RESEARCH ARTICLE

Investigating the Proteomic Profile of HT-29 Colon Cancer Cells After *Lactobacillus kefiri* SGL 13 Exposure Using the SWATH Method

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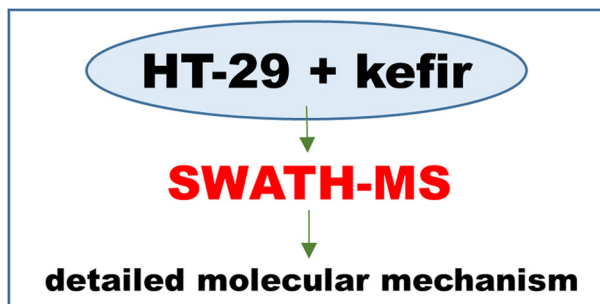
Abstract. Despite some studies revealed that kefir acts on different cancers, such as colorectal cancer, the proteomic changes that occur in the colon cancer cells remain to be explored. In this study, the proteomic analysis was combined with determination of kefir characteristics (e.g., adhesion capacity, gastrointestinal and antibiotic resistances), in order to confirm its use as a probiotic. Therefore, a label-free strategy based on SWATH-MS was applied to investigate the proteomic profile of HT-29 cells after exposure for 24 h to a specific strain of *Lactobacillus kefiri* named SGL 13. We identified a total of 60 differentially expressed proteins in HT-29 cells, among which most are located into the extracellular exosome, playing important/crucial roles in translation and cell adhesion, as indicated by the enrichment analysis. The eIF2 and retinoid X receptor activation pathways appeared to be correlated with the anti-tumoral effect of SGL 13. Immunoblot analysis showed an increase in Bax and a decrease in caspase 3 and mutant p53, and ELISA assay revealed inhibition of IL-8 secretion from HT-29 cells stimulated with LPS upon SGL 13 treatment, suggesting pro-apoptotic and anti-inflammatory properties of kefir. In conclusion, the results of this study, the first of its kind using co-culture of kefir and colon cancer cells, demonstrate that *L. kefiri* SGL 13 possesses probiotic potency and contribute to elucidate the molecular mechanisms involved in the *L. kefiri*–colon cancer cell interactions.

Keywords: SWATH-MS, Label-free proteomics, Colon cancer, *Lactobacillus kefiri*

Introduction

Colorectal cancer (CRC) is one of the commonest malignancies of the gastrointestinal tract, which represents the

third most common and the fourth most lethal cancer worldwide [1]. Human CRC is suggested to be characterized by multiple genetic alterations, of which the developing risk could be regulated by probiotics and probiotic foods [2].

Kefir is a fermented probiotic milk drink of Caucasian and Tibet origin, with a complex microbiota confined to "kefir grains," in which several lactic acid bacteria and yeasts coexist in a symbiotic association. Kefir consumption has been associated with several health-promoting properties [3], and different kefir-isolated microbial strains have also shown potentiality as probiotics. Probiotics are viable microorganisms that can confer beneficial effect on health when administrated in adequate amounts [4], and they have been extensively studied for their intestinal benefits and successfully used to improve gastrointestinal health. The interactions between commensal bacteria, intestinal epithelial cells, and immune cells play a crucial role in the maintenance of intestinal homeostasis. The microbial recognition through specific receptors induces the expression and release of various immune mediators (chemokines and pro-inflammatory cytokines), which contribute to orchestrating both the innate and adaptive immune responses [5]. The use of probiotics to modulate immune responses at the mucosal and systemic levels is today a very interesting alternative for the prevention and treatment of infectious diseases, of various immunopathies, such as inflammatory bowel diseases (IBD), allergies, and metabolic disorders.

Some studies report the immunomodulatory and anti-inflammatory properties of *Lactobacillus kefiri*. For example, the *L. kefiri* IM002 strain attenuates the pro-inflammatory response in the HT-29 intestinal epithelial cells induced by *Salmonella Typhimurium*, by reducing the secretion of IL-8 [6]. In addition, some specific strains of *L. kefiri* are able to prevent invasion by enteropathogens in the intestine, both exploiting the adhesive properties of the epithelium (thus reducing the binding sites for pathogens) and aggregating with the pathogens themselves [7]. Many studies also revealed that kefiri is a probiotic with anti-cancer properties [8], and a few of them demonstrated its anti-tumoral activity on colon cancer [9].

Although the immunomodulatory and antitumor effects of *Lactobacillus kefiri* have already been showed by few studies, to date an in-depth molecular characterization of the induced effects on colon cancer cells is still missing. In this study, we employed a sequential window acquisition of all theoretical fragmentation spectra-mass spectrometry (SWATH-MS) workflow for proteomic profiles of the HT-29 colon cancer cells exposed to a new isolated *L. kefiri* SGL 13 strain [10]. Statistics and bioinformatics analyses were undertaken to identify candidate proteins that appeared responsible for the anti-inflammatory and anti-cancer properties. The proteomic analysis was combined with the study of characteristics of *L. kefiri* SGL 13 itself (e.g., adhesion capacity, gastrointestinal and antibiotic resistances), in order to verify its use as a probiotic. The results obtained by this proteomic approach provide for the first time an insight into the molecular regulation induced by a *L. kefiri* strain in colon cancer cells and further shed light on the mechanism of counteracting the growth of colon cancer cells.

