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Lactiplantibacillus plantarum inhibits colon cancer cell proliferation as function of its butyrogenic capability

Cristian Botta [a], Katerina Spyridopoulou [b], Marta Bertolino [a], Kalliopi Rantsiou [a], Katerina Chlichlia [b], Luca Cocolin [a,*]

[a] Department of Agriculture, Forest and Food Sciences, University of Torino, Italy
[b] Department of Molecular Biology and Genetics, School of Health Sciences, Democritus University of Thrace, University Campus-Dragana, Alexandroupolis, Greece

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ABSTRACT

Lactobacilli have been shown to inhibit or suppress cancer cell growth through the release of strain-specific bioactive metabolites and their inclusion in functional foods could exert a health promoting activity on human health. Herein, we examined the antiproliferative activity of the *Lactiplantibacillus plantarum* strains S2T10D and O2T60C, which have been previously shown to exert different butyrogenic activities. Human HT-29 cells were employed as an *in vitro* colon cancer model and both bacterial strains were found to inhibit their growth. However, the strain S2T10D showed a greater antiproliferative activity which, interestingly, was correlated to its butyrogenic capability. Noteworthy, for the non-butyrogenic strain O2T60C, the growth inhibitory capability was rather limited. Furthermore, both the butyrate-containing supernatant of S2T10D and glucose-deprived cell culture medium supplemented with the same concentration of butyrate found in S2T10D supernatant, induced a pH-independent cancer cell growth inhibition accompanied by downregulation of cyclin D1 at mRNA level. The downregulation of cyclin D1 gene expression was accompanied by cell cycle arrest in G2/M phase and decrease of cyclin B1 and D1 protein levels. This *in vitro* study underlines the impact of *Lpb. plantarum* in the growth inhibition of cancer cells, and proposes butyrate-mediated cell cycle regulation as a potential involved mechanism. Since the production of butyric acid in *Lpb. plantarum* has been proven strain-dependent and differentially boosted by specific prebiotic compounds, our results open future research paths to determine whether this metabolic activity could be modulated *in vivo* by enhancing this antiproliferative effects on cancer cells.

1. Introduction

Bifidobacteria and the heterogeneous group of Lactic Acid Bacteria (LAB) are still nowadays among the most well-studied and commonly used probiotic groups in food and pharmaceutical preparations [1,2]. In particular, the former *Lactobacillus* genus is the most investigated due to several beneficial properties demonstrated *in vitro* and *in vivo* from the Metchnikoff's time to our days [3,4]. Lactobacilli are common members of human microbiota and the vagina is considered as their primary source of isolation, while in the gastrointestinal tract they are largely predominant in the small intestine [5–8]. Moreover, in the last decade, the scientific community has focused attention on traditional fermented foods of animal and vegetable origins, recognizing them as valid and heterogeneous sources for acquiring new probiotic candidates with potential health benefits [1,2,9], such as the growth inhibition of cancer cells, especially colon cancer cells [10,11].

The key risk factors for colorectal cancer are universally recognized, and they include a high fat diet, the presence of mutagens in food, folate deficiency and inflammatory disorders due to dysbiosis [16]. As proposed in several *in vitro* studies, probiotic lactobacilli might activate anticancer mechanisms in the human gut, either by preventing the carcinogenesis process or by inhibiting directly the proliferation of cancer cells [14,17,18] while certain strains have shown to possess significant immunomodulating effects [19,20]. In particular, it has been demonstrated that lactobacilli group may counteract the aberrant metabolism of cancer cells by inducing apoptosis [11,15,21,22], autophagy [23], differentiation [24], or cell cycle arrest [12,25]. Moreover, several strains of the former *Lactobacillus* genus are able to maintain the intestinal enterocytes in a healthy status, by acting on the intestinal barrier function [26] or by modulating the host immune response

* Correspondence to: Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.
E-mail address: lucasimone.cocolin@unito.it (L. Cocolin).

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towards a reduction of inflammatory status [27], which are both direct causes of carcinogenesis in the colon. Finally, lactobacilli have been shown to raise protective anticancer immune responses in preclinical cancer models [13,20]. Noteworthy, in many cases the exact mechanism of cancer cell inhibition exerted by each individual probiotic strain seems to be strain-specific and dependent on bacterial metabolites.

As far as the bacterial metabolites involved, lactobacilli may exert anticancer effects through the production of active peptides [28–30], soluble polysaccharides [31], polyphosphates [24] and short chain fatty acids (SCFAs) [32,33]. Among the SCFAs, butyric acid has the strongest influence on host physiology and gut homeostasis, by modulating the colonic inflammation status, colonic defense barrier, intestinal epithelial permeability, oxidative stress and cryptic stem cells differentiation [34–37]. This SCFA is a major energy source for the colonic epithelium *in vivo* and is thought to stimulate proliferation in normal cells, while in contrast it was shown to inhibit proliferation and induce differentiation and apoptosis in colonic epithelial cells of tumor origin via the inhibition of histone deacetylase activity [38,39].

In this context, we have previously reported a glutamine-mediated butyrogenic capability in *Lactiplantibacillus* (*Lpb*.) *plantarum* strains of food origin and theorized the metabolic pathway responsible of this SCFA production in DMEM, a common human cell growth medium [40]. The present study is therefore aimed to assess whether this differential butyrogenic capability induces a specific growth inhibitory mechanism against human colon cancer cells. Thus, we comparatively assayed the *in vitro* cancer cells growth inhibition activity exerted by the butyrate-producer strain *Lpb. plantarum* S2T10D and the not-producer strain O2T60C against HT-29 cells, and explored some potential mechanisms that might be involved.

2. Materials and methods

2.1. Cell cultures and bacterial strains

Human colon carcinoma cells HT-29 cells (91072201, ECCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM 6429; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco- Life Technologies Corporation, Carlsbad, CA, USA), 2 mM of L-glutamine, 10 U mL^{-1} of penicillin and 0.1 mg/mL of streptomycin (Sigma-Aldrich). The cell lines were grown in 25 or 75 cm^2 culture flasks (Corning, New York, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The culture media were changed routinely and once the cells reached sub-confluence (80–90%), after 3– 4 days, they were passaged further. HT-29 cells were washed thrice with Phosphate Buffered Saline (PBS) before the addition of the bacteria to remove any traces of the antibiotics.

The putative probiotics *Lpb. plantarum* S2T10D and *Lpb. plantarum* O2T60C [40] were routinely cultivated 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 the co-incubation experiments 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 the bacteria reached the early-stationary phase (18 h), and used for the experiments (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).

2.1.1. Bacterial cells and extracellular soluble fractions

In order to assay the effects of viable bacteria (Vb) on human colon carcinoma cells, the working cultures were washed twice in PBS and then resuspended in DMEM supplemented with 10% of FBS, without antibiotics. Furthermore, heat killed bacteria (HKb) were prepared by heating standardized suspensions of working culture in PBS to 95 °C for 30 min. After heat treatment, HKb were resuspended in DMEM supplemented with 10% of FBS, without antibiotics. Vb and HKb were added to HT-29 cells at different viable count levels concentrations in relation to their OD, as described before.

Bacterial cell-free supernatants (CFS) were produced form working suspensions of the strains inoculated in DMEM at 8.0 ± 0.2 Log CFU mL^{-1} (with 2 mM of L-glutamine and FBS; no antibiotics), incubated for 24 h at 37 °C, and thus collected by centrifugation (20,000 x *g* for 10 min) and filtering (0.22 μm). Viable counts and pH were detected at the beginning and at the end of the 24 h of incubation; bacterial growth has been expressed as Δ Log CFU mL^{-1} (Log CFU $mL^{-1}_{\text{after 24 h}}$ – Log CFU $mL^{-1}_{\text{initial inoculum}}$). Protein content of each CFS was determined using the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Organic acids (citric, pyruvic, lactic, acetic, butyric and propionic) and sugars (lactose, glucose and galactose) were determined with HPLC system (Thermo Finnigan Spectra System, San Jose, USA) equipped with UV detector (UV100; set to 210 nm) and a refractive index detector RI-150, following protocols and parameters previously described[40]. Part of the CFS was buffered with sterile 0.5 M NaOH solution in order to restore the initial pH of the medium (pH 7.80 ± 0.20) and obtain a neutralized cell-free supernatant (CFSn). Subsequently. a fraction of CFSn was treated with proteinase K (Sigma-Aldrich) as previously described [28], obtaining a neutralized bacterial supernatant with an inactivated enzymatic activity (CFSp). CFS, CFSn and CFSp were supplemented with 10% of FBS and tested on HT-29 cells without dilution or diluted in complete DMEM culture medium with ratios 1/2 and 1/10 (CFS/culture medium).

2.1.2. Preparation of modified culture media

Modified culture media (mCM) were produced from DMEM (Sigma; D5030) with L-glutamine supplemented with butyric acid, lactic acid, pyruvic acid and glucose at the concentrations observed in CFS after bacterial growth. The pH of mCM was subsequently neutralized at 7.80 ± 0.20 with a sterile 0.5 M NaOH solution and supplemented with 10% of FBS and antibiotics as described above for culture media.

