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Putative probiotics decrease cell viability and enhance chemotherapy effectiveness in human cancer cells: role of butyrate and secreted proteins

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ABSTRACT

Recent advances have highlighted probiotic role in preventing colorectal cancer, by promoting differentiation, inhibiting proliferation, and inducing apoptosis in colonocytes. Here, three ascertained probiotics (*L. rhamnosus* GG ATCC 53103, *L. reuterii* DSM 17938 and *L. johnsonii* LC1) and four food-isolated putative probiotics (*L. plantarum* S2, *L. plantarum* O2, *L. pentosus* S3, *L. rhamnosus* 14E4) were investigated for their ability to adhere to HT29 cancer cells and to inhibit their and the chemoresistant counterpart (HT29-dx cells) proliferation. Three putative probiotics (S2, S3 and 14E4) were able to decrease viability of both sensitive and chemo-resistant HT-29 cells. Supposing this effect related to secreted metabolites (namely short chain fatty acids (SCFA), exopolysaccharides (EPS) and extracellular proteins) we tested the efficacy of extracellular extracts and butyrate with or without the chemotherapeutic agent doxorubicin (DOXO) (10 µM, 4 hours). Increased production of mitochondrial reactive oxygen species (ROS) in HT29 and HT29-dx cells was observed. Moreover, cell exposure to DOXO (10 µM, 24 hours) and extracellular extracts (48 hours) reduced cell viability. Comparative phenotypic and secretome analyses on the effective/non effective strains, revealed quantitative/qualitative differences in EPS content and protein profiles, suggesting that P40, phage-tail-like and capsid-like proteins may be also involved. These results suggest that food-isolated bacteria releasing bioactive compounds (butyrate, EPS and peculiar proteins) may control cancer cell proliferation and improve their response to chemotherapy.

Keywords: Lactic acid bacteria, colorectal cancer, doxorubicin resistance, proteomics, phage proteins, P40-proteins.

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INTRODUCTION

Cancer is one of the primary causes of death in the western countries and a significant one in the world. Colorectal cancer (CRC) is the third most common type of cancer and has the third highest rate of cancer-associated mortality worldwide (Siegel et al., 2018). Radical surgery combined with adjuvant chemotherapy serves as the mainstream therapeutic scheme for most CRC patients. Unfortunately, the development of multidrug resistance (MDR) may result in failure of the chemotherapy treatment, leading to tumor relapse and further progression (Simon et al., 1994). The overexpression of transporters that extrude anticancer drugs, (Gottesman et al., 2002) enhanced ability of cancer cells to repair drug-induced DNA damage, altered expression of pro- or anti-apoptotic factors, and enhanced stress responses (Hanahan and Weinberg, 2011) are the best studied mechanisms of MDR.

Gut dysbiosis can contribute to colon cancer development by promoting chronic inflammatory conditions and the production of carcinogenic metabolites, resulting in activation of angiogenesis and cellular proliferation pathways (Tjalsma et al., 2012), ultimately leading to neoplasia (Sun and Kato, 2016). Metagenomic data suggests that an increased abundance of some bacteria (including *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Escherichia coli*) is associated with cancer (Kostic et al., 2012).

Several studies have shown that probiotics can have the potential to be employed in cancer prevention and therapy (Zhu et al., 2013; Mohania et al., 2013 Prisciandaro et al., 2012; Ma et al, 2021, Srikham et al., 2021; Wang et al, 2021). They can: i) degrade or quenching potential carcinogens originating from food or gut bacteria metabolism (Zhu et al., 2013), ii) modulate gut-associated and systemic immune functions (Yazdi et al., 2013), iii) control apoptosis (Mohania et al., 2013), thus reducing metastasis and attenuate chemotherapy-induced inflammation (Prisciandaro et al., 2012). In addition, studies showed a relation between gut microbiota and the efficacy and toxicity of

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chemotherapies (oxaliplatin, irinotecan) and immunotherapies (including ipilimumab), since tumor-targeting bacteria could also prompt quiescent, chemotherapy-resistant cells to enter the cell cycle and become drug sensitive (André et al., 2004; Sougiannis et al., 2019). Cousin et al. (2012) reported that fermented milk containing *Propionibacterium freudenreichii* enhanced the cytotoxicity of camptothecin that was used as a chemotherapeutic agent to treat chemically-induced gastric cancer. These results could open interesting perspectives on probiotic-based regimens as an adjuvant treatment during anticancer chemotherapy. However, no details on what occurs at the molecular level are reported, therefore a better understanding of the effect of probiotic bacteria on cancer progression and chemotherapy effectiveness is necessary.

In the present work, the role of three ascertained probiotics [*Lactobacillus (L.) rhamnosus* GG*, L. reuterii* DSM 17938 and *L. johnsonii* LC1 of human origin] and four putative probiotics [*L. plantarum* S2, O2 and *L. pentosus* S3 isolated from green table olive fermentations (Botta et al., 2014), the *L. rhamnosus* 14E4 isolated from ripened hard cheese (Bautista-Gallego et al., 2014)] was explored. We demonstrated that the addition of cell-free supernatants of some of these putative probiotics to doxorubicin treatment was associated with the increased sensitivity to doxorubicin in human colon adenocarcinoma cells (HT29 cells) and the reversion of doxorubicin resistance in their chemo-resistant counterpart, the HT29-dx cells. Some phenotypic traits of the most active strains were investigated to detect the molecular mediators of such inhibitory action namely short chain fatty acids (SCFA), exopolysaccharides (EPS) and proteins present in the secretome. In particular, we studied the effect of sodium butyrate on cell viability and on mitochondrial ROS production in HT29 and HT29-dx cells, we quantified the EPS production and analyzed by in gel proteomics and PMF the secretomes of the strains.

MATERIALS AND METHODS

Media, reagents, cell cultures and bacterial strains

Human colon adenocarcinoma (HT29) cell line obtained from the American Type Culture Collection (Rockville, MD, USA) was grown as a sub-confluent monolayer in Roswell Park Memorial

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Institute (RPMI) 1640 medium, containing 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, and 10 % (v/v) fetal bovine serum (FBS).

The chemoresistant counterpart (HT29-dx cells) line was generated by culturing parental cells in the presence of increasing concentrations of doxorubicin for 20 passages (Riganti et al., 2005). HT29-dx cells have higher Pgp, MRP1 and breast cancer resistance protein (BCRP) than HT29 cells. Moreover, compared to HT29 cells, it has been reported that HT29-dx cells have a higher half maximal inhibitory concentration (IC_{50}) for doxorubicin, irinotecan, oxaliplatin and 5-fluorouracil representing a reliable model of MDR cells (Gelsomino et al., 2013) and in addition vinblastine, etoposide, cisplatin, methotrexate, pemetrexed, gemcitabine, mitoxantrone, thapsigargin, tunicamycin, brefeldin A (Salaroglio et al. 2017). For the present work, HT29-dx cells were grown in RPMI 1640 medium containing 150 nM doxorubicin. Doxorubicin is supplied by Sigma Chemical-Aldrich; sterile material comes from Falcon (Becton Dickinson, Franklin Lakes, NJ); the protein concentration in cell lysates was assessed with the BCA kit from Sigma-Aldrich.

The putative probiotics *L. plantarum* S2 (extended strain code: S2T10D), *L. plantarum* O2 (O2T60C), *L. pentosus* S3 (S3T60C), originated by fermented olives (Botta et al., 2014), *L. rhamnosus* 14E4 originated by long seasoning cheese (Bautista-Gallego et al., 2019) and the proved probiotic strain *L. rhamnosus* GG (ATCC 53103), *L. reuterii* DSM 17938 and *L. johnsonii* LC1 were routinely grown in Man Rogosa Sharpe (MRS) broth (Lab M, Heywood, Lancashire, UK) and stored in the same medium at – 80 °C with 20 % of glycerol (Sigma-Aldrich).

Before each co-culture experiment with human colon carcinoma cells, a single fresh colony of each bacterial strain was resuspended in the appropriate culture broth, grown overnight and then inoculated at ratio 1:100 in new fresh broth. This suspension was grown until bacteria reached the early stationary phase (18 h), and then used for the evaluation of the adhesion capability (working culture). The initial concentration of each working culture was determined by OD at 630 nm with ELx880 microtiter plate reader (Savatec, Turin, Italy) and set to the same initial count (internal standard curve).

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Evaluation of *in vitro* adhesion capability

The *in vitro* adhesion, intended as the capability of the strains on an undifferentiated colon carcinoma monolayer was evaluated according to Cambronel et colleagues (Cambronel et al, 2019), with some modifications. HT-29 cells were seeded at 60,000 cells/well (c/w) in 96-well microtiter plates and grown for 2-4 days until they formed a confluent monolayer.