Materials and Methods

Bacteria and Colon Cancer Cell Growth Conditions

The *L. kefiri* SGL 13 strain was isolated from kefir grains and cultured in de Man–Rogosa–Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 30 °C for 24 h in aerobic conditions. Lyophilized stock cultures (10^9 CFU/ml) of live and heat-inactivated SGL 13 (i.e., treated for 30 min at 80 °C) were stored at −80 °C until use. Human colorectal adenocarcinoma cell line (HT-29) obtained from ATCC (American Type Culture Collection) was cultured in McCoy's medium (GibCo, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C and 5% CO_2 in a humidified chamber. The HT-29 cells were grown in 24-well tissue culture plates (Sigma-Aldrich).

Strain Identification by MALDI-TOF MS Fingerprinting

For identification of lactobacilli isolated from kefir grains, MALDI-TOF MS analysis was performed. Briefly, *L. kefiri* SGL 13 was grown overnight and then collected and centrifuged in order to have a sufficient number of cells (5×10^9). The pellet was washed with physiological solution to remove fermentation medium residues and then re-suspended with 300 µl of distilled water with the addition of 900 µl of pure ethanol. The obtained cell suspension was homogenized and centrifuged at 16,000 ×g for 2 min. Subsequently, after removal of supernatant, the sample was air-dried and stored at − 20 °C before analysis. For MALDI-TOF analysis, the sample was re-suspended in 50 µl of 70% formic acid. After resuspension, 50 µl of acetonitrile was added to the sample and mixed vigorously. The sample was centrifuged for 2 min at 16,000 ×g, and 1 µl of supernatant was deposited onto a spot of a steel target plate and air-dried. After the sample had dried, 1 µl of a matrix solution of α-cyano-4-hydroxycinnamic acid was added to the spot. MALDI-TOF MS analysis was performed with a MicroFlex LT mass spectrometer, following the user's manual. The instrument was connected to Biotyper 2.0 software (Bruker Daltonics), able to acquire the incoming data, process mass spectra, and compare to reference spectra in a database (3.740 spectra of 319 genera and 1.946 different species). For each plate containing 24 samples, a standard test was included to calibrate and validate the analysis. The degree of similarity to a given microorganism present in the database is represented by a "score value" that indicates the reliability of the identification at species level. A score value higher than 2.00 is considered a reliable species identification. Score values between 1.70 and 1.90 indicate identification to genus level. The analysis was repeated two times using four replicate cultures of *L. kefiri* SGL 13.

Characterization of L. kefiri SGL 13

For phenotypic characterization, SGL 13 was tested for Gram color stain (Liofilchem, Teramo, Italy) and catalase and

oxidase test (Liofilchem, Teramo, Italy). All tests were performed according to the manufacturer's instructions. Biochemical characterization of SGL 13 was obtained using the API 50 CH system (BioMérieux, Lyon, France), following the manufacturer's guidelines. The API 50 CH strips were read and identified, after 48 h of incubation at 37 °C, using the identification software APIWEB™.

Gastrointestinal and Antibiotic Resistance of *L. kefiri* SGL 13

The evaluation of *L. kefiri* SGL 13 survival to simulated gastric and enteric conditions was performed following the procedure described elsewhere [11] with some modifications. Briefly, simulated gastric juice, which was prepared by suspending 3 g l^{-1} of pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO), in sterile saline solution (0.5% NaCl, w/v) adjusted to pH 3, was inoculated with active overnight cultures of *L. kefiri* SGL 13 (incubated at 37 °C for 18 h in anaerobiosis). The viable cell population was determined at 0, 90, and 120 min of incubation at 37 °C in the presence of pepsin on MRS agar plates by the spread plate method. For pancreatin resistance test, simulated intestinal fluid, prepared by suspending 1 g l^{-1} of pancreatin from porcine pancreas (Sigma-Aldrich, St. Louis, MO) in sterile saline solution (0. 5% NaCl, w/v) adjusted to pH 8, was inoculated with active overnight cultures of *L. kefiri* SGL 13 (incubated at 37 °C for 18 h in anaerobiosis). The viable cell population was determined at 0, 60, and 240 min of incubation at 37 °C in the presence of pancreatin on MRS agar plates by the spread plate method. Tolerance to pancreatin and pepsin enzymes was estimated by comparing viable cell counts in test solution and control solution without enzymes at the same pH of the test solution.

For bile tolerance test, the cells were suspended in MRS broth (Oxoid, Basingstoke, UK) containing 0, 0.5, 1, 2, and 3% (w/v) bile salts (Sigma-Aldrich, St. Louis, MO). For the determination of salt (NaCl) tolerance, cells were grown in MRS broth containing 0, 2, 6, and 10% NaCl (w/v) (Sigma-Aldrich, St. Louis, MO). To determine the acid tolerance, cells were incubated in MRS broth at different pH values (6.3, 3, 2, 1.5). Hydrochloric acid 4 M was used to adjust pH values. For all tests, the viable cell population was determined immediately and after 24 h of incubation at 37 °C, by serial dilutions in maximum recovery diluent (MRD) (Liofilchem, Teramo, Italy). The selected dilutions were spread on MRS agar plates and incubated at 37 °C for 24 h.