2.2. Sulforhodamine B (SRB) assay

Cell proliferation was determined with Sulforhodamine B (SRB) assay that measures the protein content which is proportional to cell number [41]. Briefly, HT-29 cells were seeded in 96-well microtiter plates at 15,000 cells/well and were allowed to grow and attach for 24 h [42]. Different concentration of Vb, HKb, CFS, CFSp, CFSpe or mCM were co-incubated with the cells for 24 and 48 h. Cells incubated with appropriate complete media (DMEM with 10% FBS) were used as reference controls. At the end of the incubation time media were removed and cells were washed once with PBS. Following these steps the analysis proceeded as previously described [41].

Measurement of OD was performed at 600 nm and data were expressed by means of the following formula: 100 - ($OD_{\text{sample}}/OD_{\text{reference control}} \times 100$).

2.3. MTT assay

The cellular metabolic activity was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl tetrazolium bromide (MTT; Sigma-Aldrich) as previously described [43], in order to evaluate the effect of bacterial supernatants towards the metabolic activity of HT29 cells regardless of cell growth. Briefly, a confluent monolayer of HT-29 cells was obtained as described previously, and incubated with increasing concentrations of CFS, CFSp and CFSpe for 24 or 48 h in a modified atmosphere of 5% CO_2 and 95% air. Cells incubated with appropriate complete media (DMEM with 10% FBS) were used as reference controls.

Measurement of OD was performed at 490 nm and data were expressed following the formula: $OD_{\text{treated cells at each time points}}/OD_{\text{reference control at T0}} \times 100$.

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR

HT-29 cells were seeded in 6-well plates (5.0×10^5 cells/well) and grown for 36 h, until they reached 60–80% of confluence, and then cells exposed to CFSp and mCM for 4, 8 and 12 h of incubation. In parallel, A cells growth was being monitored with the SRB assay, as described previously. Cells incubated with complete culture medium were used as blank.

After the defined incubation periods, media from treated HT-29 cells were removed and total RNA was extracted using Trizol® reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Integrity of the extracts was checked using agarose gel (0.8%) electrophoresis, and their quantity and purity were determined using a Nanodrop ND-1000 spectro-photometer (Celbio, Milan, Italy). The cDNA was synthesized from 2 μg of RNA with the AMV Reverse Transcriptase System (Promega, Fitchburg, Wisconsin, USA) as previously described [44]. The resulting cDNA was stored at – 80 °C until the qPCR analysis.

The cDNA samples were analyzed with Chromo4 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.), by processing all together the samples collected from the same incubation time. The reaction mixture of 20 μL contained 1 × SsoAdvanced SYBR Green Supermix (Bio-Rad), 250 nM of each primer (Sigma Aldrich) and 1 μL of cDNA (100 ng/μL); control without template (NTC, blank) was included in each assay. The thermal cycling conditions are reported in Supplementary Table 1 for each set of primers used in this study [15,45–47]. Primer specificity was verified by performing a melting curve analysis (65–95 °C with a heating rate of 0.2 °C/s and a continuous fluorescence measurement). All reactions were performed in duplicate. Fictive Cp values are set to the highest Cp observed for a truly positive sample, which is assumed to be the level of detection (LOD), plus 1. Results were normalized using the most stable housekeeping (HK) gene among *ACTB, GAPDH, HPRT1* selected in relation to the minimal coefficient of variation [48]. The HT-29 cells condition before co-incubation treatments (time 0) was used as reference to quantify fold change ($2^{-2\Delta\Delta Ct}$) relative mRNA expression of genes *BAX*, *BIRC5a, CCND1*, and *CCNE1* [49]. Fold change were Log_2- transformed to obtain equal dimension between upregulated and downregulated values of genes expression [50].

2.5. Detection of apoptosis

Apoptosis was analyzed with the Annexin V- Propidium iodide (PI) double staining method with a commercially available kit (BD Biosciences) according to the manufacturer's protocol. HT-29 cells were seeded in 10 cm^2 cell culture plates (2×10^6 cells per plate). Cells were left to adhere overnight and subsequently treated with bacterial CFSp for 48 h. Control cells were routinely grown in complete DMEM. Following treatment, non-adherent and adherent cells were collected, washed with PBS and resuspended in Annexin binding buffer. Next, cells were labelled by adding Annexin V-FITC and PI in each sample. After a 15 min incubation period in the dark at room temperature, samples were analyzed on a flow cytometer (Calibur, BD Biosciences). Data analysis was performed with FlowJo V10 software (FlowJo LLC, Ashland, Oregon, USA).

2.6. Cell cycle analysis

For cell cycle analysis, the DNA content of CFSp-treated HT-29 cells was quantified by propidium iodide (PI) staining of fixed cells. HT-29 cells were seeded in 10 cm^2 cell culture dishes (Corning, New York, USA) at a concentration of 10^6 cells/dish and incubated overnight. Cells were treated with the bacterial CFSp for 36 h. Next, cells were harvested by trypsinization, washed twice with PBS and fixed in 70% ice-cold ethanol and stored at − 20 °C for 24 h prior to PI staining. The day of the analysis, fixed cells were washed twice with PBS and resuspended in PBS at a concentration of 10^6 cells/mL. Fifty μL of RNase A solution (100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and 400 μL of PI-stain solution (50 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were added to 100 μL of the fixed-cells and cells were incubated for 40 min at room temperature in the dark. Cell cycle distribution was then analyzed on a flow cytometer (Calibur, BD Biosciences). Further analysis was performed with FlowJo V10 software.

2.7. Western Blot assay

HT29 cells were seeded in 10 cm^2 cell culture dishes (Corning) and left to adhere overnight. Next, cells were treated with CFSp of *Lpb. plantarum* S2T60C for the indicated time points whereas control cells were routinely grown in DMEM. After the incubation period, culture medium was discarded and cells were lysed in RIPA buffer supplemented with protease inhibitors (100 μg/mL phenylmethylsulfonylfluoride (PMSF), 0.5 μg/mL leupeptin, 1.0 μg/mL pepstatin A and 0.5 μg/mL aprotinin). Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol. 60 micrograms of protein extracts were loaded on a 10% SDS- polyacrylamide gel (Bio-Rad). After electrophoresis, proteins were blotted on 0.45 μm PVDF membranes. Membranes were blocked with 5% w/v BSA/TBST solution and incubated overnight, at 4 °C with primary antibodies against cyclin B1 (1:1.000, 4138 Cell Signaling) or cyclin D1 (1:500, 2978 Cell Signaling) in 5% BSA/TBST. Next, membranes were washed with TBST and incubated with HRP-conjugated anti-rabbit secondary antibody (1:5.000). Protein bands were visualized by autoradiography using ECL/HRP chemiluminescent substrate (Life Technologies) and Kodak film. Membranes were then stripped with a stripping buffer (15 g/L glycine, 1 g/L SDS, 10 mL/L Tween20, pH 2.2) and incubated with anti-β-tubulin antibody (1:20.000, T7816, Sigma Aldrich) in 5% w/v non-fat dry milk in BST. A pre-stained protein marker was used to monitor protein molecular weight (Nippon Genetics).

2.8. Statistics

Statistical analyses and data plotting were performed using R program for Statistical Computing 3.6.0 (http://www.r-project.org) and SigmaPlot v11 (Systat Software, USA).

Data are expressed as mean of three independent experiments. Normality and homogeneity of the data were checked by means of Shapiro-Wilk's W and Levene's tests, respectively. Comparisons between individual groups were performed with Student's t-test or Wilcoxon pairs test. To assess the overall variation and differences between multiple groups the one-way ANOVA coupled with Duncan's post-hoc test was carried out for parametric data, the not parametric data were analyzed with Kruskal–Wallis test coupled with multiple comparison of mean ranks. Spearman's correlation coefficient was calculated to detect significant ($P < 0.01$) correlation between variables of interest.

3. Results

3.1. Lpb. plantarum strains S2T10D and O2T60C inhibit colon cancer cells growth in vitro

First, we investigated the potential antiproliferative activity of S2T10D (BLP, a butyrate-producing *Lpb. plantarum* strain) and O2T60C (LP, a *Lpb. plantarum* strain that does not produce butyrate) against HT-29 colon cancer cells. For this purpose, both viable and heat-killed bacteria (Vb and HKb, respectively) were examined. HT-29 cells proliferation rate was analyzed with the SRB method after 24 or 48 h of treatment. Co-incubation of HT29 cells with Vb of both strains induced a concentration- and time-dependent growth inhibition that exceeded 50% (Fig. 1A). While Vb of both strains exhibited inhibitory activity, HKb of neither BLP or LP affected growth of HT29 cells (**data not shown**).

