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Exploiting the Acidic Extracellular pH: Evaluation of *Streptococcus salivarius* M18 Postbiotics to Target Cancer Cells

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Abstract

Previously, we showed that the growth, antibiotic resistance, and biofilm formation properties of the pathogens *Pseudomonas aeruginosa* and *Klebsiella pneumonia* were tremendously inhibited by the cell-free supernatant of the oral probiotic *Streptococcus salivarius* M18. These anti-pathogenic activities of the supernatant were more efficient under acidic conditions. The present approach takes advantage of the acidic nature of the tumor microenvironment to evaluate the effect of the *S. salivarius* M18 postbiotics on colon cancer cells. In both two-dimensional (2D) and three-dimensional (3D) cell culture models, *S. salivarius* M18 cell-free supernatant showed anti-cancer actions in the pH conditions mimicking the acidity of the tumor. The inhibitory effect was more prominent when the colon cancer cells have been treated with the cell-free supernatant obtained from the inulin incubated *S. salivarius* M18. The results of this study point out the potential of the *S. salivarius* M18 functional probiotic products to be used for targeting low pH environments including the unique acidic microenvironment of tumors.

Keywords *S. salivarius* M18 · Colon cancer · Tumor microenvironment · pH · Inulin · Cancer targeting

Introduction

Over recent years, probiotics research has concentrated on the potential of probiotics in the prevention and treatment of diseases, including cancer [1]. The anti-cancerous activity of probiotics is attributed to their anti-mutagenic properties through mutagenesis inhibition, effects on the activation of the host immune system through the secretion of anti-inflammatory molecules, and intestinal pH lowering nature which inhibits the development of pathogenic bacteria [2–4]. Besides preventing and treating cancer, the use of probiotics has been also suggested as an adjuvant therapy against the common adverse effects of chemotherapeutics, including diarrhea, abdominal pain, vomiting, stomatitis, skin erythema, atrophy, and neural and vascular damages, to prevent life-threatening complications and improve quality of life [5–7].

Despite the abundance of studies suggesting the positive indications of probiotic usage, there are no clear regulations for clinical recommendations of probiotic consumptions (i.e., selection of the appropriate probiotics, control of their dietary intake, time and frequency of probiotic dosing) [8–11] and there is not any comprehensive study based on the examination of the effects of long-term probiotic use in patients [10]. Even more, probiotic consumption has been suggested to be associated theoretically with some undesirable side effects, including systemic infections, deleterious metabolic activities, and excessive immune stimulation [8, 12]. Unpredictable niche-specific actions and metabolite production, host-induced gene expressions, antibiotic resistance risks, ability to cause opportunistic infections, interfering with the colonization of commensal gut microflora, and bacterial translocation to tissue or blood are also among the proposed risks of the probiotic consumption [13]. Besides, there are technological concerns about the production of the probiotics such as decreased viability which limits the full potential applications of live cell probiotics in the food and pharmaceutical sectors [13] and microbiological and

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nonmicrobial contamination (such as allergens, various toxins, and heavy metals) risks [14]. Accordingly, FAO/WHO addressed the importance of assuring safety about the specific issues of probiotics related to their toxigenicity, pathogenicity, and allergenicity [15]. To overcome some of these obstacles in the use of live probiotics, researchers are interested in the utilization of non-viable bacteria, bacterial compounds, or probiotic metabolites showing similar or even enhanced health-improving biological activities compared to live probiotics [16]. Although the nutrition-based health strategies underline the use of commensal bacteria as probiotics [17–19], there is increasing evidence suggesting that the health benefits of some live probiotics are associated with the probiotic-derived biomolecules [17, 20]. Postbiotics is the term to describe the complex mixture of metabolic products secreted by probiotics in cell-free supernatants such as bacteriocins, enzymes, organic acids, vitamins, hydrogen peroxide, ethanol, diacetyl, peptides, cell surface proteins, quorum sensing molecules, short-chain fatty acids (SCAFs), vitamins, secreted biosurfactants, and organic acids [13, 21].

Streptococcus salivarius is one of the earliest colonizers of the epithelial lining of the human mouth and nasopharynx [22]: it was found to be established in the human oral cavity within 2 days after birth, and on the third day of the infant life, *S. salivarius* was detected as a largest taxonomic group in the fecal samples [23]. *S. salivarius* is also one of the predominant species in breast milk [24] which plays an important role in establishing the infant gut microbiome [25–27]. In adults, on the other hand, the bacterium colonizes the mucosal surfaces of the intestine including the ileum, jejunum, and colon as well as stomach [28]. M18 strain of *S. salivarius* has been isolated from the oral cavity of a healthy female adult [29] and with the report Notice No. 807, the US Food and Drug Administration (FDA) granted the strain as Generally Recognized as Safe (GRAS) (https://www.fda.gov/media/133874/download). Several numbers of nose, mouth, and throat pathogens were shown to be inhibited by the probiotic *S. salivarius* M18 [30], and recently, we showed that *S. salivarius* M18 cell-free supernatant reduced the growth of the two most common human pathogens, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, which are responsible a wide range of severe human infections. Besides, the postbiotics of the probiotic bacteria sensitized *P. aeruginosa* to antibiotic treatment and inhibited its biofilm formation ability drastically. The observed pathogen inhibitory activity of *S. salivarius* M18 cell-free supernatant was found to be more effective under acidic conditions [31]. Here, based on the need for a low pH environment for the inhibitory action of *S. salivarius* M18 cell-free supernatant, we aimed to investigate if the probiotic supernatant can be used to target cancer cells for taking the advantage of the acidic nature of the tumor microenvironment [32, 33]. The microenvironment of most solid tumors is characterized by acidosis which is considered a new hallmark of cancer [34]. The unique glycolytic metabolism of cancer cells produces an excessive amount of lactic acid, and the export of protons and lactic acid from tumor cells into the extracellular environment by acid–base regulators, such as Na^+/H^+ exchangers and monocarboxylate transporters, results in acidosis in the tumor microenvironment. Therefore, while the pH of blood and tissue is tightly controlled around pH 7.4 under normal physiological conditions [33, 35], the external pH of cancer cells typically varies between 6.7 and 7.0. Malformed tumor vasculature inhibits the removal of lactic acid and protons and therefore reduces the buffering capacity of the extracellular environment. So, the pH of a tumor microenvironment can even decrease to as low as 5.5 [33, 36]. In the tumor microenvironment, the expression of several ion/proton pumps not only in tumor cells but also in tumor-associated stromal cells finely tunes the abnormal pH gradient which has been shown to favor cancer progression by promoting local tumor invasion and also metastasis [37, 38].

By using both 2D and 3D cell culture models, we show that *S. salivarius* M18 cell-free supernatant has cytotoxic activity on colon cancer cells in a tumor pH mimicking environment, but not in pH condition of the healthy tissue. We also show that this microenvironment-dependent selective cytotoxicity of the supernatant can be enhanced further with the prebiotic inulin. Since efficient tumor-targeting therapeutics are aimed to increase the accumulation of anti-tumor agents in tumors while simultaneously reducing side effects in normal-healthy tissues [39, 40], the results of this study highlight that postbiotics of *S. salivarius* M18 have the potential to be used as tumor-targeting agents as well as anti-cancer drug delivery systems.

Materials and Methods

Bacterial Culture Conditions, Collection of Cell-Free Supernatants, and Treatments

Streptococcus salivarius M18 (BLIS M18™; Blis Technologies, New Zealand), *Pseudomonas aeruginosa* (ATCC 27,853; ATCC, Manassas, VA, USA), and *Escherichia coli* (ATCC 8739) were cultured at 37 °C with shaking in tryptic soy broth-TSB medium (cat. no.: 1.05459; Merck Millipore, Burlington, MA, USA) or on agar plates (TSA) containing 1.5%, w/v agar in TSB. For the collection of *S. salivarius* supernatants, OD_{600} of overnight cultures were adjusted to 0.1 and the bacteria were grown for 24 h at 37 °C using a shaking incubator at 160 rpm in screw cap tubes filled with 95% growth medium TSB and sealed with paraffin film to provide low oxygen conditions. In the experiments in which the effect of the prebiotic inulin was investigated, presterilized (121 °C, 15 min) TSB medium containing inulin

from the chicory plant *Cichorium intybus* (0.5% or 1.0%, w/v) was inoculated with *S. salivarius* M18, and the cultures were grown under low oxygen conditions as described before [41]. TSB, containing inulin when indicated, which has not been inoculated with bacteria but incubated under the same conditions with the *S. salivarius* M18 inoculated culture, was used as "only medium (OM) control."