Antibiogram test was performed to determine the sensitivity or resistance of SGL 13 towards several antibiotics. The MIC test strips (Liofilchem, Teramo, Italy) were used for 19 different antibiotics.

Adhesion Ability of SGL 13 to HT-29 Cells

To determine the adhesion capacity of *L. kefiri* SGL 13 to human HT-29 cells, 1×10^8 bacterial cells were added to an HT-29 monolayer (1.23×10^6 cells) and incubated for 90 min. After incubation, HT-29 cells in monolayer were washed four times with sterile PBS, and the adhered bacterial cells were quantified by real-time PCR, using species-specific primers [12]. Adherent

enteropathogenic *Salmonella Typhimurium* and *Escherichia coli* ETEC H10407 were used as positive control, while non-adherent *E. coli* B44 was used as negative control.

Co-Culture and Analysis of Cell Viability

All experiments were carried out using co-culture of HT-29 cells and *L. kefiri* SGL 13. For MS-based proteomic experiments, HT-29 cells were seeded in a 24-well plate at 2.5×10^5 cells/well. After 24 h, the medium was replaced with fresh McCoy's medium without penicillin/streptomycin, and the cells were treated with SGL 13 strain (5×10^8 CFU/ml) and incubated for 24 h. Untreated HT-29 cells were used as negative control. For quantification of IL-8 cytokine, HT-29 cells (2.5×10^5 cells/well) were pre-incubated for 2 h with live and heat-inactivated SGL 13 (5×10^8 CFU/ml), and then they were challenged with LPS (10 ng/ml) followed by incubation for 22 h. Cell viability of HT-29 was determined using Trypan Blue exclusion assay (Sigma-Aldrich, St. Louis, MO).

Protein Extraction and Trypsin Digestion

Control and treated HT-29 cells were collected, washed, and lysed in 1× PBS added with protease inhibitor cocktail 1× (Roche) and 1% SDS. Protein extraction was performed by 5–6 cycles of sonication; then, a four-fold volume of ice-cold acetone was added to the samples, and protein precipitation was conducted overnight at − 20 °C. The samples were then centrifuged at 14,000 ×g for 10 min at 4 °C, and the pellet was resuspended in 100 mM NH_4HCO_3. Protein concentration was measured with BCA Protein Assay (Sigma-Aldrich, St. Louis, MO) using bovine serum albumin as a standard. Before shotgun proteomic analysis, proteins were subjected to in-solution digestion as already described [13].

Generation of the Reference Spectral Library

The SWATH-MS-based proteomics analysis was performed as previously indicated [13] on a total number of two biological replicates (obtained by seeding and growing the cells in separate times) for both HT-29 cells treated with SGL 13 and untreated control. The biological replicates were pooled for the proteomics analysis. The digested samples were analyzed on a micro-LC (Eksigent Technologies, Dublin, CA, USA) interfaced to a 5600+ TripleTOF mass spectrometer system (AB Sciex, Concord, Canada) equipped with a DuoSpray Ion Source and a CDS (Calibrant Delivery System). The LC column was a Halo Fused C18, and the mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow rate of 15 µl min^{-1} at an increasing concentration of solvent B from 2 to 40% in 30 min. The injection volume was 4 µl at a peptide concentration of 1 µg/µl, and the oven temperature was set at 40 °C. Two technical replicates for each sample were subjected to the data-dependent acquisition (DDA) analysis to generate the SWATH-MS spectral library. Survey MS scans were acquired in the mass range of 100–1500 Da (TOF scan with an

accumulation time of 0.25 s). Thirty-five most intensive ions were chosen as precursor ions for subsequent MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps. Both the untreated and treated samples were subjected to DDA search. The resulting MS/MS search list for all identified peptides was subjected to construction of a reference spectral library for subsequent SWATH analysis.

SWATH Analysis and Targeted Data Extraction

The samples were then subjected to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s. The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. Three instrumental replicates for each sample were subjected to the DIA analysis. Thus, 6 SWATH files were acquired for subsequent data analysis. The ion source parameters in the electrospray positive mode were set as follows: curtain gas (N_2) at 25 psig, nebulizer gas GAS1 at 25 psig, and GAS2 at 20 psig, ion spray floating voltage (ISFV) at 5000 V, source temperature at 450 °C, and declustering potential at 25 V. The PeakView v.2.1 and MarkerView 1.2. (AB SCIEX; Framingham, USA) were employed for the spectral alignment and targeted data extraction.