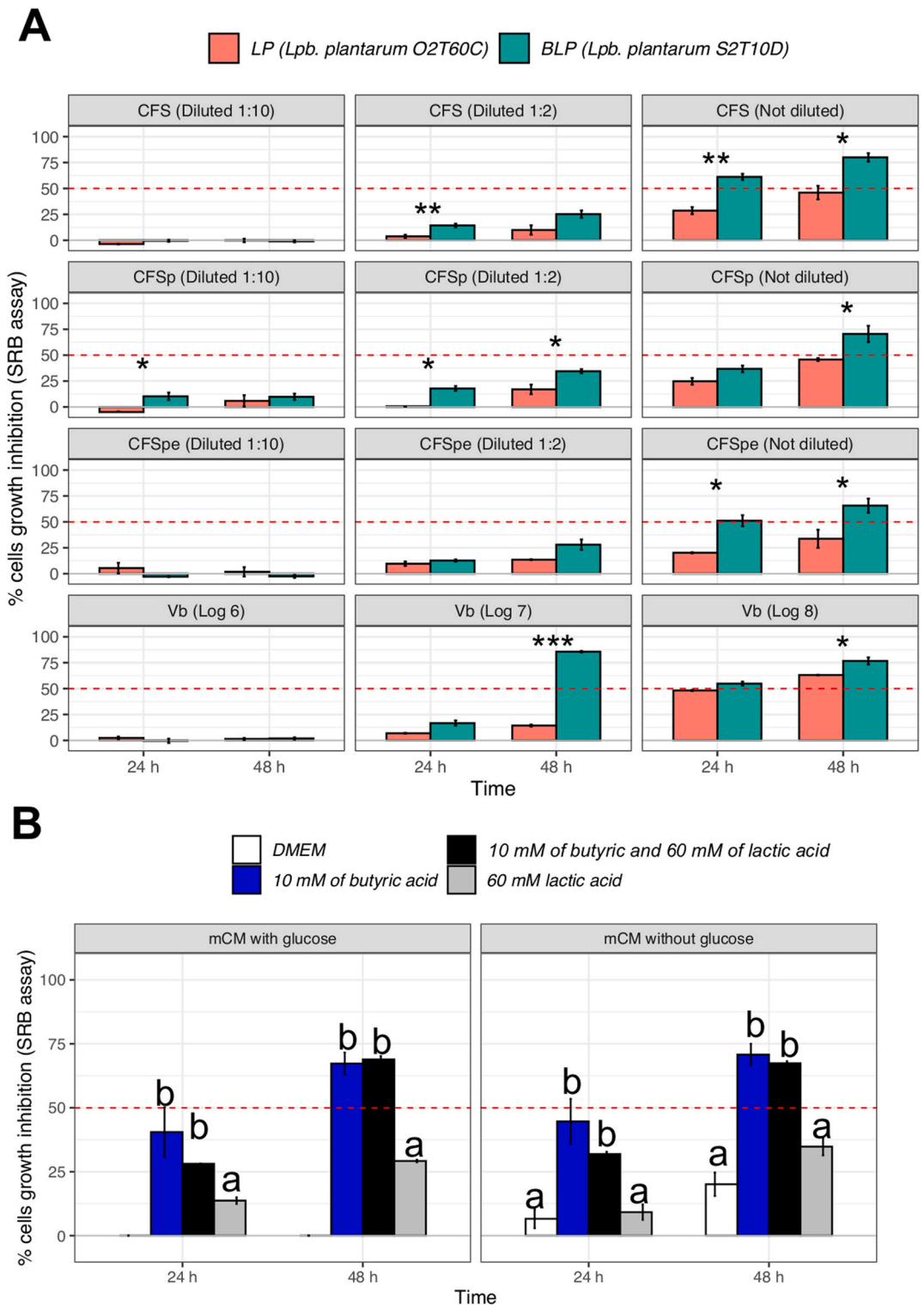

Fig. 1. Anti-proliferative activity of (A) the two *Lpb. plantarum* strains and (B) the modified culture media (cMC) incubated with HT-29 for 24 and 48 h and evaluated by means of SRB assay. (A) BLP (butyrate-producing strain S2T10D) and LP (non- producing strain O2T60C) were tested: viable bacteria (Vb); bacterial cell-free supernatants (CFS); bacterial cell-free supernatant at pH 7 (CFSp); bacterial cell-free supernatant at pH 7 with inactivation of enzymatic activity (CFSpe). Vb were tested at 6, 7 and 8 Log CFU mL^{-1} while CFS/CFSp/CFSpe were tested as such or diluted 1:2 and 1:10. (B) mCM were prepared from DMEM culture media with (4.5 g/L) or without glucose (0 g/L) supplemented with lactic and butyric acids as described in the legend. Data (means ± SEM; n = 3) are expressed in percentage (100 – [OD sample/OD reference control × 100]). At each condition tested, the asterisks (*= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$; T-test) highlighted significant (Student's T-test) differences between the antiproliferative activity of BLP (strain S2T10D) and LP (strain O2T60C), while different letters (a, b, c) highlight significant differences among the mCM ($P < 0.05$; ANOVA and Duncan's post-hoc test). Dashed red line indicate the 50% of anti-proliferation over which a tested compound is defined as cytotoxic. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to investigate whether the observed antiproliferative activity exerted by the two lactobacilli could be associated with their extracellular metabolites, we assayed cell free supernatants (CFS), which were obtained by incubating both BLP and LP in the complete DMEM culture medium (inoculum of log 7 CFU mL^{-1}) for 24 h at 37 °C. The CFS composition, in terms of proteins, glucose, lactic, acetic and butyric acid concentrations, as well as the pH decrease after bacterial growth (expressed in the table as Δ log CFU) are all displayed in Table 1 and compared to the initial composition of DMEM culture medium. Noteworthy, LP showed a significantly ($P < 0.05$) lower growth in DMEM compared to BLP, highlighted by the negative Δ log CFU value and the higher glucose and pH levels. In order to investigate the possible correlation between the antiproliferative effect of bacterial supernatants and their pH or enzymatic activity, we also assessed CFS with adjusted neutral pH (CFSp) and CFS with neutral pH and inactivated enzymes (CFSpe).

All three CFS (CFS, CFSp and CFSpe) of LP and BLP inhibited the proliferation of cancer cells in a concentration and time dependent-manner (Fig. 1A). Possibly due to their different composition, all CFS, CFSp and CFSpe of LP exhibited weaker antiproliferative activity against HT29 cells compared to CFS, CFSp and CFSpe of BLP, respectively. Interestingly, by comparatively examining the effects of CFS, CFSp and CFSpe of either LP or BLP, we observed that their growth inhibitory activity was independent from the pH and enzymatic activity (Spearman's correlation; $P > 0.05$).

Finally, to investigate whether glucose consumption or organic acids produced by bacteria were involved in the growth inhibition activity exerted by CFSp, we examined the effect of modified cell culture media (mCM). The mCM were prepared with DMEM adjusted to the neutral pH in order to simulate CFSp composition: with or without 4.5 g/L of glucose and supplemented with lactic and/or butyric acid, 30 or 60 mM and 10 mM, respectively (Table 1; Fig. 1B). Deprivation of glucose alone affected cell growth to a small extend as a 23% inhibition rate was observed in cells maintained in glucose-deprived mCM for 48 h compared to cells maintained in medium supplemented with glucose (Fig. 1B). Furthermore, both butyric and lactic acids individually inhibited cell growth in a time-dependent manner, while 10 mM of butyric acid induced a remarkable 73% decrease in cell growth rate. Noteworthy, neither glucose deprivation, nor the combination of butyric and lactic acid in concentrations equivalent to the CFSp of BLP (10 mM of butyric and 60 mM of lactic acid) enhanced the antiproliferative effect exhibited by butyric acid alone.

3.2. Cell-free supernatants of BLP and LP inhibit cancer cells metabolic activity

Based on the effects of both BLP and LP supernatants in cancer cell growth, we further investigated whether the observed antiproliferative activity was related to the inhibition of cancer cells metabolic activity. For this purpose, we treated confluent monolayers of cells (no growth state) with CFS, CFSp and CFSpe as described above, and assayed cellular metabolic activity with the MTT method. As observed for the SRB, the MTT assay revealed that CFS, CFSn and CFSp of BLP exhibited a stronger growth inhibition compared to LP supernatants (Fig. 2). Noteworthy, CFS of both BLP and LP have shown more potent repression effect on cells metabolic activity compared to their CFSp or CFSpe; i.e. low pH inhibited more cells metabolic activity (MTT assay) than cells proliferation (Supplementary Table 2).

3.3. Cell-free supernatant of BLP modulates the expression of cyclin D1 gene

In order to further investigate the mechanisms involved in the observed antiproliferative activity exerted by the CFSp of BLP and LP, we assessed the relative mRNA expression in HT29 cells of: (i) two key regulators of the apoptotic pathway, namely *BAX* and *BIRC5a*; (i) two target genes involved in cell cycle progression, namely *CCND1* and CCNE*1* (Supplementary table 1). Moreover, we analyzed the effect of modified cell culture medium (mCM) deprived of glucose and supplemented with butyric acid, lactic acid, or their combination. Differential gene expression, standardized to 0 h treatment, was compared between control untreated cells (incubated with DMEM medium complete of glucose) and CFSp-treated or mCM-treated cells after 4, 8 or 12 h of treatment (Fig. 3).