The working culture of viable bacteria was re-suspended in appropriate media as described before and added to the monolayer at a concentration of 8.0 ± 0.2 Log CFU mL^{-1}, and incubated for 90 minutes in a modified atmosphere of 5% CO_2 and 95% air. The initial amount of added bacteria was quantified in each experiment by serial dilution and plating on MRS. The confluent monolayers were washed three times with PBS and then bacterial and eukaryotic cells were homogenized with a Triton-X solution (0.25 % in PBS). After 30 minutes of incubation, the solution with released bacteria was serially diluted and plated on MRS agar. The plates were incubated for 48 hours at 30 °C in microaerophilic conditions (concentration of oxygen lower than 20 %). In parallel, assays were carried out on each bacterial strain to exclude any potential harmful effect on the survival of bacteria due to the treatment with the 0.25 % Triton-X solution.

The adhesion ability was expressed as the percentage ratio between the bacterial cells (CFU cm^{-2}) initially seeded (T_0) and those that were adherent to carcinoma monolayer after the washing steps (T_1), following the formula: $T_1 / T_0 \times 100$.

Bacterial cell-free supernatants preparation

The supernatants used came from the following bacterial strains of bacteria: *L. plantarum* S2, *L. pentosus* S3, *L. rhamnosus* 14E4. To produce bacterial cell-free supernatants (CFS) standardized suspensions of viable bacteria were washed twice in PBS and inoculated in DMEM at 8.0 ± 0.2 Log CFU mL^{-1}, supplemented with 2 mM of L-glutamine and without antibiotics and FBS (Botta et al, 2017a). After 24 hours of incubation at 37 °C the CFS were obtained by centrifugation (20,000 x g for 10 min) and subsequent sterilization with a 0.22 μm filter. At the beginning and at the end of the 24 h of incubation pH was recorded with pH-meter and concentration of bacteria was enumerated

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by serial dilution and plating on MRS agar. Part of the CFS was buffered with sterile 0.5 M NaOH solution to restore the initial pH of the medium (pH 7.80 ± 0.20) and thus, obtain a neutralized cell-free supernatant (CFSn).

The CFS and CFSn were thus supplemented with 10 % of FBS and antibiotics as described for the media preparation, kept at – 80 °C until use, and then tested on colon carcinoma cells as such or enriched with 2 g/L of glucose and 2 mM of L-glutamine and used non-diluted.

Sodium butyrate test

Concentrations of sodium butyrate here used have been chosen on the base of butyrogenic capability of the olives derived strains, previously observed in Human cells culture media, which ranged from 4 to 12 mM (Botta et al, 2017a; Botta et al 2017b).

Measurement of Cell Viability

The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a cellular culture (Repetto et al, 2008). Cells were seeded in 96-well tissue culture plates (25,000 cells/well). After 24 hours, the cells were incubated in fresh medium in absence or presence of sodium butyrate (4, 8 and 12 mM) or bacterial supernatants S2, S3 and 14E4 (not diluted) for 24 hours, followed by further 24 hours of DOXO (10 μM).

The plate was then incubated for 1 hour at 37 °C with a medium containing Neutral Red solution. The cells were subsequently washed and rinsed with the stop buffer (1:1 of 4.02 g trisodium citrate in 153 mL H_2O, 0.8 mL HCl 0.1 N in 86 mL H_2O and 25 mL of 95% v/v methanol). The absorbance was read at 540 nm and the cell viability was assessed by measuring the percentage of cells stained with neutral red dye. The viability of untreated cells was considered 100%; the results were expressed as percentage of viable cells in each experimental condition versus untreated cells.

Isolation of Mitochondria and detection of Mitochondrial ROS Production

To isolate mitochondrial fractions, 5×10^6 cells (previously treated with sodium butyrate (8, 12 and 24 mM) or bacterial supernatants S2, S3 and 14E4 (non-diluted) for 20 hours plus a bolus of DOXO (10 μM for 4 hours) were washed twice in PBS, then lysed in 0.5 mL buffer A (50 mM Tris, 100

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mM KCl, 5 mM $MgCl_2$, 1.8 mM ATP, 1 mM EDTA, pH 7.2), supplemented with protease inhibitor cocktail III (Sigma Aldrich, Milan, Italy), 1 mM PMSF and 250 mM sodium fluoride (NaF). Samples were sonicated (1 bursts of 10 seconds, amplitude 40%), clarified by centrifuging at 2000 rpm for 1 minute at 4 °C, and the supernatant was collected and centrifuged at 13,000 rpm for 5 minutes at 4 °C. This supernatant was discarded and the pellet containing mitochondria was washed in 0.5 mL buffer A and resuspended in 0.5 mL buffer B (250 mM sucrose, 15 mM K2HPO4, 2 mM MgCl2, 0.5 mM EDTA, 5% w/v BSA). Then mitochondrial fractions were incubated for 30 minutes at 37 °C with 10 μM of the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetoxymethyl ester (DCFDA-AM), centrifuged at 13,000 × g at 37 °C, washed twice with PBS and re-suspended in 0.5 mL PBS. The fluorescence of each sample, proportional to the amount of ROS, was read at 504 nm (λ excitation) and 529 nm (λ emission), using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The results were expressed as RFU/mg cell proteins. A 20 μL aliquot was sonicated and used for the measurement of protein content (Bergandi et al, 2018).

EPS: ropy phenotype and quantification. Cultures of *L. rhamnosus* GG*, L. rhamnosus* 14E4*, L. plantarum* S2 and *L. pentosus* S3 were grown in Semi Defined Medium (SDM), previously selected as the best inducer of EPS production in lactic acid bacteria (Cirrincione et al, 2018), containing: Lactose 100 g/L Yeast Nitrogen Base 5 g/L, Tryptone 10 g/L, K_2HPO_4 2 g/L, $MgSO_47{\cdot}H_2O$ 0.1 g/L, $MnSO_4$ $4{\cdot}H_2O$ 0.05 g/L, Ammonium Citrate 2 g/L, Sodium Acetate 5 g/L. After 72 hours of growth at 30°C, cultures were centrifuged (4,000 xg, 20 min, 4°C) and supernatants collected. The ropy phenotype was assessed by insertion of an inoculation loop into the cell pellet and visual inspection of filament formation when the loop was lifted (and Ruas-Madiedo and de los Reyes-Gavilan 2005). Turbidities of supernatants were assessed by measuring the optical density at 600 nm (OD600) (Spectrophotometer Ultrospec 2000, Pharmacia Biotech). For EPS quantification, the strains were grown in SDM for 72 hours at 30°C. Cell-free culture supernatants were obtained by centrifugation (4,000 xg, 20 minutes, 4°C). 95% (v/v) cold ethanol (4°C) was added to supernatants

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in 2:1 ratio. Tubes were stored for 24 h at 4°C. The precipitated polysaccharides were collected by centrifugation (4,000 xg, 20 minutes, 4°C) and the pellets were dissolved in warm ddH_2O. The precipitation step was repeated once and, finally, EPS precipitates were dissolved in warm ddH_2O and stored at -20 °C. The EPS were assayed for carbohydrate content by the phenol–sulfuric acid method, as described in (de Jesus Raposo et al., 2014), with slight modifications. Briefly, ddH_2O water was added to 20 µL of EPS solution to reach a final volume of 2 mL in a test tube, and glucose 1mg/mL was used as standard. Fifty µL of 80% (w/v) phenol and 5 mL of sulfuric acid (H_2SO_4) were added quickly to each sample. The tubes were then vortexed and incubated at room temperature for 45 minutes, afterwards, the optical density at 490 nm was measured.

Extracellular proteomic evaluation

Preparation of extracellular protein extract

L. rhamnosus GG and *L. rhamnosus* 14E4 were grown in MRS broth at 37°C and the cultures were harvested in middle exponential phase. Cells of each culture were removed by centrifugation (4,000 xg, 20 min, 4°C) and cell free supernatants were filtered in stericup 0.22 µm filters (Millipore). Protease inhibitor cocktail and 16% (p/v) trichloracetic acid was added to supernatants and incubated under shaking overnight at 4 °C allowing extracellular proteins precipitation (Mangiapane et al, 2014). The samples were then centrifuged (35,000 xg, 60 min, 4 °C). Pellets were dried and re-suspended in 70 % ethanol for two washing steps. Pellets were then re-suspended in the possible smallest volume of Tris-HCl 50 mM pH 7.3. One mL of phenol was added to 1 mL aliquot of each sample and incubated 10 min at 70 °C and then 5 min at 0°C. After centrifugation (4,000 g, 10 min) the upper- and inter-phase were discarded. One ml of milliQ water was added to the samples and incubated 10 min at 70°C and then 5 min at 0 °C. After centrifugation (4,000 g, 10 min) the lower phase was collected. One mL of ice-cold acetone was added to each sample and incubated overnight at 20°C. Precipitated proteins were collected by centrifugation (15,000 g, 20 min, 4 °C) and washed with ice-cold acetone (15,000 g, 20 min, 4 °C). Pellets were dried and re- suspended in the possible smallest volume of rehydration buffer (Tris-HCl pH 8.8, 5 mM, Tiourea 2.2 M, Urea 6.5

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M, CHAPS 4% (w/v), bromophenol Blue). Proteins were quantified by 2D Quant kit (GE-Healthcare) (Genovese et al, 2013).