Data Base Search and Data Statistical

Raw data acquired in DDA mode were searched using Protein Pilot software v. 4.2 (AB SCIEX, Concord, Canada) and Mascot v. 2.4 (Matrix Science Inc., Boston, MA, USA). Trypsin as digestion enzyme was specified for both software. For Mascot, we used two missed cleavages, the instrument was set to ESI-QUAD-TOF, and the following modifications were specified for the search: carbamidomethyl cysteine as a fixed modification and oxidized methionine as variable modification. A search tolerance of 0.08 Da was specified for the peptide mass tolerance, and 10 ppm for the MS/MS tolerance. The charges of the peptides to search for were set to $2+$, $3+$, and $4+$, and the search was set on monoisotopic mass. The UniProt Swiss-Prot-reviewed database containing human proteins (version 2015.20.07, containing 23,304 sequence entries) and *Lactobacillus kefiri* (NCBI *Lactobacillus kefiri* version 2018.10.07, containing 12,430 sequence entries) was used, and a target-decoy database search was performed. The result files from the DDA acquisitions were used for library generation using a protein FDR threshold of 1%.

For the extraction of data acquired in SWATH mode, the reference spectral library was directly loaded into the SWATH 2.0 software. The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide. The quantification was carried out with PeakView 2.0 and MarkerView 1.2. (AB SCIEX, Concord, Canada). Using the extracted fragment intensity data, the data analysis began with the normalization using total intensity sum (TIS) method to eliminate the systematic variations in chromatography across various samples. Analysis of the relative quantification was conducted to determine whether the mean was significantly different using FDR. Six peptides per protein and six transitions per peptide were extracted from the SWATH files. Shared peptides were excluded as well as peptides with modifications. Peptides with FDR lower than 1.0% were exported in MarkerView for the *t* test. Benjamini and Hochberg FDR was also calculated.

Bioinformatics Analyses

Identified proteins were annotated according to the information associated to their main functions available under gene ontology (GO) categories using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (v6.8) (http://david.abcc.ncifcrf.gov/). This analysis detected the significant ($p < 0.01$) over-representation of GO terms in the submitted dataset [14].

Moreover, in order to identify the perturbed canonical pathways associated with deregulated proteins of HT-29 cells, an Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was performed, as previously described [15]. The IPA analysis settings were as follows: (a) reference set: ingenuity knowledge base; (b) relationship to include: direct and indirect; (c) filter summary: consider only molecules and/or relationships where (species = human) AND (confidence = experimentally observed). Proteins associated with canonical pathways were estimated as significant using Fisher's exact test (*p* value at < 0.01) to determine the probability that the association between identified proteins and a canonical pathway could be explained by chance alone.

Western Blotting

Western Blot analysis was performed as previously described [16]. Briefly, after separation on 4–20% SDS-PAGE, proteins were electroblotted on PVDF membrane and subjected to immunorevelation. Incubation with specific primary antibodies anti-p53 (1:100, sc-126), anti-Bax (1:100, sc-7480), and anti-caspase 3 (1:100, sc-7272) from Santa Cruz Biotechnology Technologies was performed in blocking buffer for 3 h. Horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, sc-516102) was used as secondary antibody. The chemiluminescent signal acquisitions were done with the ChemiDoc MP Imager, a CCD imager, using the Image Lab 5.2.1 software (Bio-Rad).

Quantification of IL-8 in Cell Culture Supernatants

HT-29 cell supernatants obtained from co-culture were analyzed for expression of IL-8 using human IL-8/CXCL8 ELISA kit (RAB0319, Sigma-Aldrich) according to the manufacturer's instructions. The absorbance value of the samples was measured at 450 nm on a microplate reader. The detection limit of the assay was 1 pg/ml. All the analyses were done in duplicate.

Statistical Analysis

Each experimental condition was investigated by three independent trials. ELISA analysis was conducted in duplicate for each trial. Statistical significance between treatment and control conditions was assessed by the Student's t test. A significant difference was defined as a p value of < 0.05.

Results and Discussion

MALDI-TOF Profiling and Biochemical Identification of L. kefiri SGL 13

The potential probiotic isolated from kefir grain has been identified by a combined approach, involving MALDI-TOF MS and biochemical analyses, and concordant results have been obtained. Indeed, MALDI-TOF profiling (Table 1), as well as API gallery, oxidase–catalase negativity, and Gram + color results confirmed that SGL 13 belongs to *Lactobacillus kefiri* species.

Analysis of Probiotic Characteristics of L. kefiri SGL 13 and Its Effects on HT-29 Cell Viability

The simulation experiment of human gastrointestinal tract tolerance of SGL 13 was used to assess the tolerance of *Lactobacillus* to gastrointestinal tract conditions. We found that the number of SGL 13 was maintained above 10^6 CFU/ml, at pH 3 for 24 h (Table 2), indicating fairly good acid survival. Moreover, SGL 13 was totally resistant for 2 h after pepsin treatment. Furthermore, SGL 13 was able to multiply in the presence of 3% bile salts and it showed growth after 24 h of exposure. Sensitivity of SGL 13 to different antibiotics was determined in terms of minimum inhibitory concentration (MIC). The results are shown in Table 3.

The adhesion capacity of *L. kefiri* SGL 13 to HT-29 cells was evaluated, since the adhesion ability of probiotic lactobacilli is significant in the colonization of bacteria within the gastrointestinal tract and in the beneficial benefits of bacteria on the hosts. We found 9.32×10^3 adherent bacteria to 100 HT-29 cells, indicating that SGL 13 is able to adhere efficiently to HT-29 (Figure 1a). To assess kefiri's cytotoxicity on HT-29, we determined the percentage viability after treating the cells with live and inactivated kefiri, challenged with LPS or not. Results showed a trend of reduction in the viability of HT-29 cells after treatment with both active and inactivated kefiri (with or without LPS). However, this trend did not reach statistical significance (Figure 1b).