S. salivarius M18 (SsM18 sup.) and *E. coli* supernatants (*E. coli* sup.) were prepared as described previously [31]. Briefly, 24 h incubated bacterial cultures were centrifuged at 4 °C, 5000 rpm (1844 × *g*) for 10 min before filtration using 0.22-µm PES filters. The supernatants were used immediately or aliquoted and stored at − 80 °C for later use.

Cell Cultures and Treatments

Human umbilical vein endothelial cells, HUVECs (ATCC, England), were cultured in DMEM/F12 containing 10% FBS (fetal bovine serum), 2 mM L-glutamine, and 1% pen/strep. Human CRC epithelial cell lines HCT-116 (DSMZ, Germany) and SW-480 (ATCC) were grown in pH 7.5 RPMI-1640 complete growth medium prepared by dissolving (10.5 g/l) $NaHCO_3$-free RMPI-1640 powder medium (cat. no.: 11–100; Biological Industries, Israel) in dH_2O and supplementing it with 10% FBS, 2.8%, v/v cell culture grade $NaHCO_3$ solution (7.5%, w/v), and 1% pen/strep. Before sterilization through 0.22 µm PES-filter, 333 µl of HCl (37%) was added into the 1 l of the complete RPMI-1640 growth medium to adjust the pH to 7.5. Cells were maintained in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C. Trypsin–EDTA (0.25–0.02%, w/v) was used to detach the cells during sub-culturing or cell harvesting. Before harvesting, adherent cells were rinsed with Ca^{2+} and Mg^{2+}-free cell culture grade PBS (phosphate-buffered saline). When indicated, pH of $NaHCO_3$-free RPMI-1640 medium containing 10% FBS and 1% pen/strep was adjusted to 5.5, 6.0, 6.5, or 7.0 by using concentrated HCl. $NaHCO_3$ (7.5%, w/v) was added to the pH-adjusted complete RPMI-1640 growth media [42] to ensure pH stability during the incubation in a 5% CO_2 incubator. The required amount of $NaHCO_3$ was determined by the Henderson-Hasselbalch equation [43]. Cells were treated with 20%, v/v TSB medium (control medium; OM) or 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) in RPMI-1640 complete growth medium (pH 5.5, 6.0, 6.5, 7.0, or 7.5) for indicated period of times.

Hanging Drop and 3D On-Top Assay

Hanging drop assay was performed as described previously [44] but with some modifications. HCT-116 cells harvested and 1×10^5 cells/ml were suspended in complete RPMI-1640 medium (pH 7.5) as single cells. Drops of 30 µl were pipetted onto the inner surface of a lid of 10 cm low attachment sterile Petri dish as 30 drops per dish. The lid was inverted onto the 5 ml PBS-filled bottom chamber to maintain humidity and incubated for 48 h at 37 °C, 5% CO_2 incubator. At the end of incubation, the medium of the drops was replaced with 20%, v/v *S. salivarius* cell-free supernatant (SsM18 sup.) or 20%, v/v TSB (only medium; OM) containing complete RPMI-1640 growth medium with pH 5.5, 6.0, 6.5, 7.0, or 7.5. The plate was cultured for a further 24 h at 37 °C, 5% CO_2 incubator before the imaging of the aggregates with the inverted light microscope (Nikon) equipped with the HD camera (Toupcam).

To investigate the effects of the cell-free supernatant on cancer cell–derived spheroids, a modified version of the 3D (spheroids) on-top assay was carried out [45]. BD Matrigel™ Basement Membrane Matrix (cat. no.: 354234; BD, NJ, USA) was thawed at 4 °C overnight. Wells of the prechilled 96-well plate were coated with 15 µl Matrigel, and the plate was incubated for 20 min at 37 °C to allow the Matrigel™ gelification. 70% of confluent HCT-116 cells were detached by trypsin, and a complete growth medium was used to inhibit trypsin activity. After centrifugation at 400 × *g* for 7 min, the supernatant was discarded and the cells were suspended in the complete RPMI-1640 growth medium (ph 7.5) as 0.175×10^6 cells/ml and, then, 30 µl cell suspension was added to Matrigel™-coated wells. The plate was agitated in the x–y plane to prevent uneven cell distribution and incubated for 20 min at 37 °C. Three microliters of Matrigel™ was added into 30 µl of pre-chilled complete RPMI-1640 medium (pH 5.5, 6.0, 6.5, 7.0, or 7.5) containing 20%, v/v TSB (only medium; OM) or 20%, v/v *S. salivarius* M18 (SsM18 sup.) cell-free supernatant. After being ensured that the Matrigel™ and the medium were mixed homogenously, the mixture was pipetted down the side of the well. The culture plate was incubated at 37 °C in a 5% CO_2 incubator for 6 days by replacing the Matrigel™-medium mixture every 2 days. At the end of 6 days, the aggregates were photographed by using an inverted light microscope (Nikon) attached with an HD camera (Toupcam).

The inhibitory effect of the *S. salivarius* M18 cell-free supernatant on the CRC cells, both in 2D and 3D models, was investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described below. All culture medium ingredients were purchased from Biological Industries (Israel), and cell culture consumables were obtained from Wuxi NEST Biotechnology (China).

MTT Assay and Trypan Blue Staining

MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity [46]. The growth inhibitory effect of the *S. salivarius* M18

cell-free supernatant on the colon cancer cell lines grown in 2D cultures was analyzed by using an MTT reagent (BioVision, Pennsylvania, USA) as described before [47]. Briefly, the cells were seeded as 1×10^4 cells/well of a 96-well plate. The day after, the cells were treated with RPMI-1640 complete growth medium with pH 5.5, 6.0, 6.5, 7.0, or 7.5 containing 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or 20%, v/v TSB (only medium; OM). At the end of incubation, the medium was discarded and the cells were incubated for 4 h at 37 °C in 100 µl of complete culture medium (RPMI-1640, pH 7.5) containing 1.2 mM of MTT. One hundred microliters of the SDS-HCl solution (1 g of SDS in 10 ml of 0.01 M HCl) was added to each well, and the plates were incubated for further 16 h at 37 °C to dissolve the crystals of MTT formazan. In a microplate reader (Thermo Fisher Scientific, MA, USA), the absorbances were measured at 570 nm and MTT reduction was given as a percentage relative to the 20%, v/v TSB-treated cells in pH 7.5. Absorbance values obtained from the wells containing complete medium with MTT and SDS-HCl, but without cells were used as blanks. Before carrying out the MTT cytotoxicity assays, the treated cells were observed by Nikon Eclipse TS100 (Japan) inverted light microscope and photographed with Toupcam (China) HD camera.

MTT assay was also used to determine the metabolic activity of 3D colon cancer cell spheroids (described above). For this, at the end of 6-day culture, the Matrigel™-medium mixture was carefully discarded by pipetting and 100 µl of 1.2 mM MTT containing RPMI-1640 complete culture medium was added to each well (on top of the Matrigel). The plate was incubated at 37 °C for 4 h by shaking at 160 xrpm. At the end of incubation, 100 µl of 40 mM HCl containing acidified isopropanol was added to the wells and mixed thoroughly to dissolve the formazan crystals. The absorbances were measured at 540 nm in a microplate reader in 10 min (Thermo Fisher Scientific). Absorbance values obtained from the wells containing Matrigel™-medium mixture with MTT and acidified HCl, but without cells were used as blanks. Percent reduction of MTT was calculated with respect to the cells treated with 20%, v/v TSB (only medium; OM) in RPMI-1640 complete growth medium, pH 7.5.

To distinguish between live and dead cells, HCT-116 cells were incubated with 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or with the 20%, v/v TSB (only medium; OM) for 2 h and 4 h in RPMI-1640 complete growth medium with pH 5.5 and stained with trypan blue. For this, 50×10^4 cells were seeded in the wells of a 12-well plate in RPMI-1640 complete growth medium, pH 7.5. The day after, the medium was removed and the cells were treated with pH 5.5 complete growth medium (RPMI-1640) containing 20%, v/v probiotic supernatant (SsM18 sup.) or 20%, v/v TSB (only medium; OM) for 2 h or 4 h. At the end of incubation, the treatment medium in the wells, containing the floating cells, was collected to Eppendorf tubes separately and used to harvest the adherent cells after trypsin treatment. The cell suspensions were diluted as 1:1 (20 µl:20 µl) in 0.5% trypan blue solution, w/v (Biological Industries), and two independent samples were taken from the cell suspensions and were separately pipetted into the two counting areas of a Thoma counting chamber. The unstained live cells and stained dead cells were counted under a light microscope (Nikon), and the number of the average cell count, obtained from the counting areas, multiplied by 10^4 and the dilution factor to obtain the cell number per milliliter.