2-DE

Isoelectrofocusing (IEF) was performed using a 13 cm IPG strips (GE-Healthcare) with a linear gradient ranging from 3 to 10. A final volume of 260 µl, containing 270 µg of proteins, were loaded with 1,2 % v/v DeStreak (GE Healthcare) as reducing agent and 0,5% v/v IPG Buffer 3-10. IEF was performed using IPGphor (GE Healtcare) at 20°C, with 88000 Vhrs. After IEF, the strips were incubated at room temperature in 6 M urea, 30% v/v Glycerol, 2% w/v SDS, 50 mMTris-HCl, pH 8.6, supplemented at first with 2% w/v DTT for 15 minutes and subsequently with 4,5% w/v iodoacetamide for 15 min. The strips were then loaded at the top of 1.0 mm vertical second dimension gels. SDS-PAGE was performed on 11,5% T and 3,3% C acrylamide (Biorad Acrylamide) homogeneous gels. The running buffer was 2,5 mMTris, 192 mM glycine, 0,1% SDS The running conditions were 11 °C, 600V constant voltage, 20 mA/gel, 60 W for 15 min and 11 °C, 600-v constant voltage, 40 mA/gel, 80W for about 2,5 h. The gels were fixed (30% v/v ethanol, 10% v/v orthophosphoric acid) and stained using Processor Plus (Amersham Biosciences) with freshly prepared Colloidal Coomassie Blue (0.12% w/v Comassie G-250, 10% w/v H_3PO_4 85%, 10% w/v $(NH_4)_2SO_4$) diluted with methanol (4:1). The image of the gels was digitized with the Personal Densitometer SI (Amersham Biosciences) and then stored after dehydration in a GD Vacuum Gel Dryer System (GE Healthcare) Genovese et al, 2013).

Image analysis and statistical analysis

Image analysis was performed with the Progenesis PG 220 software (Non Linear Dynamics). Spot detection was automatically performed using the 2005 detection software algorithm and manually verified (Pessione et al, 2014). After the establishment of some user seeds, matching was automatically performed and manually checked. Three analytical replicates of all the three biological replicates were performed. A spot was considered significant when it was present in three of the technical replicates of at least two out of three biological replicates

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Protein identification

Following differential analysis, protein spots on preparative 2-DE gels were excized and analyzed by the peptide mass fingerprinting (PMF) approach with a MALDI-TOF/TOF Mass spectrometer and by mass list probabilistic matching by MOWSE score algorithms on the Swiss-Prot database. Protein spots were excized from the gel and were washed with 100% ethanol and 100 mM ammonium bicarbonate (NH_4HCO_3). Subsequently the gel was reswollen in 50 mM NH_4HCO_3 containing 12.5 ng trypsin and incubated at 37°C overnight (Gharahdaghi *et al*., 1999). Peptide extract was applied to a C18ZipTip (Millipore, Bedford, MA, USA), rinsed with a 0.1% TFA and eluted directly on the MALDI target with 0.5 ml of a saturated a-cyano-4-hydroxycinnamic acid (1:1 = ACN: 0.1% TFA) solution. All analyses were carried out in reflex positive ion mode at an accelerating voltage of 20 kV and a reflex voltage of 23 kV. The peptide mass fingerprints (PMF) obtained were used to search through the SWISS-PROT and NCBInr databases using the Mascot search engine (http://www.matrixscience.co.uk) with a tolerance of 100 ppm and one missed cleavage site.

Statistical Analyses

For the adhesion tests and EPS quantification, comparisons between individual groups were performed with Student's t-test or Wilcoxon pairs test. For the cell viability and mitochondrial ROS production tests, data were expressed as mean ± SEM of the mean. The results were checked for normal distribution and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. For proteomic data, the image analysis was performed with the Progenesis PG 220 software (Non Linear Dynamics). Spot detection was automatically performed using the 2005 detection software algorithm and manually verified. After the establishment of some user seeds, matching was automatically performed and manually checked. Two analytical replicates of all the three biological replicates were performed. A spot was considered significant when it was present in both the technical replicates of at least two out of three biological replicates. Statistical significance level was set at $p = 0.05$ for all statistical analyses.

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RESULTS

1-EFFECTS OF PUTATIVE PROBIOTIC BACTERIA ON THE SENSITIVE AND CHEMORESISTANT CANCER CELLS

1.1 Adhesion capability of the putative probiotics

All strains, three ascertained probiotics (*L. rhamnosus* GG, *L. reuterii* DSM 17938 and *L. johnsonii* LC1) and the three putative probiotics (*L. plantarum* S2, *L. plantarum* O2, *L. pentosus* S3), adhered to undifferentiated HT-29 cells (Fig. 1). Overall, the percentage of adhesion to HT-29 cells ranged from 33 % to 50 % and a low adhesion capability (< 25 %) was observed for the putative probiotic O2 and probiotics DSM 17938 and LC1. Therefore, the best adhering putative probiotics *L. pentosus* S3, *L. plantarum* S2 were selected for further studies together with a poorly adhesive strain *L.rhamnosus* 14E4, previously isolated from ripened cheese (Bautista-Gallego et al., 2019). The aim was to compare the behavior of different lactobacillus species but also to assess possible beneficial actions independent from adhesion, but rather linked to molecules secreted into the host-microbiota interface.

1.2 Effect of the three putative probiotics (S2, S3 and 14E4) extracellular extracts on cell viability in HT29 and HT29-dx cells

We investigated whether extracellular extracts (bacterial supernatants=BS) of the potential probiotics S2, S3 and 14E4 could influence cell viability. The cell viability, measured by neutral red uptake assay, was significantly lower in HT29 and HT29-dx cells cultured in presence of BS for 48 h compared to control cells cultured in RPMI medium (Figures 2A and 2C). As expected, in HT29 cells, DOXO alone significantly decreased cell viability (Figure 2B) and inversely did not modify it in HT29-dx cells (Figure 2D). Notably, cell viability was significantly further decreased in presence of bacterial supernatants plus DOXO compared to DOXO and bacterial supernatants alone (Figure 2B) and even more interestingly, when HT29-dx cells were incubated with BS plus DOXO, cell viabil-

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ity significantly decreased compared to cells incubated with DOXO alone (Figure 2D). This suggests the presence in the probiotic bacterial supernatant of compounds able to control proliferation and to reverse the chemo-resistance in these colon cancer cells.

1.3 Effect of the three putative probiotics (S2, S3 and 14E4) extracellular extracts on mitochondrial ROS production in HT29 and HT29-dx cells

To explain the observed effects on cancer cell viability exerted by the bacterial extracellular extracts, we focused our study on the ROS production analysis, since ROS are a hallmark of oxidative stress potentially leading to regulated cell death processes (Bergandi et al, 2018). Furthermore, one goal of our study was to compare the responses in chemosensitive and chemoresistant cancer cells, and the anticancer drug DOXO was shown to have important cytotoxic effect on tumor cells through ROS production (Carlisi et al, 2017). Therefore, we analyzed the levels of mitochondrial ROS, the main source of cellular ROS, in different experimental conditions, to prove that bacteria may be able to sensitize cancer cells, even chemo-resistant cells, to the cytotoxic effect of DOXO.

We found the ROS levels to be significantly higher after bolus of 10 μM DOXO for 4 h in HT29 cells compared to control cells (Figure 3B). We then investigated whether the BS of the potential probiotics S2, S3 and 14E4 could affect the mitochondrial ROS production. We first observed that, incubation of HT29 cells with some supernatants (S3 and 14E4), alone, increased the mitochondrial ROS production (Figure 3A). Furthermore, incubation of HT29 cells with each supernatant plus DOXO potentiated the effect of DOXO alone (Figure 3B), suggesting that these extracellular bacterial extracts can increase the efficiency of DOXO in the chemo-sensible HT29 cells. As expected, incubation of HT29-dx cells with DOXO did not modify the mitochondrial ROS production (Figure 3D). However, the incubation of these chemo-resistant cells with BS plus DOXO (although no significant effect on ROS production by HT29dx by BS was observed, figure 3C) induced the mitochondrial production of ROS (Figure 3D), confirming that these BS may sensitize the chemo-resistant HT29-dx cells to the cytotoxic effect of DOXO (Figure 3D).