Table 2. Assessment of Viability of the Isolated SGL 13 After Exposure to Low pH, NaCl, Pepsin, Pancreatin and Bile salts Simulated Juices

Resistance to	Time (h)	SGL 13 (log cfu/ml)
pH 1.5	24	< 2
pH 2.0	24	< 2
pH 3.0	24	6.12 ± 0.25
pH 6.0	24	7.99 ± 0.03
NaCl 0%	24	8.18 ± 0.15
NaCl 2%	24	7.95 ± 0.1
NaCl 6%	24	6.26 ± 0.18
NaCl 10%	24	5.37 ± 0.2
Pepsin 0	0	8.13 ± 0.01
Pepsin 90′	1.5	8.09 ± 0.04
Pepsin 120′	2	8.14 ± 0.05
Pancreatin 0	0	8.48 ± 0.25
Pancreatin 60′	1	8.08 ± 0.27
Pancreatin 240′	4	7.77 ± 0.27
Bile salts 0%	24	7.90 ± 0.27
Bile salts 0.5%	24	7.25 ± 0.25
Bile salts 1%	24	7.23 ± 0.28
Bile salts 2%	24	6.89 ± 0.28
Bile salts 3%	24	6.85 ± 0.25

SWATH-MS Proteomics Analysis of HT-29 Cells Exposed to L. kefiri SGL 13

Here, with the aim of deepening the understanding of SGL 13 effects on colon cancer cell biology, we carried out a SWATH-MS proteomic analysis of HT-29 cells whole-cell extracts after SGL 13 exposure. We identified a total of 573 proteins and 2437 peptides with a peptide confidence cut-off of 99% (FDR < 1%); among these, a total of 355 proteins were quantified. The identities of these proteins are presented in Supplemental Table S1. Fifteen *L. kefiri* proteins were also identified in the treated sample. Sixty proteins were differentially expressed in *L. kefir*-treated HT-29 cells as compared to the negative control (Figure 2 and Supplemental Table S2). Forty three proteins were higher accumulated in HT-29 cells treated with active *L. kefiri*, while seventeen were found down-regulated.

By GO enrichment analysis, we found that the identified proteins deregulated in HT-29 cells are mainly present at the level of extracellular exosome (24%), have predominant roles in translation (12%) and cell adhesion (12%), and exhibited "binding" activity, in particular protein (45%) and poly(A) RNA (27%) binding activity (Figure 3). Furthermore, an IPA analysis was performed to find which cellular pathways are affected by SGL 13 exposure in HT-29 cells. We found 36 different enriched pathways (p value at ≤ 0.05) (Supplemental Table S3), the top 15 of which are reported in Figure 4. The results obtained revealed

Table 1. MALDI-TOF MS Identification of *L. kefiri*-Type Strains. Genus Level (Cut-off 1.7); Species Level (Cut-off 2.0)

Analyte name	Analyte ID	Organism (best match)	Score value	Organism (best match)	Score value
D2 (+++) A	7	*Lactobacillus kefiri*	2.399	*Lactobacillus kefiri*	2.395
D3 (++) A	22	*Lactobacillus kefiri*	2.140	*Lactobacillus kefiri*	2.075
E3 (+++) A	24	*Lactobacillus kefiri*	2.369	*Lactobacillus kefiri*	2.288
E4 (+++) A	25	*Lactobacillus kefiri*	2.423	*Lactobacillus kefiri*	2.278

Table 3. MIC (μg/ml) of Antibiotics for SGL 13 as Determined by Gradient Diffusion Using M.I.C. Evaluator Strips. Values are Expressed as Mean ± Standard Deviation

Agent	SGL 13 (MIC μg/ml)
Amoxicillin	0.30 ± 0.12
Ampicillin	0.21 ± 0.03
Chloramphenicol	2.67 ± 0.47
Ciprofloxacin	> 32
Erythromycin	0.17 ± 0.03
Gentamycin	1.00 ± 0.41
Rifampin	0.21 ± 0.03
Sulphamethoxazole	12.00 ± 0.00
Tetracycline	85.33 ± 15.08
Vancomycin	> 256
Kanamycin	32.00 ± 0.00
Streptomycin	16.00 ± 0.00
Clindamycin	0.03 ± 0.01
Clarithromycin	0.06 ± 0.01
Minocycline	1.75 ± 0.25
Doxycycline	14.00 ± 2.00
Ceftriaxone	0.38 ± 0.13
Cefuroxime	0.31 ± 0.19
Cefixime	1.13 ± 0.38

the regulation of some key pathways in HT-29 cells, such as "eIF2 signaling" which is involved in tumor progression and resistance to therapy [17]. One of the interesting observations was that among the top 15 significant deregulated pathways, there are three related to the retinoid X receptor (RXR), i.e., LXR/RXR, FXR/RXR, and TR/RXR activation. RXR is a nuclear transcription factor, which represents an important drug target for the treatment of cancer, in particular, of CRC [18–20]. It has been known for decades that ligand activation of RXR is essential for the induction of apoptosis in cancer cells [21]. In colon cancer, RXRα is silenced through promotor methylation, and the impairment of its functionality plays a key role for CRC progression [20]. Recently, the down-regulation of proteins involved in "LXR/RXR activation" in colon adenocarcinoma was revealed by proteomics analysis [22].