Expression level of *BAX* mRNA was significantly downregulated after 8 h of cells incubation with mCM supplemented with butyric acid and upregulated in cells treated with 60 mM of lactic acid for 12 h**.** On the other hand, no significant modification in the *BIRC5a* expression levels were observed, either in CFSp- and mCM-treated cells, regardless from the incubation time.

Downregulation in mRNA expression of *CCNE1* encoding for the protein cyclin E1, was observed in cells treated with mCM supplemented with lactic acid after 4 h and in all mCM-treated cells after 12 h. The mRNA expression of *CCND1*, encoding for cyclin D1, was downregulated ($P < 0.01$) in HT-29 cells after either 4, 8 or 12 h of treatment with mCM supplemented with butyric acid alone and in combination with lactic acid. In parallel, the CFSp of BLP determined a significant down-regulation of *CCND1* expression HT-29 cells after 8 h of treatment, while no effect was observed in cells treated with the CFSp of LP. Interestingly, the *CCND1* mRNA expression along the incubation time was negatively correlated (Spearman's correlation; $P < 0.001$; $R = -0.52$) with the amount of butyric acid detected in CFSp and added in mCM, while the concentrations of glucose and lactic acid contents of CFSp and mCM did not show any significant correlation with the expression of this gene.

Overall, *BAX*, *CCNE1*, and *BIRC5a* mRNA expression levels did not show any significant correlation ($P < 0.05$) with glucose, lactic and butyric acids contents of mCM and CFSp (Table 1).

3.4. Cell-free supernatant of BLP exerts cell cycle-related effects on HT29 cells

Based on our observations regarding the growth inhibitory activity of

Table 1
Cell free supernatants (CFS) obtained from *Lpb. plantarum* strains S11T3E, S2T10D and O2T60C (7.0 ± 0.2 Log CFU mL^{-1}) inoculated in DMEM medium (D2426; Sigma, supplemented with 2 mM of L-glutamine) and incubated for 24 h at 37 °C. Protein content was quantify by BCA assay, quantification of glucose and organic acids were assessed with HPLC-UV-RI, and growth capability was calculated following the formula: Log CFU $mL^{-1}{}_{\text{after 24 h}}$ – Log CFU $mL^{-1}{}_{\text{initial inoculums}}$. Results are the means (± SEM) of 3 independent experiments (n = 3). Levels of significance were reported in the table for each characteristic considered.

				Concentration (mM)				
Strains	Protein (mg mL^{-1})	pH	Δ Log CFU	Glucose	Pyruvic acid	Lactic acid	Acetic acid	Butyric acid
L. plantarum S2T10D	1.13 ± 0.07	4.42 ± 0.06 a	0.21 ± 0.06 b	n.d.	0.4 ± 0.1 a	57.8 ± 3.7	0.5 ± 0.2	8.0 ± 1.2
L. plantarum O2T60C	1.57 ± 0.18	6.2 ± 0.24 b	-0.38 ± 0.16 a	10.1 ± 2.7 a	1.2 ± 0.3 b	40 ± 7.1	n.d.	n.d.
Cuture medium	1.54 ± 0.07	7.85 ± 0.06c	n.d.	25.8 ± 1.1 b	1.4 ± 0.2 b	n.d.	n.d.	n.d.
Significance:	ns	* **	* **	* **	* *	ns	/	/

a, b, c, d: Different letters indicate a significant difference among the thesis ($p < 0.05$). /: not analyzed. n.d.: not detected. ns: not significant. *$p < 0.05$. * *$p < 0.01$. * **$p < 0.001$

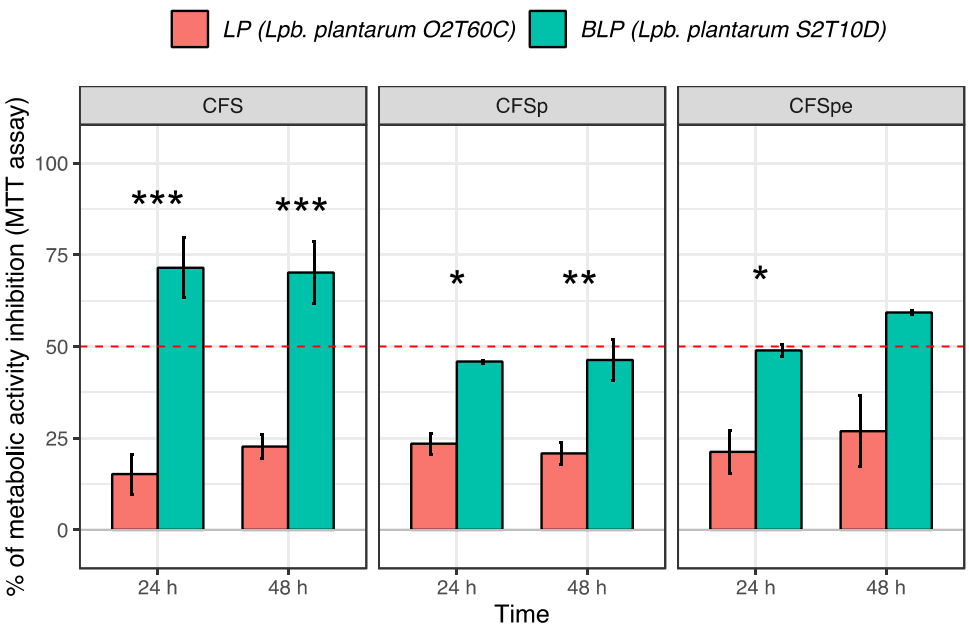

Fig. 2. Metabolic activity of confluent HT-29 (no-growth condition) cells treated with bacterial supernatants (CFS, CFSp, CFSpe) and evaluated by means of MTT assay. Inhibition of metabolic activity was detected after 24 and 48 h of incubation with bacterial supernatants. Data (means ± SEM; n = 3) are expressed in percentage (100 − [OD sample/OD reference control × 100]); At each condition tested, the asterisks (*= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$; T-test) highlighted significant (Student's T-test) differences between the anti-proliferative activity of BLP (strain S2T10D) and LP (strain O2T60C).