Caco-2 Spontaneous Differentiation for In Vitro Barrier Integrity Analysis

The human intestinal Caco-2 cell line is extensively used as a model of the intestinal barrier. Upon reaching confluency, the cells spontaneously differentiate into enterocyte-like cells expressing several morphological and functional characteristics of the mature enterocyte [48, 49]. To investigate if treatment with *S. salivarius* M18 cell-free supernatant affects the integrity of the epithelial barrier, Caco-2 cells were spontaneously differentiated for ten days as described before [49, 50]. Caco-2 cells were grown at 37 °C, 5% CO_2 incubator in EMEM-Minimum Essential Medium with Earl's salt (Thermo Fisher Scientific, Boston, MA, USA) containing 20% FBS (Biological Industries), 1% pen/strep (Biological Industries), 2 mM L-glutamine (Biological Industries), 1 × NEA-Non-Essential Amino Acids (Biochrom GmbH, Berlin, Germany), and 1 mM Na pyruvate (Biological Industries) for culturing. The cells were passaged before they reach 70% confluency (sub-confluent; un-differentiated). For the barrier model, the cells were seeded as 3.5×10^4 cells/cm^2 to the wells of 12-well plates. After about 48 h of plating, the cells reached 100% confluency. 100% of confluent cells were grown for a further 10 days during which the growth medium was refreshed every other day. After 10 days, the formation of domes was observed under a light microscope, as signs of enterocytic differentiation and transport properties [51]. The differentiated cells were treated with 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or 20%, v/v TSB (only medium; OM) in RPMI-1640 complete growth medium (pH 7.5) for 2 h or 4 h. After the treatments, CV s taining was performed as described previously by Herzog et al., with some modifications [52]. Briefly, the treatment medium was removed and the cells were washed with room temperature and cell culture grade PBS. PBS was discarded, and the Caco-2 cells were fixed at room temperature in 4.0% paraformaldehyde (v/v, in PBS). The fixation solution was removed, and the cells were rinsed with PBS before incubating for 15 min at room temperature in 0.2% CV, w/v

(Sigma-Aldrich, St. Louis, MO, USA). CV was pipetted out, and the wells were photographed after PBS washing. The CV stains in the wells were dissolved in 2 ml of 1.0% SDS (w/v, in dH_2O) and three folds diluted in dH_2O before reading the absorbances in a microplate reader (Thermo Fisher Scientific) at 550 nm. Undifferentiated Caco-2 cells (sub-confluent cells; 60–70% confluent) were analyzed by following the same procedure.

As a differentiation marker, the expression of CEA (carcinoembryonic antigen) in sub-confluent (undifferentiated; 60–70% confluent) and 10 days differentiated Caco-2 cells [47] were assayed by western blot, as described below.

Western Blot

To analyze the expression levels of XIAP (X-linked inhibitor of apoptosis protein) and caspase-3 in HCT-116 cells treated with the probiotic supernatant, the cells were seeded into the 12-well plate as 5×10^5 cells/well in the complete RPMI-1640 growth medium (pH 7.5). The day after, the medium was discarded and the cells were incubated for 2 h in the RPMI-1640 growth medium (pH 5.5) containing 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or 20%, v/v TSB (only medium; OM). At the end of incubation, the incubation medium containing the floating death cells was collected from the wells into separate Eppendorf tubes and used to collect the adherent cells after trypsin treatment. The cells were centrifuged at $400 \times g$ for 7 min, and the supernatant was discarded before washing the pellets in PBS.

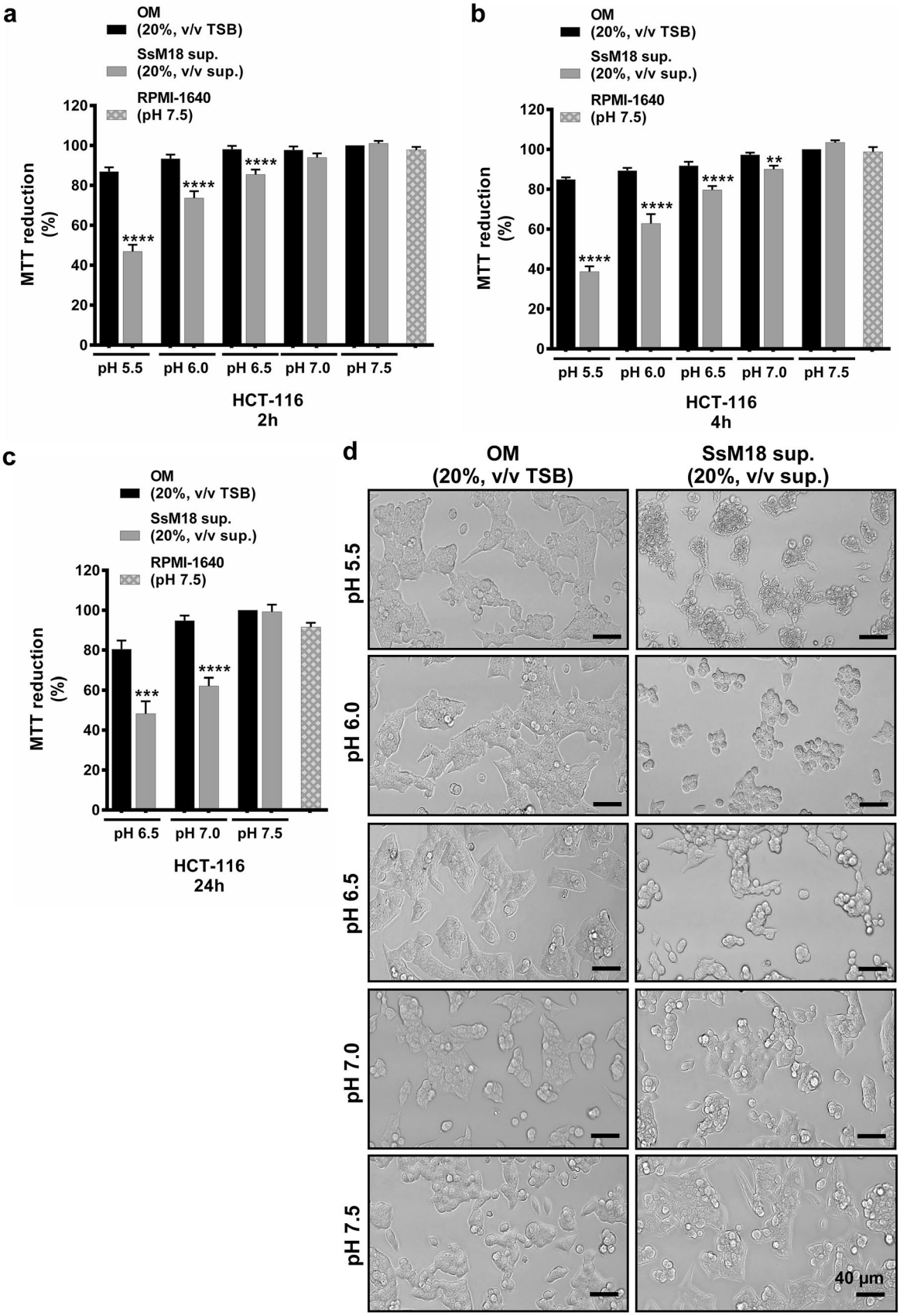
For protein extraction, 100 µl of protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor (Roche, Switzerland) containing T-PER Protein Extraction Reagent (Thermo Fisher Scientific) was used. The lysis of the pelleted HCT-116 cells was achieved by incubating the cells for 30 min on ice in lysis buffer and vortexing vigorously every 10 min. For the lysis of the Caco-2 cells, the cells were collected by scraping in lysis buffer after washing with PBS. Proteins were collected by 15 min centrifugation which was carried out at 4 °C, $14{,}000 \times g$. Pierce™ Coomassie Plus (Thermo Fisher Scientific) reagent was used to determine protein concentrations. The absorbance at 595 nm was measured and transformed into protein concentrations according to a BSA standard curve. Western blot was performed as described before [49]. Briefly, proteins (20 µg) were separated by 10% SDS-PAGE after denaturation by boiling at 95 °C for 6 min in a 6 × loading dye (12% SDS, 30% β-mercaptoethanol, 30% glycerol, 0.02% bromophenol blue, 375 mM Tris HCl pH 6.8) and then transferred to PVDF (polyvinylidene difluoride) membrane. Followed by blocking in 5% skim milk for 1 h, the membrane was immunoblotted overnight at 4 °C with the primary antibodies (XIAP, cat. no.: sc-11426, Santa Cruz Biotechnology, TX, USA; caspase-3, cat. no: STJ92021, UK; CEA, cat. no: 180057, Invitrogen, CA, USA) against the target proteins. The bands were detected by using Clarity ECL Substrate (Bio-Rad, CA, USA) in a G:BOX imaging system, equipped with GeneSys image capture software (Syngene, England) after 1 h incubation with HRP (horseradish peroxidase)-conjugated secondary antibodies (anti-rabbit, cat. no: R-05072–500 and anti-mouse, cat. no: R-05071–500, Advansta, CA, USA) at room temperature. GAPDH (cat. no: sc-25778, Santa Cruz Biotechnology) was used as a loading control.

