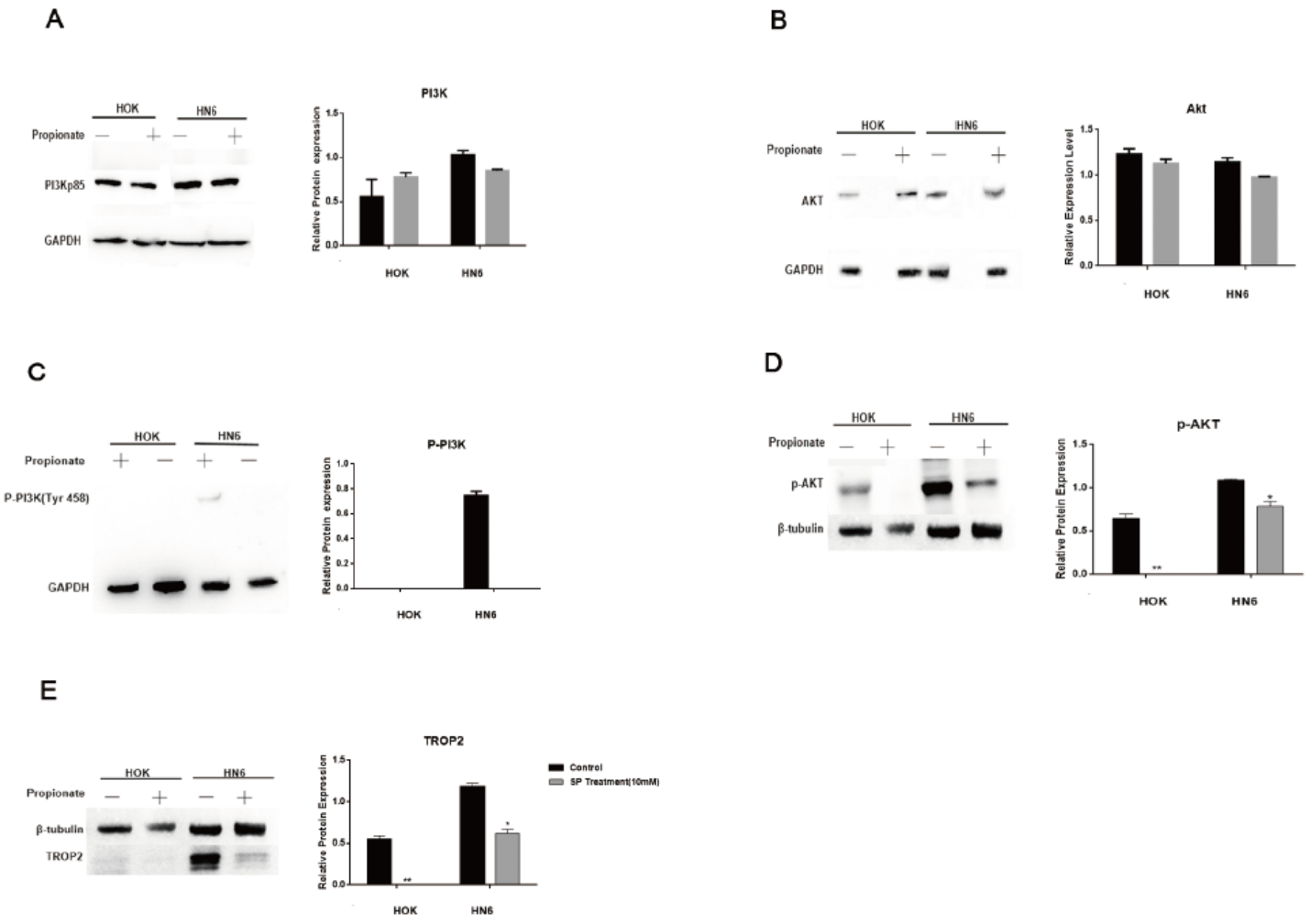



**Figure 7**

**SP inhibited the PI3K/Akt signaling pathway in OSCC cells.**

(A)Representative western blots and densitometry analysis of PI3K expression level; (B) Representative western blots and densitometry analysis of Akt expression level; (C) Representative western blots and densitometry analysis of phosphorylated PI3K expression level; (D) Representative western blots and densitometry analysis of phosphorylated Akt expression level; (E) Representative western blots and densitometry analysis of TROP2 expression level.n=3, ****p<0.0001.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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