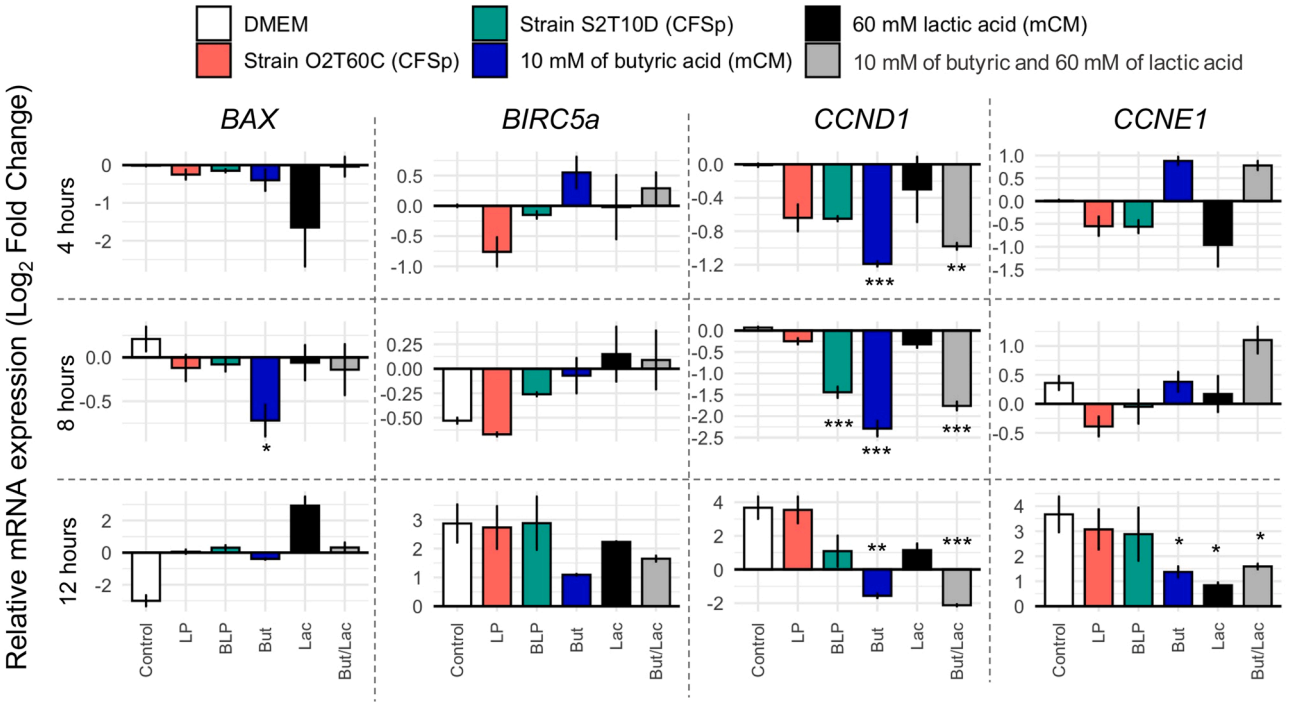

Fig. 3. Relative expression levels (mRNA) of genes related to apoptosis (*BAX, BIRC5a*) and cell cycle progression (*CCND1, CCNE1*) in HT-29 cells incubated for 4, 8 and 12 h with bacterial supernatants (CFSp) and modified culture media (mCM). Log_2 Fold change (means ± SEM; n = 3) are relative to the control cells recovered before the co-incubation treatments (time 0). The asterisks highlight significant differences (Wilcoxon's test; *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$) between the relative expression condition of the untreated control (cells incubated with complete DMEM with 4.5 g/L of glucose) and treatment condition, namely: CFSp of S2T10E (BLP); CFSp of O2T60C (LP); mCM with butyric (10 mM) and lactic (60 mM) acids (But/Lac); mCM with 10 mM of butyric acid (But); mCM with 60 mM of lactic acid (Lac). All mCM were deprived of glucose.

BLP's CFSp and its cyclin D1 downregulating effect, we investigated the potential biological mechanisms involved, by examining whether BLP's CFSp induces apoptosis and cell cycle arrest in cancer cells. Apoptosis was studied by analysing the phosphatidylserine translocation across the plasma membrane with the Annexin V-FITC/PI flow cytometry assay, while the cell cycle progression was assayed by quantification of DNA content analyzed also by flow cytometry.

We concluded that neither cell death, nor apoptosis are involved in the growth inhibitory activity of BLP's CFSp, as, even after 48 h of treatment no significant signs of apoptosis or necrosis were detected in cells (Fig. 4A). However, a significant ($p < 0.05$) cell population shift to G2/M phase compared to the untreated cells, was noted after 36 h of treatment with BLP's CFSp, reaching thereby the G2/M fraction percentage of 28.6% in treated cells, compared to 12.3% in control cells (Fig. 4B). In accordance to the G2/M arrest induced by BLP's CFSp, we observed a significant decrease in cyclin B1 protein level in the twelfth

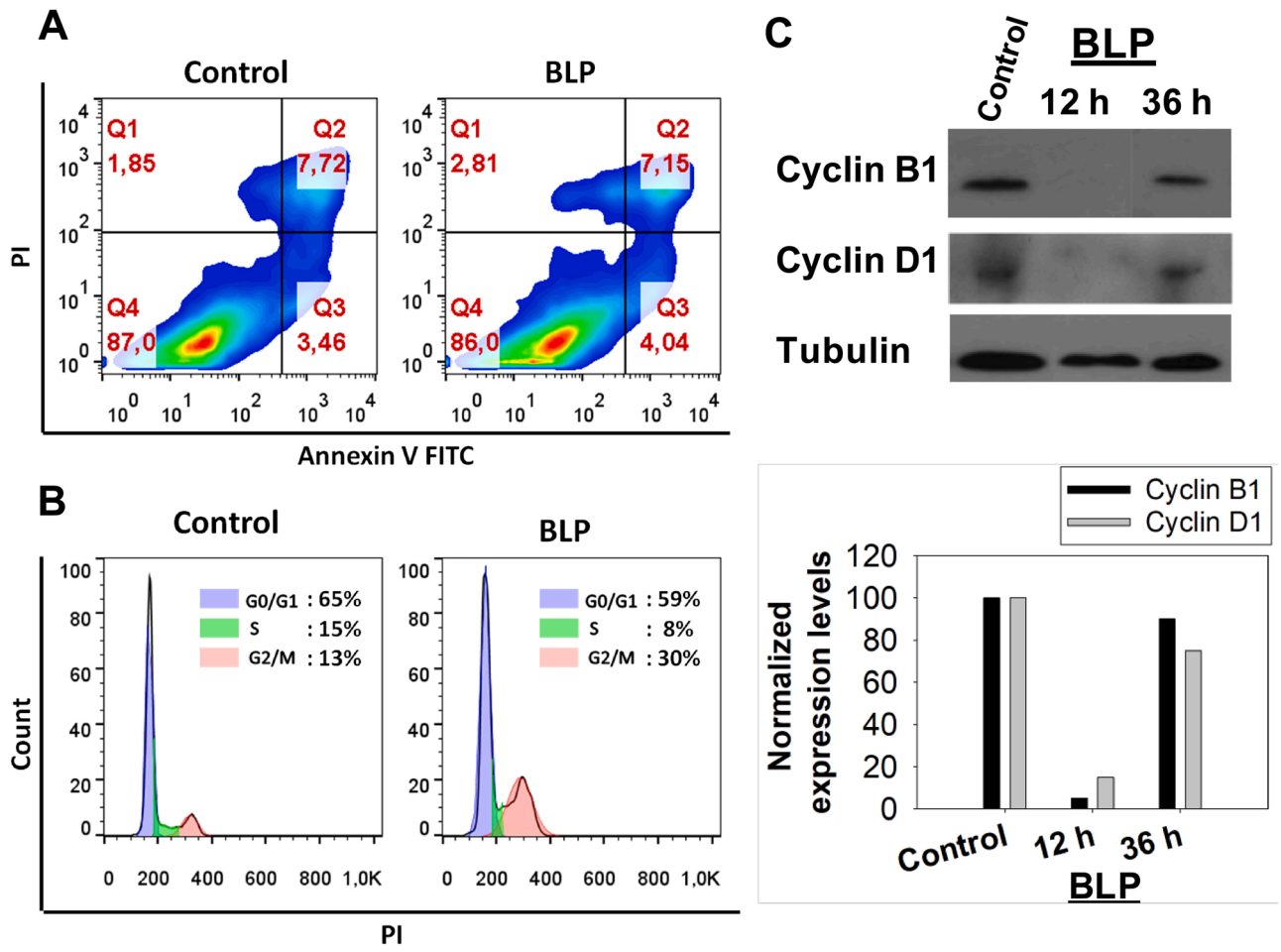

Fig. 4. Confirmation of cell cycle regulation activity by the supernatant of S2T10D. Annexin V-PI assay (A) and Flow cytometric analysis of cell-cycle progression (B) in HT29 cells treated with CFSp of *Lpb. plantarum* S2T10D for 48 or 36 h respectively. Control cells were grown in complete DMEM. Cells were fixed and stained with PI. The flow cytometric histograms are representative of three independent experiments (n = 3). Y-axis represents number of cells; X-axis represents DNA content (PI intensity). The data are presented as the means ± SD of three independent experiments ($P < 0.05$). (C) Western blot results showing cyclin B1 and D1 protein levels after 12 and 36 h of co-incubation with CFSp of *Lpb. plantarum* S2T10D.

hour of co-incubation, namely 5% compared to the control. This trend was still observable but not significant at 36 h, with a slight reduction of cyclin B1 protein expression percentage level (90%). The *CCND1* mRNA downregulation was confirmed at protein level, with cyclin D1 protein level reduced to 15% in treated cells compared to control after 12 h of treatment (Fig. 4C).

4. Discussion

Herein, the effects of two *Lpb. plantarum* against the growth of human intestinal cancer cells have been investigated to define the potential correlation between their strain-specific butyrogenic capability and the biological mechanisms underpinning these phenomena. For this purpose, the antiproliferative activities of the butyrate-producing (BLP) *Lpb plantarum* S2T10D and the not-butyrogenic strain (LP) O2T60C have been compared *in vitro*.

Viable bacteria inhibited cell growth in a time- and concentration-dependent manner, reaching the cytotoxicity cut-off threshold of 50% [41] after 24 h of co-incubation with HT-29 cells, which are considered suitable *in vitro* models to test the cytotoxicity exerted by bacteria against intestinal cancers cells and their aberrant metabolism and physiology [51,52]. Moreover, to investigate which bacterial macro-fractions were responsible for the anti-proliferating effects observed, cell-free supernatants and heat-inactivated bacteria were assayed, with the latter showing no inhibitory effects on the growth of HT-29 cells. This observation enabled us to exclude the involvement of heat-stable cell-bound components in the growth inhibitory activity exerted by the bacteria, which instead have been previously indicated among the main compounds responsible for the antiproliferative activity of several lactobacilli species [23,25,53]. Moreover, it suggests that Vb inhibit cancer cells proliferation by releasing extracellular metabolites, as it has already been observed for *Lpb. plantarum* [11,27,30].