Our analysis indicated that SGL 13 induces the deregulation of some proteins of HT-29 cells involved in RXR activation. In particular, four among five of these proteins are up-regulated (i.e., VTN, APOA1, FASN, SLC2A1), suggesting a possible trigger in this pathway as a consequence of SGL 13 exposure. Indeed, we found upregulation of vitronectin (VTNC, fold change at + 6.73), a cell adhesion protein involved in phagocyte recognition of apoptotic cells [23], tissue repair [24], bacterial adhesion
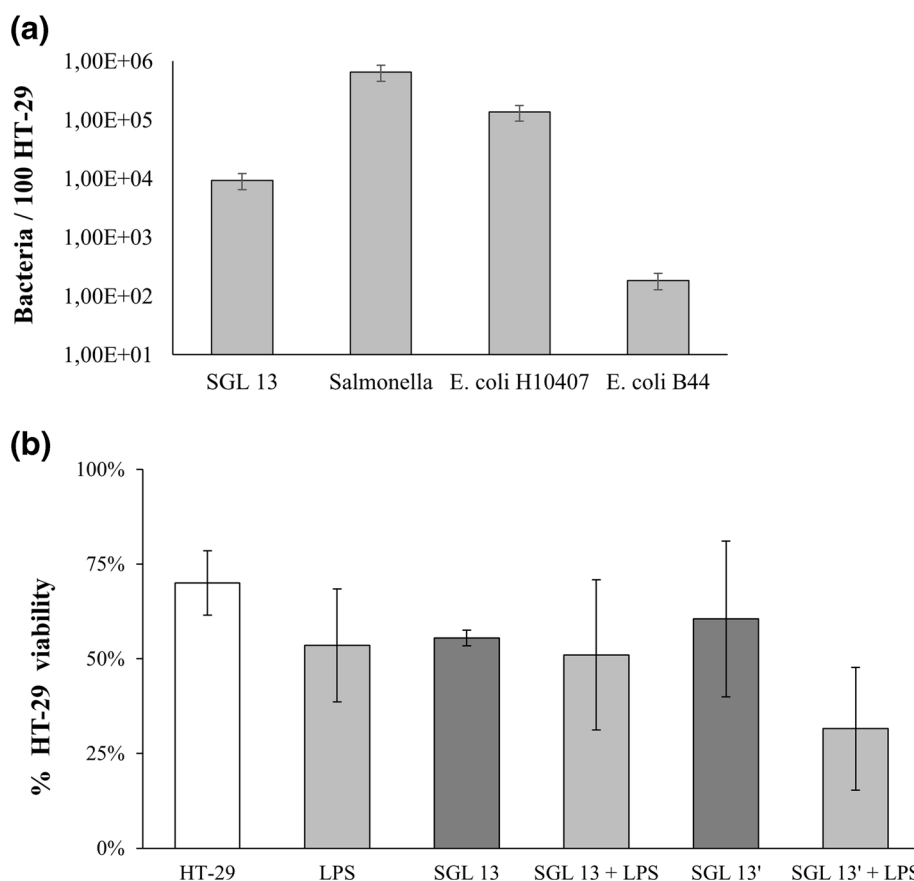
Figure 1. HT-29 cells and SGL 13: adhesion and viability. (**a**) Adhesion of *L. kefiri* SGL 13 strain and controls (i.e., the adhesive *E. coli* H10407 and the non-adhesive *E. coli* B44) to HT-29 cell monolayer, as evaluated by real-time PCR. (**b**) HT-29 cell viability after treatment with SGL 13. Trypan Blue exclusion assay for cell viability of HT-29 cancer cells after a total of 24 h of treatment with live (SGL 13) and inactivated (SGL 13') *L. kefiri* (5×10^8 CFU/ml). Results are reported as percent of viable cells out of the total number of cells (dead and alive). Mean ± standard deviation (n = 3 wells/treatment)