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All these results strongly support the evidence that some soluble factors having anti-proliferative effect also enhancing DOXO-induced toxicity are produced by the putative probiotic strains in study. Since butyrate is known for its antitumor properties (Weir et al., 2013) and two of the three strains (S2 and S3) in previous investigations displayed high butyrate production (Botta et al, 2017a; Botta et al, 2017b) we set up further experiments to establish direct effects of butyrate on HT29 and HT29-dx cells.

2- EFFECT OF SODIUM BUTYRATE ON THE SENSITIVE AND RESISTANT CANCER CELLS

2.1 Effect of sodium butyrate on cell viability in HT29 and HT29-DX cells

A dose response experiment (8, 12 and 24 mM for 48 h, since at 24 h no effect has been detected) has shown that incubation of HT29 cells with sodium butyrate even at a concentration of 8 mM decreased significantly the HT29 cell viability and reached a plateau with higher doses. Arbitrary, control condition (RPMI medium for 48 h) corresponded to 100% cell viability (Fig. 4A). We then evaluated cell viability in HT29 cells incubated with sodium butyrate at these different concentrations plus a bolus of DOXO (10 μM). Sodium butyrate was incubated alone for 24 h and DOXO was incubated together with sodium butyrate for 24 additional hours. As expected in HT29 cells, DOXO alone significantly decreased cell viability. Moreover, cell viability was furtherly significantly decreased in sodium butyrate (8 and 24 mM) plus DOXO conditions compared to DOXO condition (Fig. 4B).

We then observed that the incubation of HT29-dx cells with sodium butyrate at 8, 12 and 24 mM significantly decreased cell viability in a dose dependent manner (Fig. 4C). As expected, DOXO alone did not modify cell viability in HT29-dx cells (Figure 4D). Interestingly, when HT29-dx cells were incubated with sodium butyrate (8 and 24 mM) plus a bolus of DOXO cell viability significantly decreased compared to cells incubated with DOXO alone (Fig. 4D), suggesting that sodium butyrate is able to reverse the chemo-resistance in these colon cancer cells.

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2.2-Effect of sodium butyrate on mitochondrial ROS production in HT29 and HT29-dx cells

As accomplished with BS, we then investigated whether sodium butyrate could affect the mitochondrial ROS production. A dose response experiment (8, 12 and 24 mM for 20 h) has shown that a significant increase in ROS production in HT29 cells at 12 and 24 mM of sodium butyrate occurs. No dose-dependent effect was observed (Figure 5A). As expected, DOXO alone (10 µM, 4h) increased significantly mitochondrial ROS production in HT29 cells (Figures 5B). Moreover, we observed that the incubation of cells with sodium butyrate (8, 12 and 24 mM for 20h) plus DOXO (10 µM for 4 additional hours) potentiated the effect of DOXO alone on mitochondrial ROS production in HT29 cells (Figures 5B), suggesting a synergic effect of sodium butyrate on DOXO action. It is worth noting that sodium butyrate (at all doses) plus DOXO incubation also significantly increased the mitochondrial ROS production compared to sodium butyrate alone (Figure 5B). As expected, incubation of HT29-dx cells with DOXO (10 µM, 4 h) did not modify the mitochondrial production of ROS (Figures 5D). Neither sodium butyrate alone (at any dose) modified this production in HT29-dx cells (Figure 5C). Interestingly, the presence of sodium butyrate, even at a concentration of 8 mM, together with DOXO, increased the mitochondrial ROS production in these cells, suggesting that sodium butyrate may sensitize resistant cells to the cytotoxic effect of DOXO (Figures 5D). Even if not complete, this reversion was close to values of ROS produced by HT29 cells stimulated by DOXO plus sodium butyrate (146 *vs.* 186 for 8 mM; 175 *vs.* 202 for 12 mM and 178 *vs.* 205 for 24 mM.

Based on these results, butyrate seems to be involved in the biological effects observed with bacterial supernatants. We previously observed the production of butyrate by the strains S2 and S3 in the human cells culture media used in this study (Botta et al., 2017 a; Botta et al., 2017 b). However, further molecules could be involved since strain 14E4, not displaying high butyrate content was one of the most active in controlling tumor cells viability. Therefore, considering literature data, we also explored other biological compounds by quantifying, in the three putative probiotic strains, EPS

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and, in the 14E4 strain, the secreted protein profiles. In these experiments, LGG was used as reference strain.

3. QUANTIFICATION OF EPS AND ANALYSES OF THE PROTEIN PROFILES IN THE THREE PUTATIVE PROBIOTICS CONTROLLING CANCER CELLS

3.1-Quantification of EPS in the responder and control strains

The four tested strains (*L. rhamnosus* GG*, L. rhamnosus* 14E4*, L. plantarum* S2*, L. pentosus* S3) did not display a ropy phenotype (*i.e*. EPS composition giving rise to a filament when a culture pellet is pulled up by a loop). The EPS quantification reported in Tab. 1 shows that the amount of EPS produced is comparable among the tested strains, with the exception of *L. rhamnosus* GG, which polysaccharides were hardly soluble in water, so it was hard to collect a representative sample for the assay, affecting the reproducibility of the quantification.

3.2-Extracellular protein profiles of the responder and non-responder *L. rhamnosus* strains.

The extracellular proteomic maps of the responder strains *L. plantarum* S2 and *L. pentosus* S3 have been published in a previous work (Pessione et al., 2015). In the present investigation we wanted to compare the secretomes of the two strains *L. rhamnosus* 14E4 and *L. rhamnosus* GG that were able or not to revert chemotherapy resistance and affect cell viability, respectively. For both *L. rhamnosus* strains, extracellular proteins were recovered from middle exponential phase cultures to avoid cytosolic contaminations due to cell lysis. 2-DE gels were performed in 3–10 pI range (Fig. 6) and after image analysis, 38 spots were present in at least two out of three biological replicates deriving from both bacteria. All spots were analyzed by MALDI TOF-TOF/MS. By considering the different isoforms, 10 and 17 proteins were identified for *L. rhamnosus* 14E4 and *L. rhamnosus* GG, respectively. Table 2 shows the proteins identified in this study, and the proteonic data are available in the Electronic Supplementary information 1 and 2. Among the identified proteins, having demonstrated extracellular localization, P75, P40, GAPDH, L-lactate DH were identified in both strains, while surface antigen proteins, enolase, elongation factors, phage major capsid protein and phage-tail-like

protein were identified only in one of the two strains. It is worth of mentioning that the phage major capsid protein and the phage-tail-like protein were detected only in the *L. rhamnosus* 14E4 responsible for the reduction of cell proliferation and of the revertant effect on chemotherapy-resistance.

For what concern the proteins shared by both strains, the ratio between the volume of all the spots identifying the same protein and the total spot volume in the gel was defined as relative abundance. According to this parameter, P75 and the antigen surface proteins showed no differences in relative abundances between strains. Conversely, GAPDH and L-lactate DH were more abundant in GG compared to 14E4 (6 and 13-fold respectively) while P40 was double in 14E4 compared to GG.

DISCUSSION

Experimental research has demonstrated that probiotics possess anti-proliferative or pro-apoptotic activities on human cancer cells. Cytoplasmic fractions of *Bifidobacterium adolescentis* SPM0212 (Kim et al., 2008) *as* well as *Lactobacillus rhamnosus* strain GG (LGG) and *Lactobacillus paracasei* (Orlando et al. 2012) showed significant antiproliferative activities in cancer cell lines including HT-29, SW 480, and Caco-2 cells. In particular, recent evidence suggests that probiotic bacteria, like *Lactobacillus brevis* and *Lactobacillus paracasei,* can induce apoptosis of HT-29 colon adenocarcinoma cell lines by inducing caspases and the Bax/Bcl2 pathway (Karimi Ardestani et al, 2019) as well as *L. rhamnosus* can cause cell cycle arrest and apoptosis on the same cell lines (Dehghani, et al, 2021). This general anticancer role of the probiotic component of gut microbiota has been recently confirmed in a very comprehensive report by Sehrawat et al (2021). However, not only cytoplasmic fractions exerted antitumor effects, but also other probiotic-derived molecules included those from *Bacillus polyfermenticus* (Ma et al., 2010), *Lactobacillus acidophilus* 606 (Kim et al., 2010), LGG/Bb12 (Borowicki et al., 2011).