In this frame, the organic acids and glucose profiles of bacterial supernatants reflected the growth capability of each strain in the DMEM culture medium. Accordingly, the viable cells of *Lpb. plantarum* O2T60C and its supernatant showed a modest anti-proliferative profile due to its low metabolic activity and inability to produce butyric acid. This SCFA was produced by *Lpb. plantarum* S2T10D, by confirming previous results in cell culture media, cheeses and bacterial culture broth [40,54,55]. Overall, *Lpb. plantarum* is known to produce butyric acid in relation to the media composition and, for instance, the fructo-oligosaccharides (FOS) and the inulin induce its production [56,57]. In our case, we identified in a prior study the L -glutamine as the main inducer of butyrogenic capability in S2T10D (BLP), by linking the butyrate metabolism of the species *Lpb. plantarum* to the complementary activities of a medium-chain thioesterase and the Fatty Acid Synthase (FASII) pathway [40]. This characterization of the butyrogenic pathway in *Lbp. plantarum* has been recently confirmed by another genomic investigation [58]. Regardless from the butyrogenic metabolism of *Lpb. plantarum*, according to the literature the concentrations of butyric acid found in

S2T10D supernatant is adequate to inhibit significantly the proliferation of cancer cells [59,60]. This was confirmed by the reduced growth rates we observed in HT-29 cells treated with mCM supplemented with 10 mM of butyric acid and tested at neutral pH. To the best of our knowledge no studies to date have attributed to *Lpb. plantarum* the production of butyrate as a possible antiproliferative mechanism independently of media acidification.

Together with butyric acid, SCFAs like propionate and acetate have been proposed as the molecules responsible for cancer cells inhibition exerted respectively by propionibacteria and bifidobacteria [33,61–64], but mainly in relation to the pH decrease.

In our study the media acidification had a negligible effect on HT-29 growth inhibition, but it significantly impacted the human cells metabolic activity, independently from their proliferation. This second observation is in accordance with the previously reported association between metabolic activity of human cells and the parallel consumption of sugar and acidification produced by bacterial growth [62]. Besides, the mCM tested with or without glucose demonstrated that the sugar consumed by bacteria during the co-incubation with HT-29 cells is not the main reason behind the antiproliferative effect exerted by the two strains.

Furthermore, antiproliferative activities of *Lpb. plantarum* supernatants have been attributed to the bacterial pheromone PlnA [30] and to other diffusible bacteriocin-like peptides [26,28,30], however here the negligible differences observed between pH-neutralized supernatants (CFSp) and their protease-treated counterparts (CFSpe) suggest a limited involvement of proteinaceous components for both strains.

Flow cytometry cell cycle and apoptosis analysis, as well as the decreased mRNA expression levels of Cyclin D1 in HT29-cells treated with the CFSp of BLP or the mCM supplemented with butyric acid, indicate that cell growth inhibition might be due to cell cycle arrest rather than apoptotic cell death, which is often reported for various lactobacilli strains with anticancer activity [65–67]. This conclusion is also supported by the similar expression levels of BAX gene between treated and control cells; the oligomerization of this pro-apoptotic protein triggers the breaking of mitochondrial outer membrane, the release of cytochrome C and the subsequent apoptogenic cascade [68]. Interestingly, this pathway can be activated *in vitro* by nutrients deprivation, like the one induced by the direct (viable cells) or indirect (supernatants) fermentative activities of these two *Lpb. plantarum* strains. Strains of this species have indeed shown the *in vitro* capability to enhance Bax protein level in a strain- dependent manner [69,70], however we did not detect such an effect in our experiments.

On the other hand, the significant downregulation of cyclin D1 expression in HT-29 cells exerted by both butyrate-producer S2T10D and butyrate-supplemented media, confirm the indirect but key role of butyric acid in the transcriptional downregulation of this gene [38,71,72]. Noteworthy, cyclin D1 is considered a hub gene of the protein-protein interaction network described in colon cancer cells metabolism and has been proposed as a valid target for developing novel therapeutic strategies [73].

The transcriptional evidences of a butyrate-related effect of S2T10D in the cells cycle regulation were thus confirmed by the G2/M cell cycle arrest and the reduction of cyclin D1 and cyclin B1 protein levels observed in HT-29 cells treated with its supernatant (CFSp). Similarly to its effect in cyclin D1 regulation, butyrate has also been shown to increase the hyperacetylation of histone and to downregulate downstream the expression of cyclin B1 [74].

In summary, the mechanisms involved in the antiproliferative properties induced by the two lactobacilli strains are multifaceted and generally correlated to bacterial growth. However, the greater inhibitory activity of the strain S2T10D seems to be related to its butyrogenic capability and induction of cell cycle arrest. Taking into account the current limited knowledge about butyrogenic capability of *Lbp. plantarum* in the intestine, this study highlights the importance to explore in depth this substrate- and strain-dependent metabolic activity and its biological potential. Finally, considering the key role of butyric acid in the maintenance of gut homeostasis and the ubiquity of *Lpb. plantarum* in fermented foods, we propose that synbiotic combinations between *Lpb. plantarum* species and targeted pro-butyrogenic prebiotics could potentially improve the clinical efficacy of cancer preventive/therapeutic approaches.

CRediT authorship contribution statement

Katerina Chlichlia, **Kalliopi Rantsiou**, **Luca Cocolin** and **Cristian Botta** designed and conducted the research. **Cristian Botta** and **Katerina Spyridopoulou** performed co-culturing experiments with human cancer cells and bacteria. **Marta Bertolino** carried out HPLC analysis. **Cristian Botta** and **Katerina Spyridopoulou** conducted statistical analysis and generated the manuscript figures. **Cristian Botta** wrote the main manuscript text. **Katerina Chlichlia** and **Katerina Spyridopoulou** contributed to the manuscript preparation. All authors read and approved the final manuscript..

Conflict of interest statement

Authors declare no no financial/personal interest or belief that could affect the objectivity of the study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112755.

References

[1] M.I. Hossain, M.F.R. Mizan, M. Ashrafudoulla, S. Nahar, H.J. Joo, I.K. Jahid, S. H. Park, K.S. Kim, S. Do, Ha. Inhibitory effects of probiotic potential lactic acid bacteria isolated from kimchi against *Listeria monocytogenes* biofilm on lettuce, stainless-steel surfaces, and MBECTM biofilm device, LWT Food Sci. Technol. 118 (2020), https://doi.org/10.1016/j.lwt.2019.108864.

[2] T. Touret, M. Oliveira, T. Semedo-Lemsaddek, Putative probiotic lactic acid bacteria isolated from sauerkraut fermentations, PLoS One 13 (2018), https://doi.org/10.1371/journal.pone.0203501.

[3] N. Saad, C. Delattre, M. Urdaci, J.M. Schmitter, P. Bressollier, An overview of the last advances in probiotic and prebiotic field, LWT Food Sci. Technol. 50 (2013) 1–16.

[4] J. Zheng, S. Wittouck, E. Salvetti, C.M.A.B. Franz, H.M.B. Harris, P. Mattarelli, P. W. O'Toole, B. Pot, P. Vandamme, J. Walter, K. Watanabe, S. Wuyts, G.E. Felis, M. G. Gänzle, S. Lebeer, A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* (Beijerinck 1901), and union of *Lactobacillaceae* and *Leuconostocaceae*, Int. J. Syst. Evol. Microbiol. (2020), https://doi.org/10.1099/ijsem.0.004107.

[5] C. Huttenhower, J. Fah Sathirapongsasuti, N. Segata, D. Gevers, (Human Microbiome Project Consortium), et al., Structure, function and diversity of the healthy human microbiome, Nature 486 (2012) 207–214.

[6] Y. Nami, N. Abdullah, B. Haghshenas, D. Radiah, R. Rosli, A.Y. Khosroushahi, Probiotic potential and biotherapeutic effects of newly isolated vaginal *Lactobacillus acidophilus* 36YL strain on cancer cells, Anaerobe 28 (2014) 29–36.

[7] K.C. Anukam, J.M. Macklaim, G.B. Gloor, G. Reid, J. Boekhorst, B. Renckens, S.A.F. T. van Hijum, R.J. Siezen, Genome sequence of *Lactobacillus pentosus* KCA1: vaginal isolate from a healthy premenopausal woman, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0059239.

[8] G.P. Donaldson, S.M. Lee, S.K. Mazmanian, Gut biogeography of the bacterial microbiota, Nat. Rev. Microbiol. 14 (2015) 20–32.

[9] X. Xu, D. Luo, Y. Bao, X. Liao, J. Wu, Characterization of diversity and probiotic efficiency of the autochthonous lactic acid bacteria in the fermentation of selected raw fruit and vegetable juices, Front. Microbiol. 9 (2018), https://doi.org/10.3389/fmicb.2018.02539.