Acetone Precipitation of Proteins and Treatments

To obtain proteins in the *S. salivarius M18* cell-free supernatant, acetone precipitation was applied following the procedure defined by Matikevičienė et al. with some modifications [53]. Four times the sample volume of cold (− 20 °C) acetone (ISOLAB, Germany) was added to the cooled (4 °C), filtered probiotic supernatant (SsM18 sup.) or bacteria growth medium (Tryptic Soy Broth, TSB) mixed for 15 min at 4 °C using a rotator, and then incubated 60 min at − 20 °C. After centrifugation for 10 min at $15{,}000 \times g$ at 4 °C, the supernatant was carefully discarded and the remaining acetone was allowed to evaporate from the uncapped tubes at room temperature for 30 min. The pellets were dissolved in ice-cold, sterile dH_2O (25-folds concentrated respect to the initial volume of the supernatant or the control medium TSB) and used immediately or aliquoted and stored at − 80 °C for further use.

The pathogen inhibitory potential of the protein fractions was investigated on *P. aeruginosa* by inoculating the overnight culture of the pathogen as 10^6 CFU/ml in a pH 5.0 or pH 7.0 TSB medium containing 25%, v/v SsM18 sup. or TSB acetone precipitated fractions. After 24 h incubation at 37 °C with shaking, the optical densities of the cultures were measured at 600 nm. The serial dilution-spotting assay was applied to monitor the viability of the pathogen at the end of 24 h incubation. The dilutions were spotted on both TSA plates and *P. aeruginosa* selective Cetrimide agar plates (cat. no.: 1052840500, Merck). The plates were incubated for 12 h at 37 °C. TSA plates were photographed by G:BOX imaging system, equipped with GeneSys image capture software (Syngene), and the Cetrimide agar plates were observed and photographed under a UV transilluminator (DAIHAN Scientific, South Korea).

To evaluate the cytotoxic effect of the precipitated fraction of *S. salivarius* M18 cell-free supernatant (SsM18 sup.), HCT-116 cells were seeded in a 96-well plate as described before. The day after, the cells were treated with the SsM18 sup. or TSB acetone fractions as 5 µl of protein fraction in 100 µl of RPMI-1640 complete growth medium with pH 5.5, 6.0, 6.5, 7.0, or 7.5. At the end of 2 h incubation at 37 °C in a 5% CO_2 incubator, cells were photographed under the


a
OM
(20%, v/v TSB)
SsM18 sup.
(20%, v/v sup.)
RPMI-1640
(pH 7.5)
MTT reduction
(%)
120
100
80
60
40
20
0
****
****
****
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
HCT-116
2h
b
OM
(20%, v/v TSB)
SsM18 sup.
(20%, v/v sup.)
RPMI-1640
(pH 7.5)
MTT reduction
(%)
****
****
****
**
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
HCT-116
4h
c
OM
(20%, v/v TSB)
SsM18 sup.
(20%, v/v sup.)
RPMI-1640
(pH 7.5)
MTT reduction
(%)
***
****
pH 6.5
pH 7.0
pH 7.5
HCT-116
24h
d
OM
(20%, v/v TSB)
SsM18 sup.
(20%, v/v sup.)
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
40 µm

◂**Fig. 1** Treatment with *S. salivarius* M18 cell-free supernatant reduced the viability of HCT-116 cells in lower pH conditions. **a** The inhibitory effect of the probiotic supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium), achieved by detecting the reduction level of MTT, was evident after 2 h and **b** 4 h incubation, and **c** enhanced further after 24 h incubation. **d** The morphology of HCT-116 colon cancer cells after 2 h treatment with *S. salivarius* M18 cell-free supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium) at different pH observed with a light microscope. RPMI-1640 complete growth medium treated cells at physiological pH were used to investigate if the cell viability was affected by the pH change during the incubation periods. The results (mean ± SEM) were normalized to 20%, v/v tryptic soy broth (TSB) containing RPMI-1640 complete growth medium treated cells at pH 7.5 and given as percent (%) change. The experiments were repeated three times each with at least four technical replicates. The statistical significances were calculated by using t-test between each pH treatment group (**$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$)

microscope and the effect of the SsM18 sup. acetone precipitant was determined by MTT assay.

Statistical Analysis

Results are expressed as the mean ± SEM (standard error of the mean). t-test was applied for comparisons using Prism 6.01 (GraphPad, CA, USA) and differences at $p \leq 0.05$ were considered significant (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). Experiments were repeated independently at least two times with technical replicates.

Results

Cell-Free Supernatant of *Streptococcus salivarius* M18 Inhibits the Viability of Colorectal Cancer Cells, and the Inhibitory Effect Is Enhanced by the Prebiotic Inulin

The anti-cancer activity of *S. salivarius* M18 cell-free supernatant was investigated on HCT-116 and SW-480 colon cancer cell lines. First, the amount of the bacterial growth medium, TSB, that can be used to treat the cancer cells was determined. As shown in Fig. S1, 20%, v/v TSB (only medium-OM) in complete RPMI-1640 growth medium did not affect the cell viability and, thus, this supernatant to growth medium ratio was used for further analysis in which the anti-cancer potential of the probiotic supernatant was investigated.

The viability of HCT-116 cells after 2 h and 4 h incubation with the cell-free supernatant of *S. salivarius* M18 (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium) was analyzed by using MTT assay. As shown in Fig. 1a, b, the supernatant inhibited the viability of HCT-116 cells in lower pH environments, but not at physiological pH, pH 7.5, and the inhibition was more prominent in more acidic pH values. A similar effect was also observed with the other colon cancer cell line, SW-480 (Fig. S2). The morphological changes that occurred in the cancer cells treated with the probiotic supernatant were evident even after 2 h of incubation (Fig. 1c; Fig. S2). Although the viability of the cancer cells was not affected much after 4 h incubation at pH 6.5 and pH 7.0, prolonged incubation (24 h) was enhanced the inhibitory effect of the supernatant (Fig. 1d). On the other hand, treatment with the cell-free supernatant collected from the commensal *E. coli* (20%, v/v in RPMI-1640 complete medium with pH 5.5, 6.0, 6.5, 7.0, or 7.5) did not show any inhibition on the viability of colon cancer cells (Fig. S3), indicating that the observed activity was caused specifically by the cell-free supernatant of *S. salivarius* M18.

Inulin is a fermentable carbohydrate by *S. salivarius* M18, and it has been previously shown as a prebiotic for the strain M18 [41]. We found that treatment of HCT-116 colon cancer cells with the supernatant (20%, v/v in RPMI-1640 complete medium) collected from the inulin-incubated (0.5% and 1.0%, w/v) *S. salivarius* M18 enhanced the anti-cancer activity of the probiotic supernatant further (Fig. 2).

3D tumor spheroids, typically applied in the screening of tumor therapies to simulate the 3D structure of tumors in vitro, are compact aggregates of cells that resemble the structure of solid tumors or tumor tissues [54]. Since 3D cancer cell systems represent more physiological models of cancer cell behaviors [55], hanging drop and scaffold-based 3D on-top assays were used to monitor the effect of the probiotic supernatant on the colon cancer cells. In the hanging drop experiments, the spheroids were observed as compact cell aggregates in a pH 7.5 environment while the spheroids of HCT-116 cancer cells dispersed and lost their homogenous, dense, and compact structure after the incubation with the cell-free supernatant of *S. salivarius* M18 (20%, v/v SsM18 sup. in RPMI-1640 complete medium) at lower pH levels (Fig. 3a). Besides, in the 3D Matrigel™ model, the cancer cells incubated with the probiotic supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete medium) at pH 7.5 formed larger aggregates while at lower pH values, the cells made smaller aggregates when they were treated with the probiotic supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete medium) as shown in Fig. 3b. Accordingly, incubation with the cell-free supernatant of the M18 strain (20%, v/v SsM18 sup.) in the Matrigel™ based 3D culture reduced the viability and proliferation capacity of HCT-116 colorectal cancer cells at lower pH conditions (Fig. 3c).