By adhering to intestinal surfaces *Lactobacillus spp.* can influence the host through direct contact or by releasing extracellular metabolites (Orlando et al., 2012; Thirabunyanon and

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Hongwittayakorn, 2013). Our results have shown strain-dependent adhesion capabilities on cancer cells, with a percentage similar to those observed for other lactobacilli (Orlando et al., 2012; Thirabunyanon and Hongwittayakorn, 2013), but significantly lower than those obtained for the same strains on primary human colon cells (Botta et al., 2014)**.** However, it is interesting to underline that the low-adherent putative probiotic 14E4 is one of the best performants in controlling cell proliferation and ROS production, suggesting that even a weak interaction can promote significant effects mediated by secreted compounds.

In the past decades, *in vivo* studies demonstrated that both *Lactobacillus acidophilus* alone (Urbanska et al., 2009) and its combination with *Lactobacillus casei,* and *Bifidobacterium longum* (Lee et al., 2004), or also *Bifidobacterium bifidum* (Mohania et al., 2014) significantly inhibited the colon cancer development in mouse and rat models respectively. As far as studies on human volunteers are considered, consumption of synbiotic preparations containing oligofructose-enriched inulin (SYN1) plus *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (BB12), significantly reduced colorectal cancer risk (Rafter et al., 2007) and administration of *L. casei* for 4 years prevented atypia of colorectal cells (Ishikawa et al., 2005).

Emerging evidence suggests that gut microbiota may influence the response to chemotherapy. Fecal microbiota transplantation from 5-fluorouracil (5FU) treated to control mice altered functional performance and colon inflammatory markers (Sougiannis et al. 2019). Our *in vitro* results confirmed this evidence, since addition of cell-free supernatants from putative probiotics (*L. plantarum* S2, *L. pentosus* S3 and *L. rhamnosus* 14E4) to doxorubicin was associated with a reduction in cell viability. This is associated with the increased sensitivity to doxorubicin in human colon adenocarcinoma cells (HT29 cells) and the reversion of resistance in their chemoresistant counterpart, the HT29-dx cells.

Concerning probiotic ability to counteract cancer, the ultimate goal is the characterization at a molecular level of the key-factors displaying control on cell proliferation, differentiation and

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apoptosis. However, the mechanisms by which probiotics can enhance cancer therapy or revert chemotherapy resistance remain unknown. Among probiotic-derived metabolites with attributed anticancer benefits, short-chain fatty acids (i.e. acetate, lactate, propionate and butyrate) mainly originating from indigestible vegetable polysaccharide chains are the best studied (Matsuki et al, 2013).

Butyrate, the best studied SCFA, helps to regulate the balance between proliferation, differentiation, and apoptosis of colonocytes (Serban, 2014). It can be found in higher quantities in the feces of healthy individuals compared with patients with colorectal cancer. It is estimated that reducing 1 μg/L of butyric acid concentration in feces increases the risk of developing CRC by 84.2% (Weir et al., 2013). In addition, butyrate is capable to increase colonocyte's mucus production and a decrease in the number of butyrate-producing bacteria relates with a mucosal inflammatory status (Rios Covian et al, 2016). Actually, butyrate also stimulates the production of anti-inflammatory cytokines, such as interleukin (IL)-10, regulates the activity of proteins involved in apoptosis, such as Bcl-2, Bak, and caspases 3 and 7; increases the activity of the antioxidant enzyme glutathione S-transferase (GST); suppress cyclooxygenase (COX)-2 activity (Vipperla et al., 2012). Butyrate has also epigenetic properties: it is able of inhibiting the deacetylation of histones, thus overexpressing inhibitory gene p21, which is connected with cell cycle arrest. (Serban, 2014).

As far as butyrate production is concerned, strains belonging to *L. rhamnosus* species have been demonstrated incapable to produce it (LeBlanc et al., 2017). Conversely, *L. pentosus* S3 and *L. plantarum* S2 have previously proved to produce butyrate in semi-hard cheeses (Botta et al., 2015) and in the human cell culture medium DMEM (Botta et al, 2017a; Botta et al, 2017b) respectively, hence this molecule can be partly involved in the antitumor action observed in the present study. Indeed, our results have shown that sodium butyrate decreased cell viability even in HT29-dx cells and induced mitochondrial ROS production, thus reverting the doxorubicin resistant phenotype of HT29-dx cells.

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A second important class of molecules controlling cancer are exopolysaccharides (EPS). LAB can produce EPS loosely bound to the cell by non-covalent interactions that can be released into the surrounding environment (Pingitore et al, 2016). EPS produced by *L. acidophilus* showed a powerful antitumor effect both *in vitro* and *in vivo* (El Ghany et al., 2015) and were found to inhibit the growth of CaCo-2 colon cancer cell line in a dose-dependent manner (Deepak et al., 2016). EPS from *Lactobacillus helveticus* MB2-1 have been described to inhibit Caco-2 growth as well (Li et al, 2015). EPS from *Lactobacillus gasseri* strains isolated from human vagina induced apoptosis in cervical tumor cells (HeLa) and also showed an anti-inflammatory impact on HeLa cells by decreasing the production of TNF-α and increasing the IL-10 production (Sungur et al. 2017). In a more recent evaluation, EPS from *L. paracasei* and *L. brevis* were demonstrated to be able to induce apoptosis and DNA fragmentation in HT-29 cancer cell lines (Mojibi et al, 2019).

In the present work, no significant differences in the amounts of produced EPS were observed in the strains positively affecting cell proliferation. For the control strain *L. rhamnosus* GG (not affecting cell proliferation) the test was of hard interpretation because of the low water solubility of the produced EPS. Although more detailed structural studies, that are out of the scope of the present investigation, are needed to better clarify the nature of the EPS produced, the detected amounts (Tab. 1) suggest that the three effective strains (*L. rhamnosus* 14E4 *L. plantarum* S2, *L. pentosus* S3) synthesize hetero-EPS since their levels are relatively low. These considerations are based on the fact that it has been reported that homo-EPS can reach several grams per liter (e.g., *L. reuteri* producing 10 g/L) (Ruas-Madiedo and de los Reyes-Gavilán, 2005) whereas hetero-EPS are generally produced in lower amounts (few mg/L) (Staaf et al., 2000).

Quantification of EPS in bacteria displaying antitumor activities was recently reported by Sungur et al. (2017), revealing amounts ranging from 100 to 200 mg/L. This is consistent with the detected concentrations of the three cell proliferation-inhibiting strains *L. rhamnosus* 14E4, *L. plantarum* S2, *L. pentosus* S3 (Tab. 1). As a confirm of this statement, all EPS displaying antitumor activity described in the literature are hetero-polysaccharides (El Ghany et al., 2015; Deepak et al.,

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2016; Li et al, 2015; Sungur et al. 2017). The fact that for *L. rhamnosus* GG was not possible to establish a correct quantification may also indicate that these EPS are probably more hydrophobic and thus not effective against cancer growth as experimentally proved in the present study. It is worth mentioning that the LAB displaying EPS-mediated antitumor activity reported in the literature so far are: *L. acidophilus* (El Ghany et al., 2015)*, L. gasseri* (Sungur et al. 2017) *and L. helveticus* (; Li et al, 2015). To our knowledge, this is first report concerning *L. rhamnosus, L. plantarum and L. pentosus*. In our opinion, the strain specificity has proved to be important as shown by the absence of efficacy of *L. rhamnosus* GG as respect to *L. rhamnosus* 14E4.

Finally, a pivotal role in the host–probiotic relationship is played by cell-envelope and secreted proteins, which, being in the interface between bacteria and the host, have the ability to interact with enterocytes and extracellular matrix proteins. Although the main function of most surface-displayed or secreted proteins is tissue adhesion or immune stimulation (Sanchez et al., 2011) some proteins, such as P40 can be involved in the homeostatic regulation of gut epithelium controlling both inflammation and tumorigenesis (Yan et al, 2013).

The present proteomic results obtained comparing the secretomes of two different strains of *Lactobacillus rhamnosus*, the ascertained probiotic strain GG having neither effects on tumor cell viability nor chemotherapy-resistance revertant action, and the putative probiotic strain 14E4 displaying both effects, revealed interesting features.

As reported in the Results section, the secretomes seem to be very similar in the two strains**.** However, a significant variation in the relative abundance of the P40 protein was observed (Fig. 6, Tab.2). Actually, the detected amount of this protein is double in the putative probiotic 14E4 strain (spot 5) (where alone accounts for the 30% of the overall strain 14E4 secretome) compared to GG strain (spot 8) (where it is consistent with only the 17% of the overall secretome) suggesting its possible role in the proliferation-controlling effects observed. This result is consistent with data indicat-

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ing that P40 is able to inhibit colitis-associated tumorigenesis by activating the epidermal growth factor, EGR-R (Yan et al., 2013).