[10] G. Aindelis, P. Ypsilantis, K. Chlichlia, Alterations in faecal microbiota and elevated levels of intestinal iga following oral administration of *Lacticaseibacillus casei* in mice, Probiotics Antimicrob. Proteins (2021), https://doi.org/10.1007/s12602-021-09864-7.
[11] G. Saxami, A. Karapetsas, E. Lamprianidou, I. Kotsianidis, A. Chlichlia, C. Tassou, V. Zoumpourlis, A. Galanis, Two potential probiotic *Lactobacillus* strains isolated from olive microbiota exhibit adhesion and anti-proliferative effects in cancer cell lines, J. Funct. Foods 24 (2016) 461–471.
[12] M. Uccello, G. Malaguarnera, F. Basile, V. D'Agata, M. Malaguarnera, G. Bertino, M. Vacante, F. Drago, A. Biondi, Potential role of probiotics on colorectal cancer prevention, BMC Surg. 12 (2012), https://doi.org/10.1186/1471-2482-12-s1-s35.
[13] E.S.L. Chong, A potential role of probiotics in colorectal cancer prevention: review of possible mechanisms of action, World J. Microbiol. Biotechnol. 30 (2014) 351–374.
[14] I. Kahouli, C. Tomaro-Duchesneau, S. Prakash, Probiotics in colorectal cancer (CRC) with emphasis on mechanisms of action and current perspectives, in: J. Med. Microbiol., 62, 2013, pp. 1107–1123.
[15] A. Górska, D. Przystupski, M.J. Niemczura, J. Kulbacka, Probiotic bacteria: a promising tool in cancer prevention and therapy, Curr. Microbiol. 76 (2019), https://doi.org/10.1007/S00284-019-01679-8.
[16] G. Aindelis, K. Chlichlia, Modulation of anti-tumour immune responses by probiotic bacteria, Vaccines 8 (2020), https://doi.org/10.3390/vaccines8020329.
[17] B. Haghshenas, Y. Nami, D. Radiah, Different effects of two newly-isolated probiotic *Lactobacillus plantarum* 15HN and *Lactococcus lactis* subsp. *lactis* 44Lac strains from traditional dairy products on cancer cell lines, Anaerobe 30 (2014) 51–59, https://doi.org/10.1016/j.anaerobe.2014.08.009.
[18] A. Tiptiri-Kourpeti, K. Spyridopoulou, V. Santarmaki, G. Aindelis, E. Tompoulidou, E.E. Lamprianidou, G. Saxami, P. Ypsilantis, E.S. Lampri, C. Simopoulos, I. Kotsianidis, A. Galanis, Y. Kourkoutas, D. Dimitrellou, K. Chlichlia, *Lactobacillus casei* exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells, PLoS One 11 (2016), e0147960, https://doi.org/10.1371/journal.pone.0147960.
[19] N.M. El-Deeb, A.M. Yassin, L.A. Al-Madboly, A. El-Hawiet, A novel purified *Lactobacillus acidophilus* 20079 exopolysaccharide, LA-EPS-20079, molecularly regulates both apoptotic and NF-κB inflammatory pathways in human colon cancer, Microb. Cell Factor. 17 (29) (2018), https://doi.org/10.1186/S12934-018-0877-Z.
[20] U. Tukenmez, B. Aktas, B. Aslim, S. Yavuz, The relationship between the structural characteristics of lactobacilli-EPS and its ability to induce apoptosis in colon cancer cells in vitro, Sci. Rep. 9 (2019) 8268, https://doi.org/10.1038/s41598-019-44753-8.
[21] Y. Kim, S. Oh, H.S. Yun, S. Oh, S.H. Kim, Cell-bound exopolysaccharide from probiotic bacteria induces autophagic cell death of tumour cells, Lett. Appl. Microbiol. 51 (2010) 123–130.
[22] A.K.I. Sakatani, M. Fujiya, N. Ueno, S. Kashima, J. Sasajima, K. Moriichi, K. Ikuta, H. Tanabe, Y. Kohgo, Polyphosphate derived from *Lactobacillus brevis* inhibits colon cancer progression through induction of cell apoptosis, Anticancer Res. 598 (2016) 591–598.
[23] L. Huang, Y.-J. Shan, C.-X. He, M.-H. Ren, P.-J. Tian, W. Song, Effects of *L. paracasei* subp. *paracasei* X12 on cell cycle of colon cancer HT-29 cells and regulation of mTOR signalling pathway, J. Funct. Foods 21 (2016) 431–439.
[24] P. Hu, W. Song, Y. Shan, M. Du, M. Huang, C. Song, L. Zhang, *Lactobacillus paracasei* subsp. *paracasei* M5L induces cell cycle arrest and calreticulin translocation via the generation of reactive oxygen species in HT-29 cell apoptosis, Food Funct. 6 (2015) 2257–2265.
[25] J. Escamilla, M.A. Lane, V. Maitin, Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion in vitro, Nutr. Cancer- Int. J. 64 (2012) 871–878.
[26] A. Borthakur, A.N. Anbazhagan, A. Kumar, G. Raheja, V. Singh, K. Ramaswamy, P. K. Dudeja, The probiotic *Lactobacillus plantarum* counteracts TNF-{alpha}-induced downregulation of SMCT1 expression and function, Am. J. Physiol. Gastrointest. Liver Physiol. 299 (2010) 928–934.
[27] G. Aindelis, A. Tiptiri-Kourpeti, E. Lampri, K. Spyridopoulou, E. Lamprianidou, I. Kotsianidis, P. Ypsilantis, A. Pappa, K. Chlichlia, Immune responses raised in an experimental colon carcinoma model following oral administration of *Lactobacillus casei*, Cancers 12 (2020) 1–16, https://doi.org/10.3390/cancers12020368.
[28] D. Dimitrovski, A. Cencič, E. Winkelhausen, T. Langerholc, *Lactobacillus plantarum* extracellular metabolites: In vitro assessment of probiotic effects on normal and cancerogenic human cells, Int. Dairy J. 39 (2014) 293–300.
[29] B. Di Luccia, N. Manzo, L. Baccigalupi, V. Calabrò, E. Crescenzi, E. Ricca, A. Pollice, *Lactobacillus gasseri* SF1183 affects intestinal epithelial cell survival and growth, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0069102.
[30] S.L. Sand, T.M. Haug, J. Nissen-Meyer, O. Sand, The bacterial peptide pheromone plantaricin A permeabilizes cancerous, but not normal, rat pituitary cells and differentiates between the outer and inner membrane leaflet, J. Membr. Biol. 216 (2007) 61–71, https://doi.org/10.1007/s00232-007-9030-3.
[31] J. Wu, Y. Zhang, L. Ye, C. Wang, The anti-cancer effects and mechanisms of lactic acid bacteria exopolysaccharides in vitro: a review, Carbohydr. Polym. 253 (2021), https://doi.org/10.1016/J.CARBPOL.2020.117308.
[32] M. Thirabunyanon, P. Hongwittayakorn, Potential probiotic lactic acid bacteria of human origin induce antiproliferation of colon cancer cells via synergic actions in adhesion to cancer cells and short-chain fatty acid bioproduction, Appl. Biochem. Biotechnol. 169 (2013) 511–525.
[33] T. Matsuki, T. Pédron, B. Regnault, C. Mulet, T. Hara, P.J. Sansonetti, Epithelial cell proliferation arrest induced by lactate and acetate from *Lactobacillus casei* and *Bifidobacterium breve*, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0063053.
[34] H.M. Hamer, D. Jonkers, K. Venema, S. Vanhoutvin, F.J. Troost, R.J. Brummer, Review article: the role of butyrate on colonic function, Aliment. Pharmacol. Ther. 27 (2008) 104–119, https://doi.org/10.1111/j.1365-2036.2007.03562.x.
[35] G.E. Kaiko, S.H. Ryu, O.I. Koues, P.L. Collins, L. Solnica-Krezel, E.J. Pearce, E. L. Pearce, E.M. Oltz, T.S. Stappenbeck, The colonic crypt protects stem cells from microbiota-derived metabolites, Cell 167 (2016) 1708–1720.
[36] R.X. Wang, J.S. Lee, E.L. Campbell, S.P. Colgan, Microbiota-derived butyrate dynamically regulates intestinal homeostasis through regulation of actin-associated protein synaptopodin, Proc. Natl. Acad. Sci. USA 117 (2020) 11648–11657, https://doi.org/10.1073/PNAS.1917597117.
[37] D. Parada Venegas, M.K. De la Fuente, G. Landskron, M.J. González, R. Quera, G. Dijkstra, H.J.M. Harmsen, K.N. Faber, M.A. Hermoso, Short Chain Fatty Acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases, Front. Immunol. 11 (2019), https://doi.org/10.3389/FIMMU.2019.00277.
[38] J.R. Davie, Inhibition of histone deacetylase activity by butyrate, J. Nutr. 133 (2003) 2485S–2493S.
[39] F.M. Ruemmele, S. Dionne, I. Qureshi, D.S. Sarma, E. Levy, E.G. Seidman, Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP), Cell Death Differ. 6 (1999) 729–735.
[40] C. Botta, A. Acquadro, A. Greppi, L. Barchi, M. Bertolino, L. Cocolin, K. Rantsiou, Genomic assessment in *Lactobacillus plantarum* links the butyrogenic pathway with glutamine metabolism, Sci. Rep. 7 (2017), https://doi.org/10.1038/s41598-017-16186-8.
[41] V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening, Nat. Protoc. 1 (2006), https://doi.org/10.1038/nprot.2006.179.
[42] R. Zanabria, A.M. Tellez, M. Griffiths, M. Corredig, Milk fat globule membrane isolate induces apoptosis in HT-29 human colon cancer cells, Food Funct. 4 (2013) 222–230.
[43] C. Botta, T. Langerholc, A. Cencič, L. Cocolin, *In vitro* selection and characterization of new probiotic candidates from table olive microbiota, PLoS One 9 (2014), https://doi.org/10.1371/journal.pone.0094457.
[44] M. Mataragas, A. Greppi, K. Rantsiou, L. Cocolin, Gene transcription patterns of pH- and salt-stressed *Listeria monocytogenes* cells in simulated gastric and pancreatic conditions, J. Food Prot. 77 (2014) 254–261.
[45] Q. Zhao, Y. Yu, X. Zuo, Y. Dong, Y. Li, Milk fat globule-epidermal growth factor 8 is decreased in intestinal epithelium of ulcerative colitis patients and thereby causes increased apoptosis and impaired wound healing, Mol. Med. 18 (2012) 497–506.
[46] B. Lallemant, A. Evrard, C. Combescure, H. Chapuis, G. Chambon, C. Raynal, C. Reynaud, O. Sabra, D. Joubert, F. Hollande, J.-G. Lallemant, S. Lumbroso, J.-P. Brouillet, Reference gene selection for head and neck squamous cell carcinoma gene expression studies, BMC Mol. Biol. 10 (2009), https://doi.org/10.1186/1471-2199-10-78.
[47] V.C. Janbandhu, A.K. Singh, A. Mukherji, V. Kumar, p65 negatively regulates transcription of the cyclin E gene, J. Biol. Chem. 285 (2010) 17453–17464, https://doi.org/10.1074/jbc.M109.058974.
[48] T. Remans, E. Keunen, G.J. Bex, K. Smeets, J. Vangronsveld, A. Cuypers, Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy, Plant Cell. 26 (2014) 3829–3837.
[49] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[50] M. Kubista, R. Sindelka, The prime technique, G. I. T. Lab. J. 9 (10) (2007) 33–35.
[51] W. Grajek, A. Olejnik, Epithelial cell cultures in vitro as a model to study functional properties of food, Pol. J. Food Nutr. Sci. 13 (2004) 5–24.
[52] V. Lievin-Le Moal, A.L. Servin, Pathogenesis of human enterovirulent bacteria: lessons from cultured, fully differentiated human colon cancer cell lines, Microbiol. Mol. Biol. Rev. 77 (2013) 380–439.
[53] S.S. Choi, Y. Kim, K.S. Han, S. You, S. Oh, S.H. Kim, Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress *in vitro*, Lett. Appl. Microbiol. 42 (2006) 452–458.
[54] A. Pessione, G. Lo Bianco, E. Mangiapane, S. Cirrincione, E. Pessione, Characterization of potentially probiotic lactic acid bacteria isolated from olives: evaluation of short chain fatty acids production and analysis of the extracellular proteome, Food Res. Int. 67 (2015) 247–254, https://doi.org/10.1016/j.foodres.2014.11.029.
[55] C. Botta, M. Bertolino, G. Zeppa, L. Cocolin, Evaluation of Toma Piemontese PDO cheese as a carrier of putative probiotics from table olive fermentations, J. Funct. Foods 18 (2015) 106–116.
[56] F. Nazzaro, F. Fratianni, P. Orlando, R. Coppola, Biochemical traits, survival and biological properties of the probiotic *Lactobacillus plantarum* grown in the presence of prebiotic inulin and pectin as energy source, Pharmaceuticals 5 (2012) 481–492, https://doi.org/10.3390/ph5050481.
[57] M. Bosch, M.C. Fuentes, S. Audivert, M. a Bonachera, S. Peiró, J. Cuñé, *Lactobacillus plantarum* CECT 7527, 7528 and 7529: probiotic candidates to reduce cholesterol levels, J. Sci. Food Agric. 94 (2014) 803–809.
[58] W. Widodo, A.L. Ariani, D. Widianto, D. Haltrich, Genomic comparison of *Lactobacillus casei* AP and *Lactobacillus plantarum* DR131 with emphasis on the butyric acid biosynthetic pathways, Microorganisms 9 (2021), https://doi.org/10.3390/microorganisms9020425.
[59] J. Hofmanová, A. Vaculová, Z. Koubková, M. Hýzd'alová, A. Kozubík, Human fetal colon cells and colon cancer cells respond differently to butyrate and PUFAs, Mol.