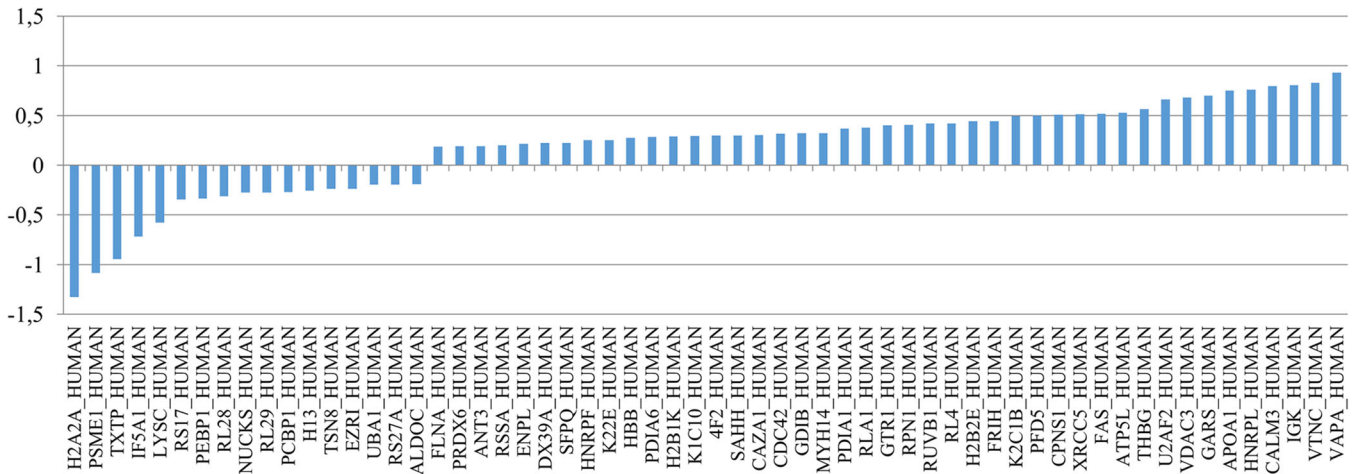
Figure 2. Differentially expressed proteins of HT-29 cells after SGL 13 treatment. Graphical representation of fold change of modulated proteins in label-free SWATH-MS comparative analysis. Bars indicate the values of fold change expressed in logarithmic scale
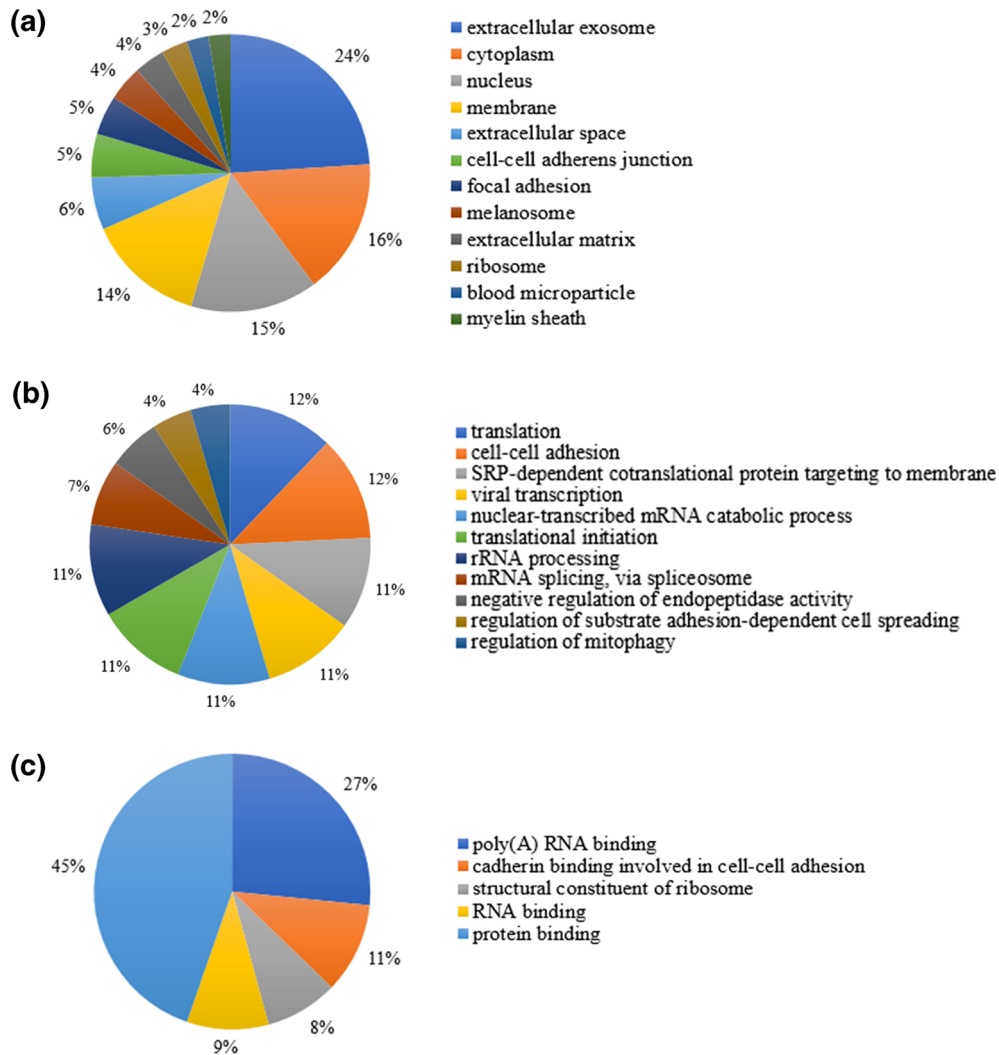
Figure 3. Functional GO annotation of HT-29 deregulated proteins. Pie chart of the enriched cellular component (**a**), biological process (**b**), and molecular function (**c**) terms generated from the differentially expressed proteins of HT-29 cells are reported. The proportion of each specific subcategory is also provided
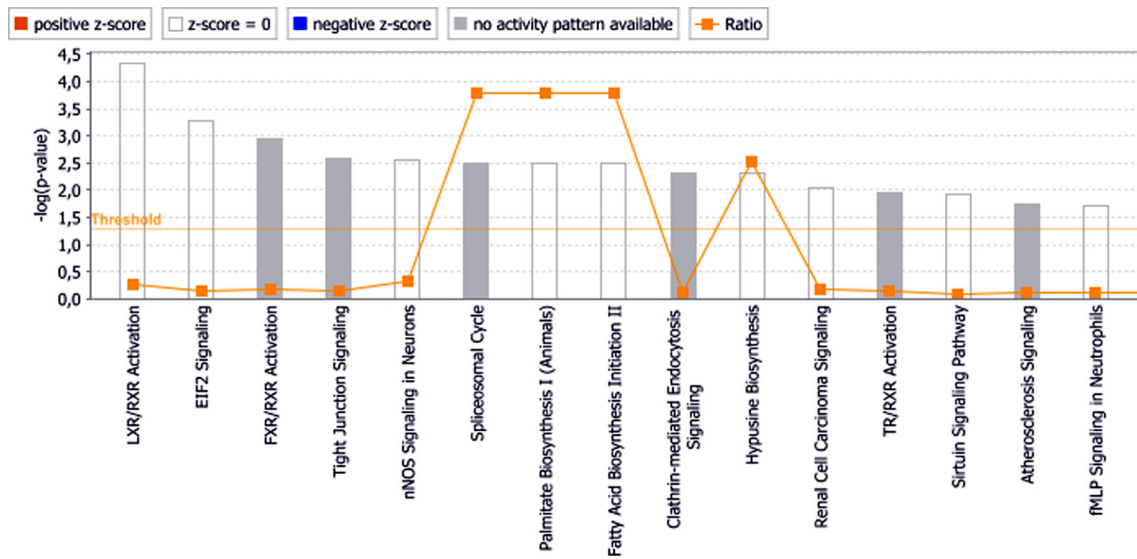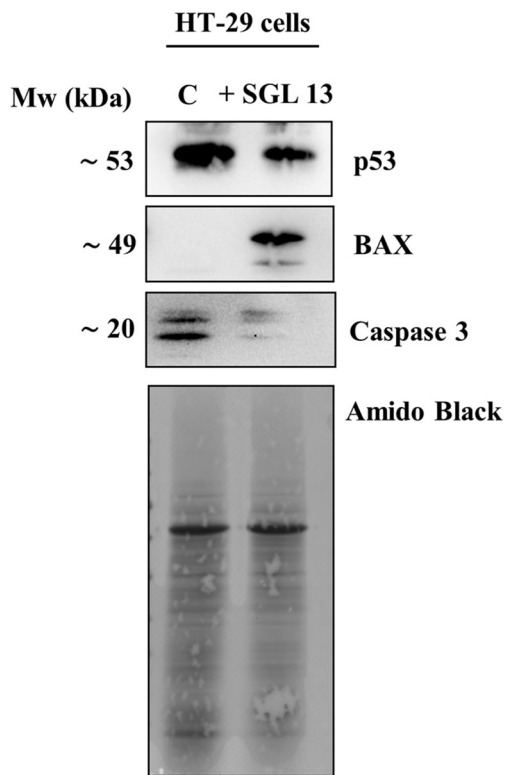
Figure 4. Identification of deregulated canonical pathways in HT-29 cells. The top 15 significantly altered canonical pathways associated with deregulated proteins of HT-29 cells. The y-axis corresponds to the –log of the *p* value (Fisher's exact test) and the ratio (orange points) the number of genes in a given pathway that meet the cut-off criteria, divided by the total number of genes that map to that pathway