Very interesting is the presence in the secretome of strain 14E4 of a phage-tail-like protein (spot 11) and a phage major capsid protein (spot 9) both missing in the *L.rhamnosus* GG strain. Although we do not have information on the presence of a prophage in the 14E4 genome, temperate phage colonization is known to be common in Lactobacilli where the presence of lytic phages is relatively low (Durmaz et al, 2008). In the species *L. rhamnosus*, lysogenic prophages are positively selected since they provide essential functions, for bacterial survival (Durmaz et al, 2008), in particular, phage-associated genes encoding for proteins important for host colonization and interaction have been detected (Kankainen et al, 2009). However, the presence of prophages is strain-related, due to different susceptibility and to different opportunities that each bacterial strain must interact with bacteriophages (Pessione, 2020). The antitumor-activity of bacteriophages was assessed in animal models as early as 1940 (Bloch, 1940). In addition, bacteriophage potential in cancer therapy has been proposed (Merril et al, 2003; Budynek et al, 2010). To shed light on the molecular mechanisms underlying this effect, experiments were performed (Dabrowska et al, 2004a) revealing that phages proteins containing a KGD (Lys-Gly-Asp) motif are ligands for the *β*3 integrins on animal cells. However, this sequence is lacking in both the proteins differently abundant (spots 9 and 11) detected in the present research. Binding of phage T4 to the membrane of cancer cells was observed in both confocal and electron microscopy and proved with direct binding tests in human melanoma and lung cancer cells. Anti-β3 antibodies and β3 synthetic ligands can inhibit this binding (Dabrowska et al, 2004b). Furthermore, it has been demonstrated in *in vivo* that the efficacy of bacteriophage T4 in counteracting melanoma development is much higher in mutants lacking the gp Hoc protein (Dabrowska et al, 2007). Therefore, some phage proteins can prevent tumor progression while other can slower this effect. These authors speculate on the fact that phages, being important parasites of gut bacteria belonging to the animal microbiota, during evolution have acquired the ability to cross talk with their "environment" thus interacting with host cells and tissues and

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controlling possible physio-pathological events *(*Dabrowska et al, 2005). Other reports describe phage-induced tumor regression occurring when contact between phage and TLR occurs (Eriksson et al, 2007). Similarly, also flagellar proteins from Firmicutes and gamma-proteobacteria can interact with TLR. We found a flagellar protein in the secretome of the cell proliferation inhibiting strain 14E4 (spot 19) but not in that of the ineffective strain GG. A question arises: why a flagellar protein in a non-motile organism? May be a remnant protein (probably acquired by horizontal transfer) useful for interaction with the host exactly as what hypothesized by Dabrowska and co-workers (2005) for phage protein remnants? A possible answer comes from the more recent report of Ghesquire and De Mot (2015) on tailocins, particles similar to phage tails that display different activities such as acting as type VI secretion system and as bacteriocins, as well as agents for interaction with eukaryotic cells. It is interesting to underline that bacteria can use exogenous genetic elements (in this case from viruses and from motile bacteria) for their own benefit, for maintaining a balanced interaction with their host and in ultimate analysis to ensure themselves a healthy ecological niche (Pessione, 2020).

Although proteomic analyses were performed only on the two *L. rhamnosus* strains, it must be underlined that in a previous proteomic investigation on the same *L. pentosus* S3 strain studied in the present work and displaying the beneficial activities against tumor growth, an NlpC/P60 protein was found (Pessione et al, 2015). No comments about this protein were reported in the previous paper since the focus of the research was not on cancer. NlpC/P60 proteins belong to a highly conserved peptidase superfamily present in different kingdoms such as RNA and DNA viruses, bacteria, archea and Eucarya. NlpC/P60 proteins bear three conserved catalytic residues (a cysteine, a histidine and a polar residue) and perform distinct catalytic activities, such as murein degradation, acyltransfer and amide hydrolysis (Anantharaman and Aravind, 2003), some of which related to cancer (*i.e.* the eukaryotic candidate tumor suppressor H-rev107). However, in these eukaryotic proteins (also detected in bacteria like *Vibrio cholerae*) a serine was found as substitute of the cata-

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lytic cysteine (Sers et al 1997). It has been suggested that H-rev107 could potentially act as an acyl-transferase that might modify membrane components (Anantharaman and Aravind, 2003).

CONCLUSIONS

Microbial-based cancer therapy using live and heat-killed bacteria is an old concept with reported effectiveness that was discontinued in the 1930's because of the advancement of radiotherapy and later chemotherapy. However, the huge amount of new scientific knowledge about cancer and microorganisms makes it timely to revisit microbial-based cancer therapy from new perspectives.

In the present investigation, a decrease in cell viability and an increase in the effectiveness of doxorubicin treatment in human colon adenocarcinoma cells by food-isolated putative probiotic LABs have been demonstrated. Such an effect is even able to revert a chemo-resistant phenotype. The action is not species- but rather strain-related and it is based upon released metabolic products since not only intact cells, but also cell-free supernatants are effective. Several factors, such as short-chain fatty acids, EPS and cell-envelope or extracellular proteins, can account for the biological modulation observed. Among secreted proteins present in higher abundance in the effective *L. rhamnosus* 14E4 strains, the most significant (p40, phage-tail-like and phage major capsid proteins) were previously suggested to be in some way involved in the control of tumor cell growth, and, in general, the relationship between bacteriophages and tumor control has attracted significant interest in the last two decades.

The overall results of this study, by depicting mechanisms possibly involved in the control of cell proliferation and attenuation of chemo-resistance exerted by beneficial bacteria, open the way for promising field of investigation both to improve human health and to shed light on aspects concerning co-evolution of bacteria and their potential hosts. Differently to what occurs with conventional bacterial immunotherapy that has some risks linked to the pathogenicity of the strains used (*Salmonella, Listeria, Klebsiella*) (Bazett et al, 2018), the anti-proliferative effect exerted by probiotics

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might represent a potentially valuable tool to specifically target cancer cells including cells that are resistant to current therapies. New therapeutic strategy combining both chemotherapy and/or immunotherapy with an adjuvant treatment targeting the gut microbiota (probiotics, post-biotics, parabiotics, fecal microbiota transplantation) are now available and can be developed to improve treatment response and tolerance.

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FIGURE'S CAPTIONS

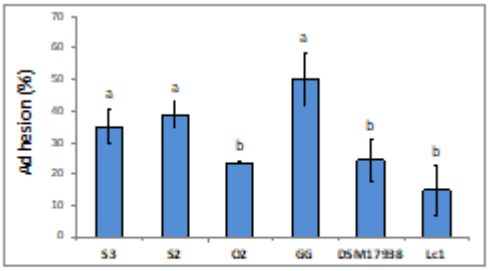

Fig.1

Figure 1. Adhesion profiles of the bacterial strains on confluent HT-29 cells. Data are expressed as the percentage (%) mean ratio (mean ± SEM; n=3) between the bacteria recovered from the human cells after 90 min of incubation/washing steps and the initial bacterial count added on the cells (8.0±0.2 Log CFU mL^{-1}). Different letters shown significant differences between the strains adhesion by means of ANOVA and Duncan's post hoc test ($p < 0.05$).

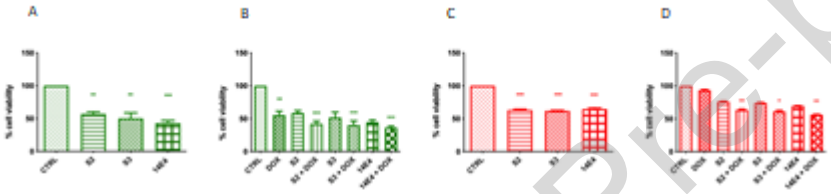

Fig.2

Figure 2. A: Effect of bacterial supernatant (S2, S3 and 14E4) on cell viability in HT29 cells after 48 h of incubation. **p< 0.01, vs. CTRL and ***p< 0.001, vs. CTRL.

B: Cell viability after incubation of HT29 cells with vehicle alone (CTRL, RPMI medium) for 48 h, doxorubicin (DOX 10 µM, 24 h), bacterial supernatant S2 (non-diluted, 24 h), S2 plus doxorubicin (S2+DOX), bacterial supernatant S3 (non-diluted, 24 h), S3 plus doxorubicin (S3+ DOX), bacterial supernatant 14E4 (non-diluted, 24 h) and 14E4 plus doxorubicin (14E4+ DOX). **p< 0.01, vs. CTRL. °°° p< 0.001, vs. DOX

C: Effect of bacterial supernatant (S2, S3 and 14E4) on cell viability in HT29-DX cells after 48 h of incubation. ***p< 0.001, vs. CTRL.