Nutr. Food Res. 53 (Suppl 1) (2009) 102–113, https://doi.org/10.1002/mnfr.200800175.
[60] A. Rada-Iglesias, S. Enroth, A. Ameur, C.M. Koch, G.K. Clelland, P. Respuela-Alonso, S. Wilcox, O.M. Dovey, P.D. Ellis, C.F. Langford, I. Dunham, J. Komorowski, C. Wadelius, Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes, Genome Res. 17 (2007) 708–719.
[61] G. Jan, A.-S. Belzacq, D. Haouzi, A. Rouault, D. Métivier, G. Kroemer, C. Brenner, Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria, Cell Death Differ. 9 (2002) 179–188.
[62] F.J. Cousin, S. Jouan-Lanhouet, M.-T. Dimanche-Boitrel, L. Corcos, G. Jan, Milk fermented by *Propionibacterium freudenreichii* induces apoptosis of HGT-1 human gastric cancer cells, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0031892.
[63] A. Lan, D. Lagadic-Gossmann, C. Lemaire, C. Brenner, G. Jan, Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria, Apoptosis 12 (2007) 573–591.
[64] K. Śliżewska, P. Markowiak-Kopeć, W. Śliżewska, The role of probiotics in cancer prevention, Cancers 13 (2021) 1–22, https://doi.org/10.3390/CANCERS13010020.
[65] F. Maghsood, B. Johari, M. Rohani, H. Madanchi, Z. Saltanatpour, M. Kadivar, Anti-proliferative and anti-metastatic potential of high molecular weight secretory molecules from probiotic *Lactobacillus reuteri* cell-free supernatant against human colon cancer stem-like cells (HT29-ShE), Int. J. Pept. Res. Ther. 118 (2020) 2619–2631, https://doi.org/10.1007/S10989-020-10049-Z.
[66] Y. Guo, T. Zhang, J. Gao, X. Jiang, M. Tao, X. Zeng, Z. Wu, D. Pan, *Lactobacillus acidophilus* CICC 6074 inhibits growth and induces apoptosis in colorectal cancer cells in vitro and in HT-29 cells induced-mouse model, J. Funct. Foods 75 (2020), https://doi.org/10.1016/J.JFF.2020.104290.
[67] R. Ghanavati, F. Mohammadi, A. Javadi, Z. Shahosseini, M. Talebi, M. Rohani, The effects of a mixture of *Lactobacillus species* on colorectal tumor cells activity through modulation of Hes1 pathway, PharmaNutrition 13 (2020), https://doi.org/10.1016/J.PHANU.2020.100207.
[68] G. Dewson, R.M. Kluck, Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis, J. Cell Sci. 122 (2009) 2801–2808.
[69] I. Khan, S.C. Kang, Apoptotic activity of *Lactobacillus plantarum* DGK-17-fermented soybean seed extract in human colon cancer cells via ROS-JNK signaling pathway, J. Food Sci. 82 (2017) 1475–1483.
[70] Z.-Y. Chen, Y.-M. Hsieh, C.-C. Huang, C.-C. Tsai, Inhibitory effects of probiotic *Lactobacillus* on the growth of human colonic carcinoma cell line HT-29, Molecules 22 (2017), https://doi.org/10.3390/molecules22010107.
[71] S. Maier, M.C. Daroqui, S. Scherer, S. Roepcke, A. Velcich, S.M. Shenoy, R. H. Singer, L.H. Augenlicht, Butyrate and vitamin D3 induce transcriptional attenuation at the cyclin D1 locus in colonic carcinoma cells, J. Cell. Physiol. 218 (2009) 638–642.
[72] M.C. Daroqui, L.H. Augenlicht, Transcriptional attenuation in colon carcinoma cells in response to butyrate, Cancer Prev. Res. 3 (2010) 1292–1302.
[73] F. Liu, F. Ji, Y. Ji, Y. Jiang, X. Sun, Y. Lu, L. Zhang, Y. Han, X. Liu, In-depth analysis of the critical genes and pathways in colorectal cancer, Int. J. Mol. Med. 36 (2015) 923–930.
[74] J.K. Fauser, L.D. Prisciandaro, A.G. Cummins, G.S. Howarth, Fatty acids as potential adjunctive colorectal chemotherapeutic agents, Cancer Biol. Ther. 11 (2011) 724–731.