Effect of *Streptococcus salivarius* M18 Cell-Free Supernatant Is Specific to Acidic Environment

2D and 3D colon cancer cell models showed that *S. salivarius* M18 cell-free supernatant has an inhibitory effect in an environment representing the acidic pH nature of the tumors,

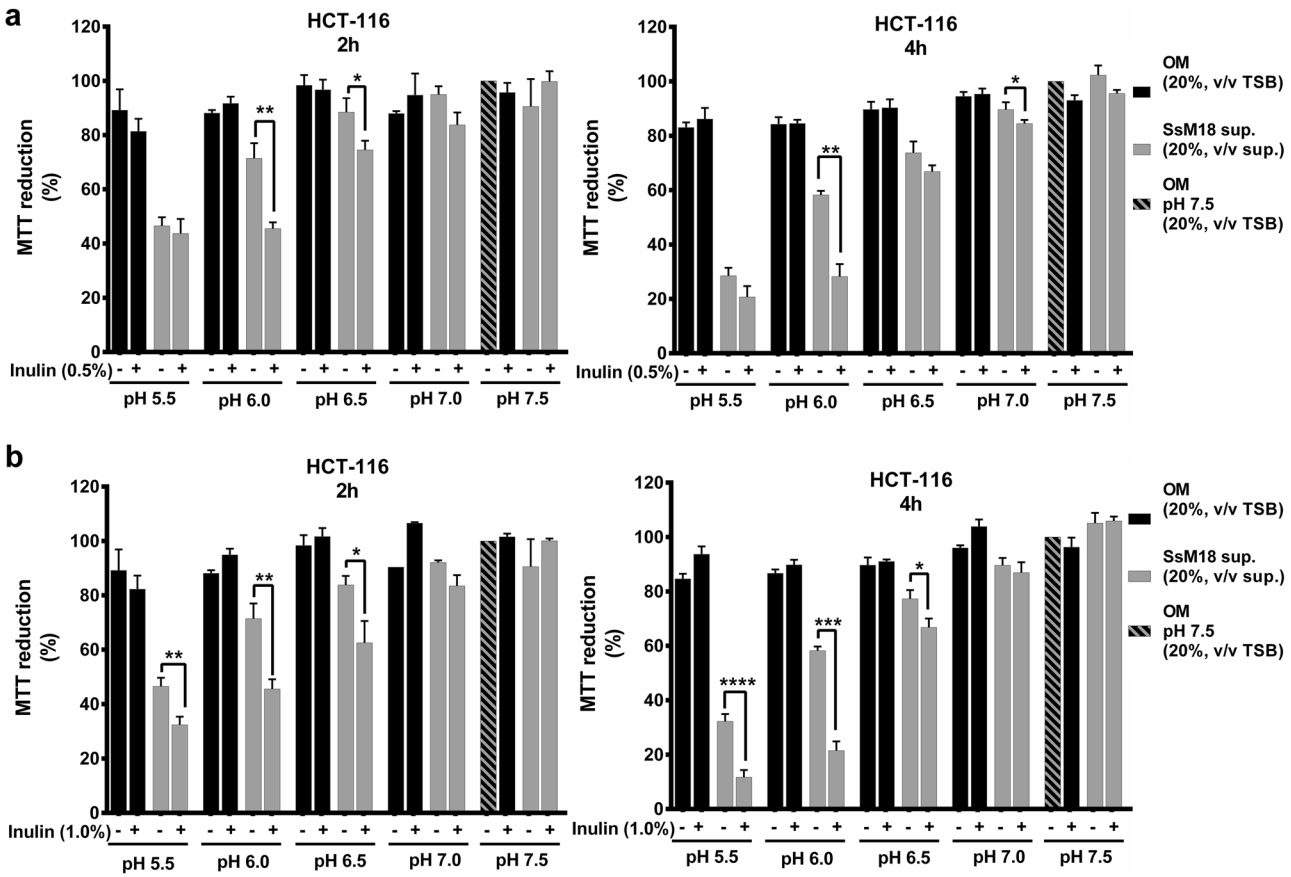


Fig. 2 In the tumor pH mimicking environment, the cell-free supernatant collected from *S. salivarius* M18 incubated with inulin showed an enhanced inhibitory activity on colon cancer cells. Cell-free supernatant collected from (**a**) 0.5%, w/v or (**b**) 1.0%, w/v inulin incubated *S. salivarius* M18 showed an enhanced inhibitory activity on HCT-116 colon cancer cells. The results were normalized to MTT reduction levels in 20%, v/v tryptic soy broth (TSB) containing RPMI-1640 complete growth medium treated cells at pH 7.5 and given as percent (%) change. T-test was used to compare the results obtained from *S. salivarius* M18 supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium) treated HCT-116 cells with the results obtained from the HCT-116 cells treated with the supernatant collected from the *S. salivarius* M18 (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium) incubated with inulin. The data, represented as mean ± SEM, belong to two biological replicates, each with four technical replicates (**$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$)

while the cancer cells were not affected by the treatment at the normal pH of healthy tissue. Targeting the acidic microenvironment should not represent aggression against normal tissues since healthy tissues lack this feature [56]. Therefore, to evaluate if *S. salivarius* M18 cell-free supernatant has any deleterious effect on the healthy, normal cells under the physiological pH condition, the effect of the probiotic supernatant on HUVECs, the primary, non-immortalized cells of human origin was investigated in the pH 7.5 environment. Furthermore, we also asked if *S. salivarius* M18 cell-free supernatant impairs the integrity of the colon epithelial barrier under physiological pH.

Although acidic pH can be toxic to healthy, normal cells, cancer cells, as well as the cells in the tumor microenvironment, can adapt and survive under acidic conditions [36, 57]. As shown in Fig. 4a, 2 h and 4 h treatments with *S. salivarius* M18 supernatant (20%, v/v SsM18 sup.) did not show any cytotoxicity on HUVECs at pH 7.5, but HUVECs, even control medium treated cells, did not survive for a prolonged time (24 h) under acidic conditions (data not shown). On the other hand, as observed previously [58, 59], HUVECs also did not endure the growth factor reduced conditions caused by the addition of the bacterial medium (20% TSB, v/v or 20% SsM18 sup., v/v) for 24 h (data not shown), unlike colon cancer cells (Fig. 1c).

By using Caco-2 cells, we aimed to investigate the effect of the probiotic supernatant on the epithelial barrier integrity under physiological pH. These cells are widely used as a model for intestinal differentiation and barrier function as well as for screening of drugs, delivery systems, and for carrying out cytotoxicological evaluations [60]. Upon reaching confluency, Caco-2 colorectal cancer cells spontaneously

differentiate into enterocyte-like cells, synthesize intestinal enzymes, and form domes [61]. Exhibiting the signs of enterocytic differentiation and transport properties [51, 62], functional polarization of Caco-2 cells was confirmed by dome formation (Fig. 4b, upper panel) and evaluating the expression of the cell density-dependent increase in the expression of CEA after 10 days following confluency [50] (Fig. 4b, lower panel). On day 10, the epithelial cell layers were treated with either the control medium (20% TSB, v/v) or *S. salivarius* M18 cell-free supernatant (20% SsM18 sup., v/v) and the integrity of the epithelial layers determined by CV staining. As shown in Fig. 4c, the supernatant did not damage the cell layer at pH 7.5.

Taken together, these results emphasize that *S. salivarius* M18 cell-free supernatant does not have cellular toxicity and does not harm the integrity of the intestinal epithelium under the normal physiological pH of healthy tissue. Of note, the pH of the large intestine in healthy humans ranges between 7.5 and 8.0 [63].

Streptococcus salivarius M18 Cell-Free Supernatant Triggers Apoptosis of Colon Cancer Cells

In addition to the MTT assay, the effect of the probiotic supernatant on the viability of the cancer cells was also investigated by the trypan blue dye exclusion assay. The negatively charged trypan blue only stains cells with a compromised cell membrane, and so, it can be used to distinguish dead cells from live cells. HCT-116 cells were incubated with the control medium (20%, v/v TSB) or with the probiotic supernatant (20%, v/v SsM18 sup.) for 2 h and 4 h at pH 5.5, and the adherent and floating cancer cells were collected and stained with trypan blue. The results of the staining showed that the probiotic supernatant induces cell death: in Fig. 5a, the numbers of live and dead cells after the treatments are shown on the left and the representative microscope images of the stained cells are given on the right panel.

Although trypan blue dye exclusion assay is a simple and rapid method for visually identifying cells with disrupted cell membranes, the method cannot be used to differentiate between apoptosis and necrosis [64]. To investigate if the cell-free probiotic supernatant induces apoptotic cell death, the expression of the anti-apoptotic marker XIAP and the expression and the activation of caspase-3 were analyzed in HCT-116 cells incubated 2 h with the *S. salivarius* M18 supernatant (20%, v/v SsM18 sup.) or the control medium (20%, v/v TSB) at pH 5.5. Treatment with the probiotic supernatant decreased the expression of XIAP and enhanced the expression and cleavage of caspase-3 (Fig. 5b), indicating the apoptosis promoting activity of *S. salivarius* M18 cell-free supernatant.