D: Cell viability after incubation of HT29-DX cells with vehicle alone (CTRL, RPMI medium) for 48 h, doxorubicin (DOX 10 µM, 24 h), bacterial supernatant S2 (non-diluted, 24 h), S2 plus doxorubicin (S2+DOX), bacterial supernatant S3 (non-diluted, 24 h), S3 plus doxorubicin (S3+DOX), bacterial supernatant 14E4 (non-diluted, 24 h) and 14E4 plus doxorubicin (14E4+ DOX). °p < 0.05, vs. DOX; °° p< 0.01, vs. DOX

Bacterial supernatants were incubated alone for 24 h and doxorubicin was incubated together with them for 24 additional hours.

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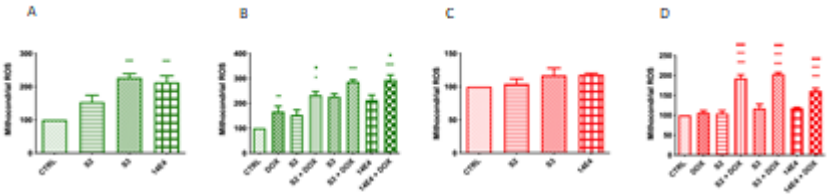

Fig. 3

Figure 3: A: Effect of bacterial supernatant (S2, S3 and 14E4) on mitochondrial ROS production in HT29 cells after 20 h of incubation. ***p< 0.001, vs. Ctrl.

B: Mitochondrial ROS production after incubation of HT29 cells with vehicle alone (Ctrl, RPMI medium), doxorubicin (Dox., 10 µM, 4 h), bacterial supernatant S2 (non-diluted, 20 h), S2 plus doxorubicin (S2+Dox.), bacterial supernatant S3 (non-diluted, 20 h), S3 plus doxorubicin (S3+Dox.), bacterial supernatant 14E4 (non-diluted, 20 h) and 14E4 plus doxorubicin (14E4+Dox.). **p< 0.01, vs. Ctrl; ***p< 0.001, vs: Ctrl; °p< 0.05, vs: Dox., °°°p< 0.001, vs. Dox. and ■ p< 0.01 vs: S2 or 14E4, respectively.

C: Effect of bacterial supernatant (S2, S3 and 14E4) on mitochondrial ROS production in HT29-DX cells after 20 h of incubation.

D: Mitochondrial ROS production after incubation of HT29-DX cells with vehicle alone (Ctrl, RPMI medium), doxorubicin (Dox., 10 µM, 4 h), bacterial supernatant S2 (non-diluted, 20 h), S2 plus doxorubicin (S2+Dox.), bacterial supernatant S3 (non-diluted, 20 h), S3 plus doxorubicin (S3+Dox.), bacterial supernatant 14E4 (non-diluted, 20 h) and 14E4 plus doxorubicin (14E4+Dox.). ***p< 0.001, vs. Ctrl; °°°p< 0.001, vs. Dox.; ■■■ p< 0.001, vs: S2, S3 or 14E4, respectively.

Bacterial supernatants were incubated alone for 20 h and doxorubicin was incubated together with them for 4 additional hours.

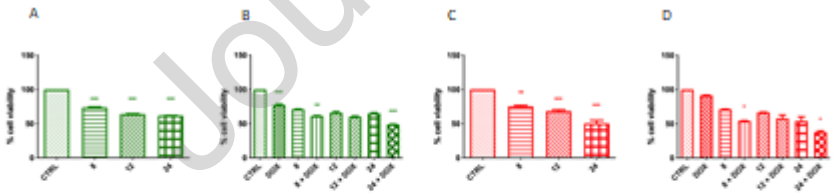

Fig.4

Figure 4. A: Dose response effect of sodium butyrate (8, 12 or 24 mM) on cell viability in HT29 cells after 48 h of incubation. ***p< 0.001, vs. CTRL.

B: Cell viability after incubation of HT29 cells with vehicle alone (CTRL) for 48 h, doxorubicin (DOX 10 µM, 24 h), sodium butyrate (But. 8, 12 or 24 mM, 48 h) or sodium butyrate (8, 12 or 24 mM, 24 h) plus doxorubicin(10 µM, 24 h.) (8 + DOX, 12 + DOX, and 24 + Dox). °°p< 0.01, vs. DOX; °°°p< 0.001, vs. DOX

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C: Dose response effect of sodium butyrate (8, 12 or 24 mM) on cell viability in HT29-DX cells after 48 h of incubation. **p< 0.01 vs. CTRL and ***p< 0.001, vs. CTRL.

D: Cell viability after incubation of HT29-DX cells with vehicle alone (CTRL) for 48 h, doxorubicin (DOX 10 µM, 24 h), sodium butyrate (But. 8, 12 or 24 mM, 48 h) or sodium butyrate (8, 12 or 24 mM, 24 h) plus doxorubicin (10 µM, 24 h.) (8 + DOX, 12 + DOX and 24 + DOX). °p< 0.05, vs. DOX. Sodium butyrate was incubated alone for 24 h and doxorubicin was incubated together with sodium butyrate for 24 additional hours.

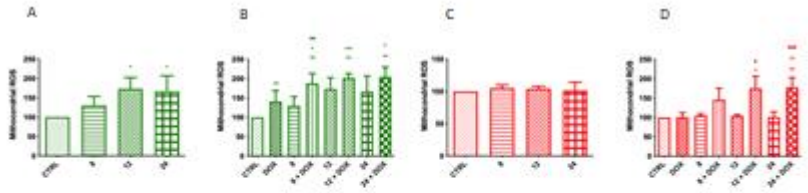

Fig.5

Figure 5. A: Dose response effect of sodium butyrate on mitochondrial ROS production in HT29 cells after 20 h of incubation. *p < 0.05, vs. CTRL.

B: Mitochondrial ROS production after incubation of HT29 cells with vehicle alone (CTRL), doxorubicin (DOX, 10 µM, 4 h), sodium butyrate (But., 8, 12 or 24 mM, 20 h) and sodium butyrate plus doxorubicin (8 + DOX, 12 + DOX And 24 + DOX). Sodium butyrate was incubated alone for 20 h and doxorubicin was incubated together with sodium butyrate for 4 additional hours. **p < 0.001 vs. CTRL; ° p < 0.05 vs DOX; °°°p< 0.001, vs. DOX; ■■ p< 0.01,vs. But.

C: Dose response effect of sodium butyrate on mitochondrial ROS production in HT29-DX cells after 20 h of incubation.

D: Mitochondrial ROS production after incubation of HT29-DX cells with vehicle alone (CTRL.), doxorubicin (DOX, 10 µM, 4 h), sodium butyrate (But., 8, 12 or 24 mM, 20 h) and sodium butyrate plus doxorubicin (8 + DOX, 12 + DOX And 24 + DOX). Sodium butyrate was incubated alone for 20 h and doxorubicin was incubated together with sodium butyrate for 4 additional hours. *p< 0.05, vs. CTRL; **p < 0.001 vs. CTRL °°p< 0.01, vs. DOX; ■■■ p< 0.001,vs. But.

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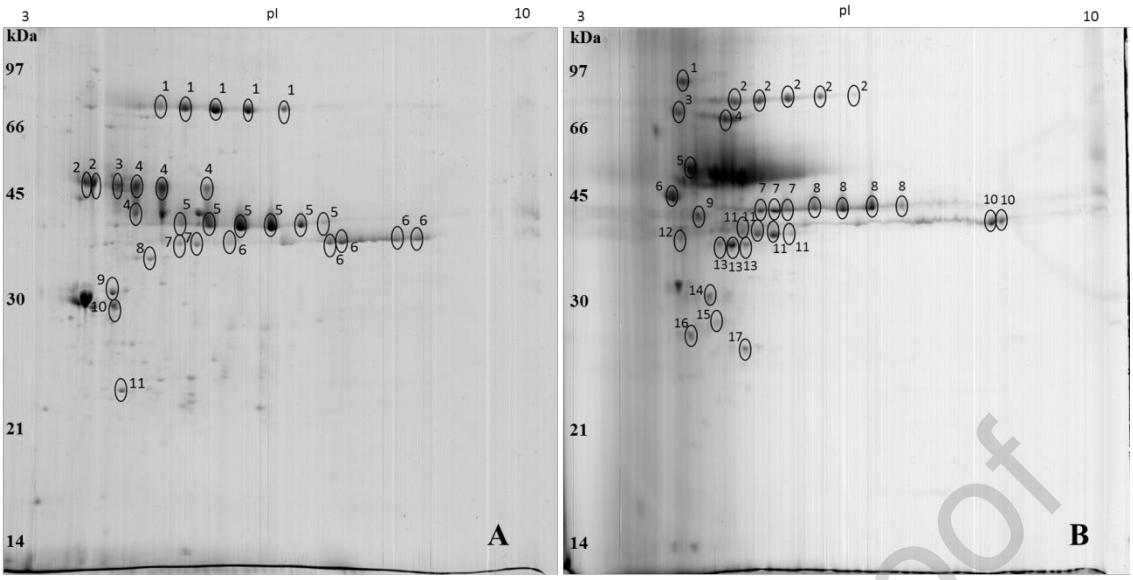

Fig. 6

Figure 6. 2-DE gels of the extracellular proteins recovered from *L. rhamnosus* 14E4 (A) and *L. rhamnosus* GG (B). Numbers reported in the part A of the figure refer to the spot number of protein identifications listed in Table 2a. Numbers reported in the part B of the figure refer to the spot number of protein identifications listed in Table 2b.

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Tab 1	ROPY PHENOTYPE	QUANTIFICATION (mg/L of cultures)
L. rhamnosus GG	-	nd
L. rhamnosus 14E4	-	**142.9** (± 5.1)
L. plantarum S2	-	**159.6** (± 10.2)
L. pentosus S3	-	**154.3** (± 0.3)

Table 1: EPS characterization and quantification in different culture supernatants of LAB strains

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Table 2a: proteins identified by MALDI-TOF/TOF from the maps of extracellular proteins of *L. rhamnosus* 14E4.

Spot number	Protein name	NCBI accession	MW/pI Theoretical	Mr Obs (kDa)	MS/MS Identified peptides	Score Sequence	coverage %
1	P75 (glycosyl hydrolase)	WP_046783173.1	49323/6.90	75000	R.YVTFGDTVQLLGK.T K.SGATTVWNSPSWHQVK.R	125	22
2	Hypothetical protein HMPREF0541_01875	EHJ29416.1	45800/5.53	50000	K.YTALAYFMPDFAVRPSL U K.LEQLAQNVDDRDQK.K K.NVLPLVQVAR.N	158	35
3	NAD(FAD)-dependent dehydro-genase	WP_064533598	49332/5.01	50000	R.GYDWATSIK.A R.QWSLLNLAAQHALGEPR.F K.GTVSLDDRGFINTDPYLR.T	142	32
4	Hydrolase	WP_085320180.1	32015/6.84	480000	R.ITMSNGQVLER.T K.LRITMSNGQVLER.T	120	42
5	P40 (CHAP-domain containing protein)	WP_094069269.1	41054/6.45	400000	K.VYAEIWYETSDHGWVPER.Y K.IASLSGEITAAQK.N K.QIEAANTEVINLNK.Q	110	33
6	Surface antigen	WP_029607431.1	40960/8.62	37500	K.IASLSGEITAAQKNVAAR.K K.LSQASAEALQAVK.D K.SNLKDQLISLQK.R	152	50
7	Type I glyceraldeidhyde-3-phosphate dehydrogenase	WP_016364194.1	36790/5.54	37500	K.LSQASAEALQAVKDSEAK.V K.AIGLVIPELNGK.L R.VYAEPQAQNIPWVK.N	175	56
8	L-lactate dehydrogenase	EPC53562.1	30633/5.24	35000	K.TVAWYDNEYGFTCQMIR.T R.VVGSGTSLDTAR.F R.QSIAEMVNVDAR.S	110	58
9	Phage major capsid protein	KRM25919.1	31081/4.61	31000	R.FRQSIAEMVNVDAR.S R.YLGAAFRFGVEK.A K.HILNVDLDPAYSR.S	137	35
10	Hydrolase	WP_003563024.1	23013/4.86	29000	R.QQVTLEWEDSKIYGR.Y U K.ITLSDGTVWYR.T	71	
11	Tail protein	WP_048488424.1	21861/4.82	24000	K.EVFGGYVLSPSLGK. K.AHVALLITTQTIDR.T M.ATVGLYQIQLALVDAQQK.L	225	55

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Table 2b: proteins identified by MALDI-TOF/TOF from the maps of extracellular proteins of *L. rhamnosus* GG.

Spot number	Protein name	NCBI accession	MW/pI Theoretical	Mr Obs (kDa)	Identified peptides	Score Sequence	coverage %
1	Elongation factor G	WP_029607645.1	76910/4.78	80000	K.VATDPFVGR.L K.VGEPQVAYR.E K.GFEFENAIVGGVVPR.E R.YVTFGDTVQLLGK.T	110	56
2	P75 (glycosyl hydrolase)	WP_046783173.1	49323/6.90	75000	K.SGATTVWNSPSWHQVK.R K.YTALAYFMPDFAVRPSL.	312	22
3	Molecular Chaperon DnaK	WP_005713948.1	67190/4.82	72000	K.IAGLNVQR.I R.FELTDIPPAPR.G	160	61
4	Pyruvate kinase	WP_064460591.1	64710/5.37	70000	R.KPQDVLDIR.E K.TIVAATESGYTAR.M K.NITEAVGQSVAHTAR.N	210	46
5	Elongation factor Tu	WP_020751927.1	43530/4.86	50000	K.TLDLGEAGDNVGVLLR.G K.VGDEVEIIGLKPDVLK.S	135	66
6	Phosphopyruvate hydratase	WP_005711903.1	47130/4.71	45000	K.EAGYTAVVSHR.S K.AVIGLDVTEQR.L R.ALVPSGASTGEHEAVELR.D	165	54
7	Phosphoglycerate kinase	WP_085054267.1	42000/5.63	40000	K.LTLKPVAER.L K.KPVTFVPATR.G	95	67
8	P40 (CHAP-domain containing protein)	WP_020752118.1	42640/6.97	40000	K.IASLSGEITAAQK.N K.QIEAANTEVINLNK.Q	159	44
9	Elongation factor Ts	WP_046782965.1	31570/5.03	38000	K.DVAMHVAAINPEYLDR.S K.WLSEISLVDQEFVKDPDQTVAK.Y	125	63
10	Surface protein	WP_048482352.1	40860/8.62	37000	K.LSQASAEALQAVK.D K.SNLKDQLISLQK.R K.LSQASAEALQAVKDSEAK.V	220	58
11	Glyceraldehyde-3-phosphate dehydrogenase	WP_048488143.1	36940/5.53	36000	K.AIGLVIPELNGK.L R.VYAEPQAQNIPWVK.N K.TVAWYDNEYGFTCQMIR.T	123	51
12	Manganese dependent inorganic pyrophosphatase	WP_049179904.1	33500/4.84	36000	R.IANFHTASPLYYR.A R.IGQVNTVDLDDIYAR.Q	168	57

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					R.VVGSGTSLDTAR.F		
13	L-lactate dehydrogenase	EPC53562.1	30630/5.33	35000	R.QSIAEMVNVDAR.S	225	53
					R.FRQSIAEMVNVDAR.S		
14	Phosphoglycerate kinase	WP_085054267.1	42000/5.63	31000	K.LTLKPVAER.L	95	67
					K.KPVTFVPATR.G		
15	Fructose-1,6-biphosphate aldolase	WP_031545718.1	31610/5.08	29000	K.GLHFDRLQELNDAVK.M	150	68
					K.MPLVLHGGSGIPQEQVQK.A		
16	Triose-phosphate isomerase	WP_064517249.1	26980/4.91	27000	K.TATADQAQEVVAHIR.A	158	46
					K.AIFKNNLLPIICCGESLAQR.E		
					K.HAGELIK.Q		
17	Phosphoglyceromutase	KMO66326.1	24190/5.40	26000	R.YADLDPR.I	100	40
					R.RYADLDPR.I		

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CRediT authorship contribution statement

Doublier Sophie: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Writing original draft, Writing reviewing and editing. **Cirrincione Simona**: Formal analysis, Investigation, Methodology, Software, Visualization. **Scardaci Rossella**: Data curation, Investigation, Software, Visualization, Writing original draft, Writing reviewing and editing. **Botta Christian**: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing original draft, Writing reviewing and editing. **Lamberti Cristina**: Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Di Giuseppe Francesco, Angelucci Stefania:** Data curation, Formal analysis. **Rantsiou Kalliopi**: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation. **Cocolin Luca**: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision. **Pessione Enrica**: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing original draft, Writing reviewing and editing.

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Highlights

- Bacterial supernatants from lactobacilli modulate viability in HT-29 cancer cells
- Effects on HT-29 cells are linked to an increase in mitochondrial ROS production
- Butyrate could influence tumor viability for only two of the three strains
- Among others, the extracellular protein P40 could trigger the detected effects
- Chemotherapy effectiveness in resistant CRC could be improved by bacterial compounds

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