Streptococcus salivarius M18 Cell-Free Supernatant Contains Inhibitory Biomolecules Proteinous in Nature

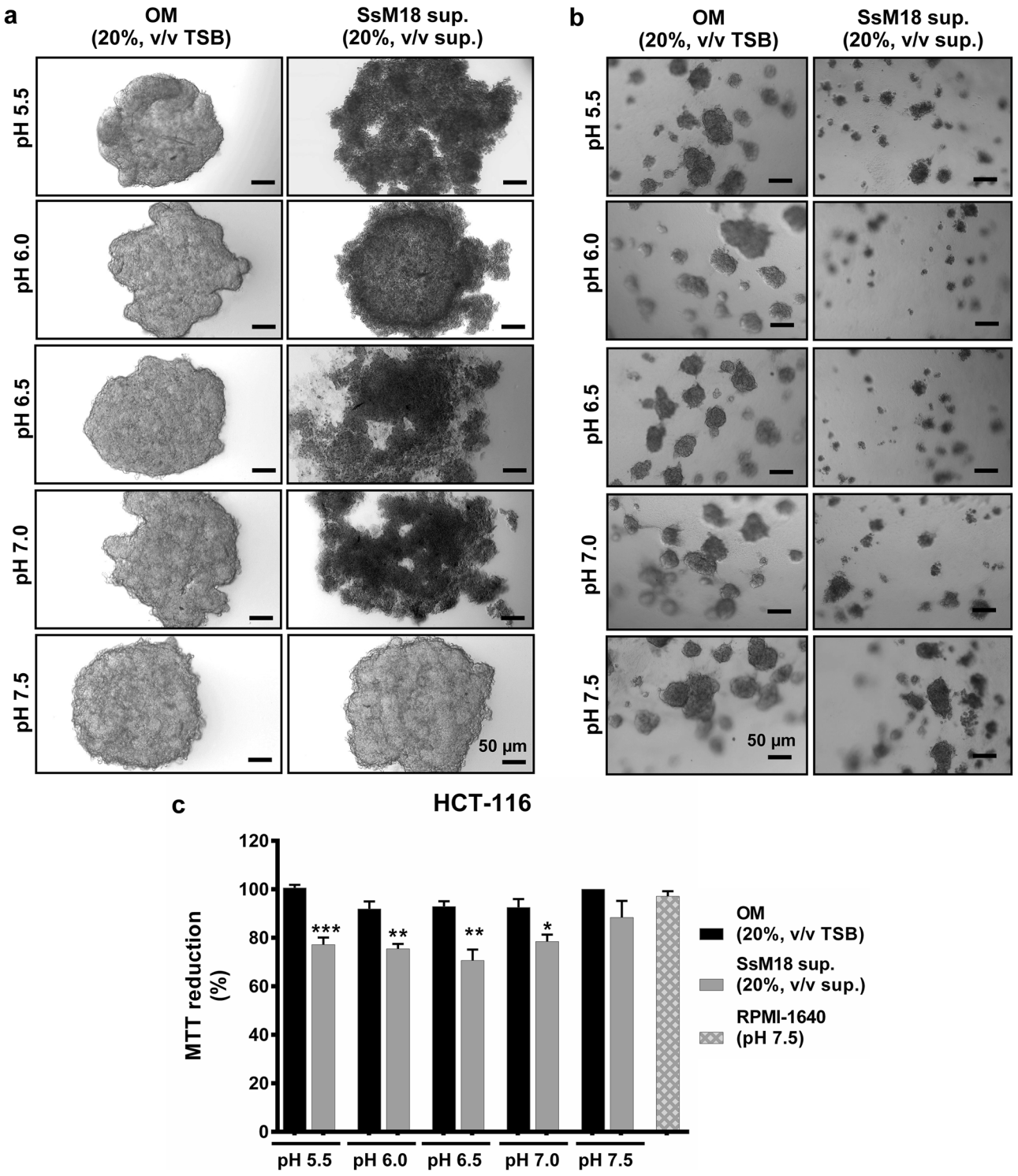
To investigate if the nature of the biomolecules responsible for the anti-cancer and anti-pathogen activities of *S. salivarius* M18 supernatant is proteinous, proteins present in the cell-free supernatant of *S. salivarius* M18 (SsM18 sup.) or control medium (TSB) precipitated using acetone and HCT-116 cells and *P. aeruginosa* were treated with the precipitated protein fractions.

The acetone precipitation fraction of the probiotic supernatant showed a striking growth inhibitory effect on *P. aeruginosa.* According to our previous findings [31], this inhibitory activity of the SsM18 sup. protein fraction was more evident at pH 5.0; an acidic environment. The inhibition was determined by measuring optical densities at 600 nm (Fig. 6a) as well through agar-spotting assay which was performed by spotting multiple dilutions of *P. aeruginosa* onto the Cetrimide Agar (Fig. 6b, on the left) and TSA plates (Fig. 6b, on the right).

The acetone precipitation fraction of the probiotic supernatant was also analyzed for its cytotoxic effect on HCT-116 colon cancer cells. The protein fraction of the probiotic supernatant showed moderate cytotoxicity after 2 h treatment (Fig. 6d); however, the morphological changes caused by the treatment were obvious (Fig. 6e). On the other hand, the inhibitory activity of the protein precipitated fraction of *S. salivarius* M18 cell-free supernatant was less than the cytotoxic activity of the non-precipitated supernatant (Fig. 1a). This result might be caused by protein and/or protein activity loss during the precipitation [65].

Discussion

Because of glycolytic cell metabolism, hypoxia, insufficient blood perfusion, and inflammation, the external pH of the tumor is acidic [33] and tumor acidosis is becoming increasingly recognized as one of the most distinctive hallmarks of solid tumors [66]. Although adaptation to the acidic environment provides a fitness advantage to cancer cells and low pH has been implicated in enhancing malignancy in several tumor types, this chemical nature also exposes tumors to novel therapeutic vulnerabilities. For instance, small molecules and antibodies targeting pH regulators or metabolic products of acidosis have been developed; neutralization of tumor-derived acid with systemic buffers was investigated as a therapeutic approach to reduce the aggressiveness of tumor cells by directly interfering with tumor acidification; and acid-activatable drugs and nanomedicines have been designed to selectively deliver therapeutic agents [67, 68]. To the best of our knowledge, this is the first study



evaluating the anti-cancer potential of the postbiotic products of a probiotic strain in tumor pH mimicking conditions.

Probiotics have gained attention due to their beneficial effects on the prevention and management of several diseases, including cancer [69]. On the other hand, because of theoretical risks related to the use of the live form of probiotic cells, there is a current attempt to use non-viable probiotic-derived biomolecules, namely postbiotics instead of viable probiotic bacteria. The results presented here exhibit that postbiotics of the oral probiotic *S. salivarius* M18 has an anti-cancer activity on CRC cell lines in low pH environments mimicking the external pH of tumors. In 2D

◂**Fig. 3** Effect of *S. salivarius* M18 cell-free supernatant treatment on colon cancer cells were investigated in 3D cultures. **a** In hanging drop assays, HCT-116 cells were allowed to form aggregates for 48 h and then the medium was replaced with the RPMI-1640 complete growth medium (pH 5.5, 6.0, 6.5, 7.0, or 7.5) containing 20%, v/v *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or 20%, v/v tryptic soy broth-TSB. The aggregates were observed and photographed under a light microscope at the end of 24 h incubation. **b** In the scaffold 3D culture model, HCT-116 cells were allowed to form spheroids in 6 days by replacing Matrigel™-medium (pH 5.5, 6.0, 6.5, 7.0, or 7.5 RPMI-1640 complete growth medium containing 20%, v/v SsM18 sup. or 20%, v/v bacteria growth medium-TSB) mixture every 2 days. The spheroids were photographed under an inverted light microscope, and **c** the inhibitory activity of the *S. salivarius* M18 cell-free supernatant on the HCT-116 spheroid formation was investigated by MTT assay. The MTT assay was performed two times each with six technical replicates. The results (mean ± SEM) were normalized to 20%, v/v tryptic soy broth-TSB containing RPMI-1640 (pH 7.5) treated samples and represented as percent (%) change. T-test was used to determine statistical significance between each pH treatment group (**$p \leq 0.01$; ***$p \leq 0.001$). The 3D culture images are representative of two independent experiments

in vitro studies, prominent morphological changes accompanying the viability reducing and apoptosis promoting effects of the probiotic supernatant were shown. Additionally, the supernatant collected from the inulin incubated *S. salivarius* M18 enhanced the observed anti-cancer activity further, which may be caused by the inulin-driven increased production of probiotic end-products [70], including antimicrobial peptides [71]. Since 3D cell culture provides a useful platform for further identifying the biological features of cancer cells, we also evaluated the effect of the probiotic supernatant by using scaffold independent and scaffold dependent 3D culture techniques. In hanging drop experiments, treatment with *S. salivarius* M18 cell-free supernatant under lower pH conditions resulted in the dispersion of the pre-formed colon cancer cell aggregates: as an indication for cell loss, the aggregates lost their integrity after the treatment [72]. However, the control medium treated aggregates preserved their structure: the aggregates remained tight and compact with clear boundaries as evidence of aggressive invasive phenotype [73]. Therefore, it can be suggested that the probiotic supernatant has the potential to antagonize the pre-formed tumor. In the Matrigel™ based 3D culture model, control medium treated cells assembled as larger spheroids which were reported as a feature of chemoresistance [74, 75] while colon cancer cells treated with the probiotic supernatant formed smaller aggregates and showed reduced proliferation capacity in lower pH environments, suggesting that the biomolecules present in the probiotic supernatant can limit the tumor-forming capacity of pre-transformed cells.

The tumor microenvironment most commonly includes cells of the immune system, fibroblasts, pericytes, and endothelial cells of the vasculature and lymphatics [76, 77]. In this microenvironment, normal cells are modified and co-opted by the cancer cells to produce a variety of tumor-promoting factors such as chemokines, growth factors, and matrix-degrading enzymes that extend the proliferation and invasion capabilities of the tumor [78]. For instance, in a tumor microenvironment, tumor endothelial cells (TECs) can survive and proliferate under low pH conditions through enhanced expression of vascular endothelial growth factor (VEGF) signaling–driven carbonic anhydrase 2 (CAII) and they support tumor angiogenesis [57]. Although tumor cells together with the co-opted cells in the tumor microenvironment are aimed to be simultaneously inhibited for efficient therapies [78], off-target adverse effects on the healthy, normal cells can cause toxic effects and also limit the efficacy of cancer therapeutics by lowering the dose of an anti-cancer molecule administered [79]. In this study, primary endothelial cells were used to investigate if the probiotic supernatant has any deleterious effects on normal, healthy cells, except the tumor-associated stromal cells undergone tumor-driven re-programming. We showed that at physiological pH, the probiotic supernatant did not show any inhibitory activity on normal, healthy cells; besides, the supernatant did not damage the integrity of the colon epithelial barrier in vitro.

Finally, we investigated that to a considerable extent, the observed anti-pathogen and anti-cancer effects of the *S. salivarius* M18 cell-free supernatant rely on the postbiotics proteinous in nature. Even though proteins and peptides derived from bacteria arise as an alternative strategy to combat cancer, decreasing or eliminating cytotoxic effects on normal, healthy cells and increasing the specificity of the targeting are still important issues [80, 81]. Therefore, fast effective and tumor microenvironment-specific action of the *S. salivarius* M18 postbiotics make them good candidates for further characterization. The active biomolecule(s) responsible for the observed anti-pathogen and anti-cancer effects is aimed to be elucidated in more detail in future studies by concentrating on the bacteriocins of *S. salivarius*, referred to as salivaricins [31].

The colonization pattern of *Streptococcus salivarius* suggests that the bacterium can play an important role not only in the oropharyngeal but also in digestive tract ecology [82]. Although the oral cavity and the colon are distant anatomic regions, recent studies indicate that oral bacteria can disseminate into the colon through saliva or by the bloodstream and systemic circulation [83, 84]. Epidemiologic studies suggest that periodontal disease is associated with an increased risk of CRC and periodontal disease-causing oral pathogens propounded to be involved in the pathogenesis of a variety of systemic diseases as well as CRC [85, 86]. The associations between oral and intestinal microbiota compositions and CRC carcinogenesis were very well-reviewed by Koliarakis et al. [83] and Olsen and Yamazaki [87]. As a periodontal pathogen, *Fusobacterium nucleatum* and also other *Fusobacterium* species, especially phylotypes with the greatest similarity to *F. nucleatum*, *F.*

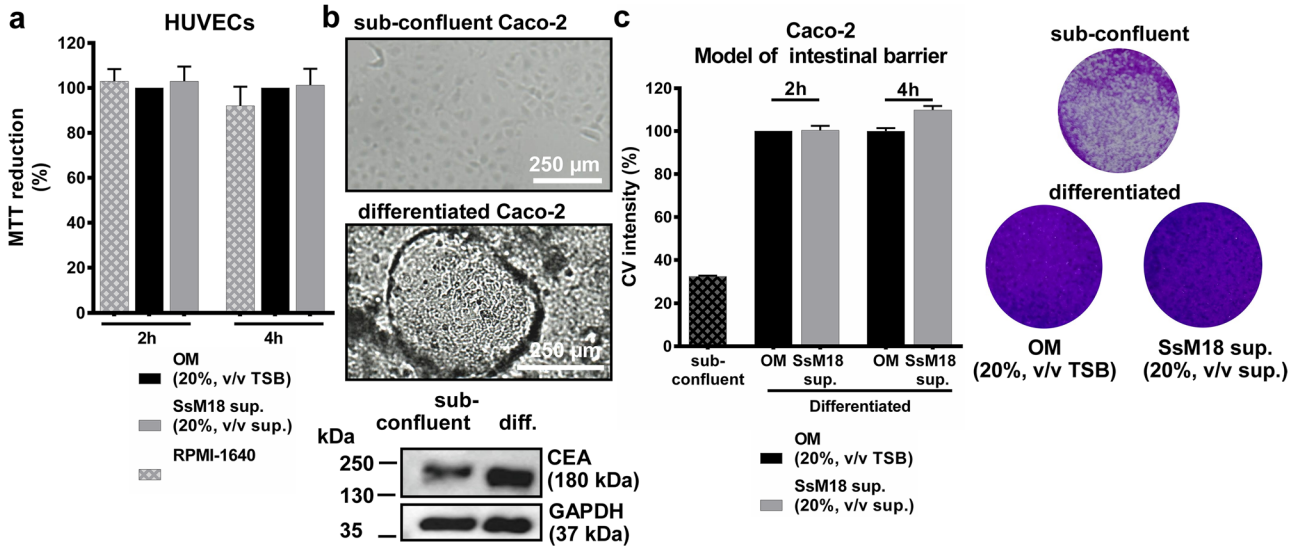


Fig. 4 The effect of *S. salivarius* M18 cell-free supernatant was investigated at physiological pH on normal, healthy endothelial cells (HUVECs) by MTT assay and on epithelial barrier integrity by crystal violet (CV) staining. **a** The results of the MTT assays (2 h and 4 h after treatment) were normalized to control medium (20%, v/v bacteria growth medium-TSB in RPMI-1640 complete growth medium) treated cells and shown as percent (%) change. **b** In spontaneously differentiated cultures of Caco-2 cells, dome formations were observed under a light microscope (upper panel). The confluency-dependent increase in CEA expression was detected by western blot in which GAPDH was used as a loading control (lower panel). **c** Effect of the *S. salivarius* M18 cell-free supernatant (20%, v/v SsM18 sup.) treatment (2 h and 4 h) on barrier integrity at pH 7.5 was evaluated using Caco-2 spontaneous differentiation model through CV staining. Intensities of the CV stain were normalized to 2 h 20%, v/v TSB-treated cells and given as percent (%) change (on the left). Representative well images after CV staining are shown on the right panel. The results (mean ± SEM) belong to two biological replicates with technical replicates

mortiferum, and *F. necrophorum*, were found to be enriched in cancer tissue or stool samples of CRC patients [84, 88]. The isolation of identical clones of *F. nucleatum* from the oral cavity and tissue specimens of CRC in the same subjects has further confirmed the association of oral *F. nucleatum* with CRC [89]. Besides, *Fusobacterium* spp. were suggested to be associated

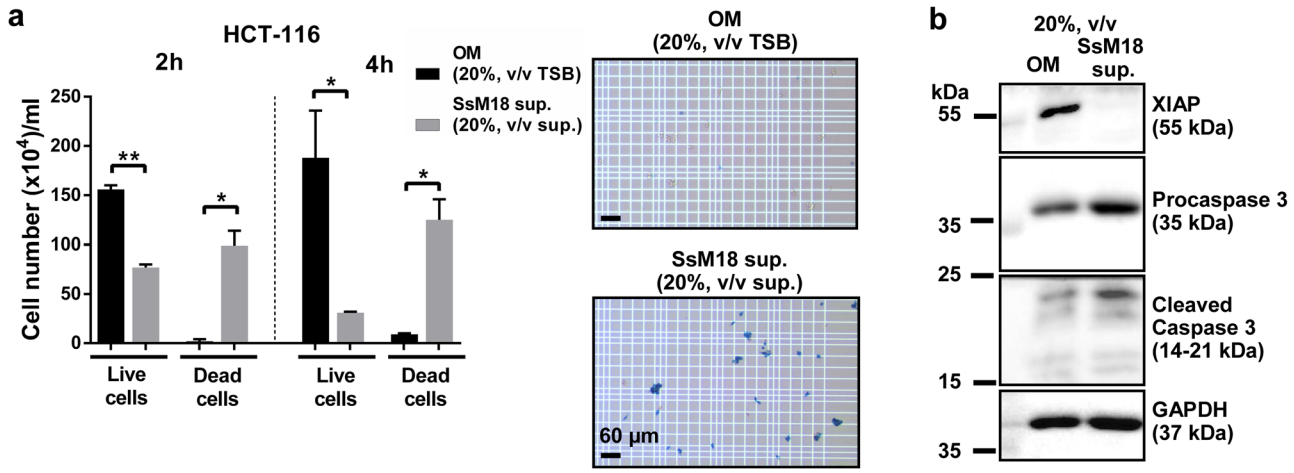


Fig. 5 Cell death promoting effect of *S. salivarius* M18 cell-free supernatant on HCT-116 colon cancer cells was investigated. **a** After 2 h and 4 h treatments with the bacteria growth medium-TSB (20%, v/v in RPMI-1640 complete growth medium) or with the probiotic supernatant (20%, v/v SsM18 sup. in RPMI-1640 complete growth medium) at pH 5.5, HCT-116 cells were stained with trypan blue and counted by Thoma slide. Numbers of live and dead cells are shown on the left and the representative images of the Thoma slide are shown on the right. The results belong to two independent experiments. Two counting chambers were counted per slide for technical replicates. **b** Western blot was used to detect changes in the expressions of XIAP and caspase-3 after 2 h treatment with the probiotic supernatant (20%, v/v SsM18 sup.) at pH 5.5. GAPDH was used as a loading control

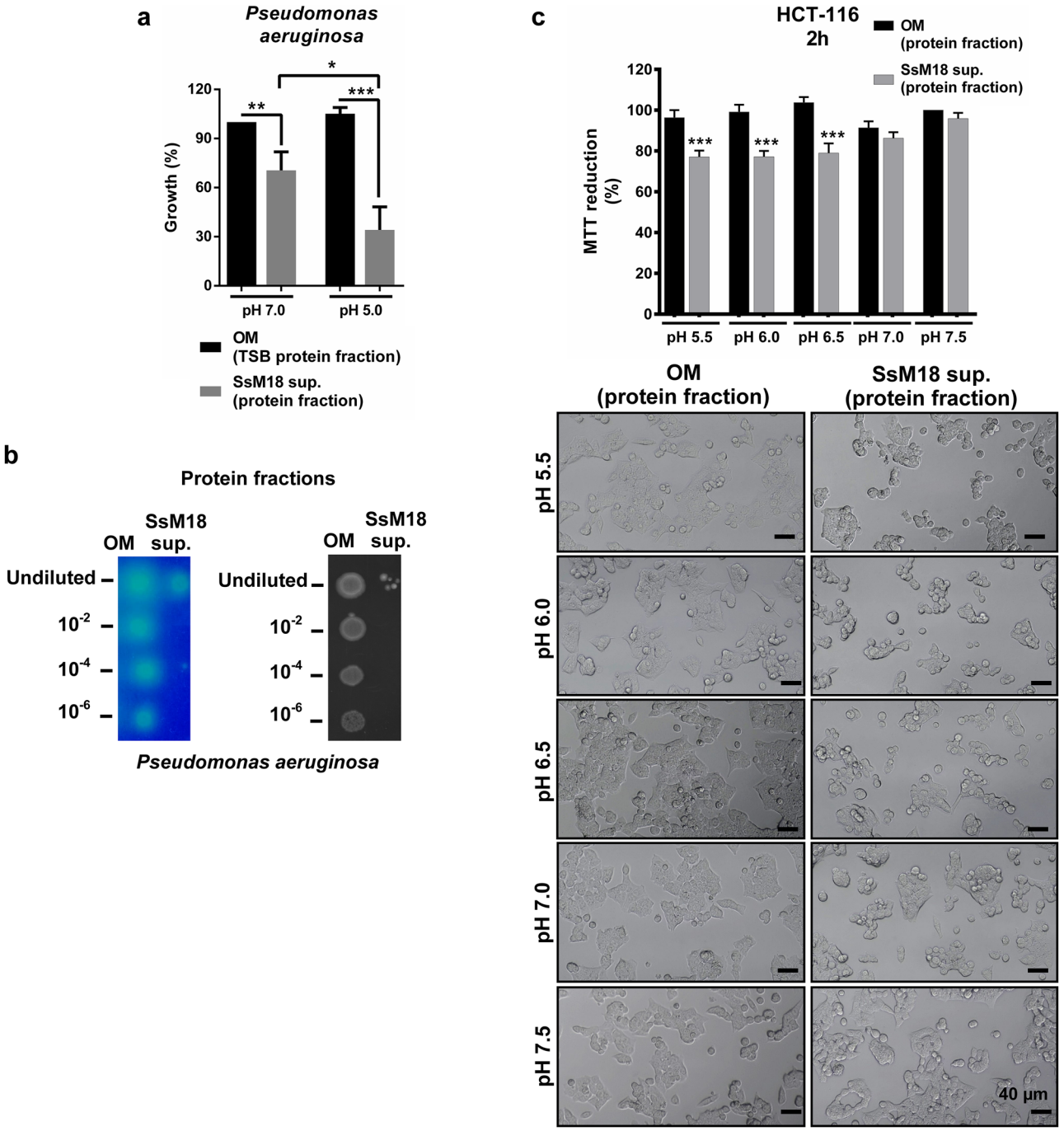


Fig. 6 The growth inhibitory potential of the protein fraction of *S. salivarius* M18 cell-free supernatant was investigated on *P. aeruginosa* and HCT-116 cells. **a** *P. aeruginosa* treated with the acetone precipitated fractions of the probiotic supernatant (SsM18 sup.) or control medium (TSB) at pH 7.0 or pH 5.0. After 24 h incubation, OD_{600} values were measured and normalized to *P. aeruginosa* treated with TSB protein fraction (pH 7.0) and represented as percent (%) change. The results, which belong to three independent replicates, are shown as mean ± SEM. T-test was used to determine statistical significance. **b** *P. aeruginosa* was grown at pH 5.0 in the presence of OM (TSB) or SsM18 sup. acetone precipitated fractions for 24 h and then spotted on the Cetrimide agar (on the left) or TSA plates (on the right). **c** HCT-116 cells were treated for 2 h with the RPMI-1640 complete growth medium (pH 5.5, 6.0, 6.5, 7.0, or 7.5) containing TSB or SsM18 sup. acetone precipitation fractions. The changes in the MTT reduction levels (upper panel) were normalized to TSB precipitation fractions treated cells at pH 7.5 and given as percent (%). The morphologies of HCT-116 cells after 2 h treatment with the TSB or SsM18 sup. acetone precipitation fractions are shown in the lower panel. The experiments were repeated three times with technical replicates and shown as mean ± SEM. T-test was used to determine statistical significance between each pH treatment group (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$)

with inflammatory bowel disease, one of highest risk factors for CRC [88]. Accordingly, in the Apc(Min/+) mouse model of intestinal tumorigenesis, *F. nucleatum* increased tumor multiplicity and supported the neoplasia progression through recruitment of tumor-infiltrating immune cells and generating a proinflammatory microenvironment [90]. The periodontal pathogen *Porphyromonas gingivalis* was also proposed as a risk factor for gastrointestinal cancers, including CRC [91, 92]. In another study in which the risk of developing CRC was investigated in pre-diagnostic mouth rinse samples in a large cohort by using 16S rRNA gene sequencing, pathogenic taxa including *Treponema denticola* and *Prevotella* (*P. denticola*, *P. intermedia*, *Prevotella* sp. oral taxon 300) were significantly and positively associated with increased risk of CRC development [93]. Interestingly, *Streptococcus* and *Fusobacterium* were found to be negatively correlated in saliva [94, 95] and *S. salivarius* M18 was shown to have an inhibitory effect on the oral pathogens *P. gingivalis*, *P. intermedia* [96, 97], and *T. denticola* [97]. To functionally explore if there is a link between the oral and colon abundance of *S. salivarius* M18 and defined pathophysiological processes in the host, strain-specific genome-wide association studies are needed. Results obtained from the studies designed to investigate oral-colon interactions, especially on the strain-specific identification level, can be promising for the development of functional probiotic products capable of fostering a healthy oral microbial diversity for preventing and treating periodontal diseases as well as systemic diseases and malignancies of the gastrointestinal system.

In summary, the results of this study show that under low pH conditions, the postbiotics of the oral probiotic *S. salivarius* M18 have anti-cancer activities on colon cancer cells in vitro. This property of the probiotic supernatant merits investigation in more detail as a potential therapeutic exploitation approach in the treatment of solid tumors.

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Data Availability All data generated during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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