[25], as well as bacteria internalization into host cell [26]. The proteomic data also showed the upregulation in HT-29 cells of apolipoprotein AI (APOA1, + 5.67 fold), a protein involved in


Figure 5. Western blotting analysis. Expression profile of p53, Bax, and caspase-3 proteins in HT-29 cells. Proteins were resolved in 4–20% SDS-PAGE gels, transferred onto PVDF membranes, and probed with specific antibodies against the indicated targets. Amido black staining was used as total loading control

cholesterol homeostasis whose gene has a retinoic acid-response element that responds to the RXR [27]. Interestingly, increased levels of APOA1 mRNA have been detected in colon cancer cells cultured with LXR agonist [28], and ApoA-1 protein has a protective role in colitis and inhibits colitis-associated colorectal carcinogenesis [29]. Also, fatty acid synthase was upregulated (FASN, + 3.33 fold) in HT-29 after SGL 13 co-culture. FASN is a LXR target gene [30] whose expression increased in all-trans retinoic acid-treated cell [31].

Analysis of Pro-Apoptotic and Immunomodulatory Effects of SGL 13 on HT-29 Cells

Importantly, SWATH-MS analysis revealed deregulated proteins of HT-29 involved in cell death and inflammatory processes, suggesting a pro-apoptotic and anti-inflammatory effect of SGL 13. For example, of particular interest are some proteins strongly repressed after kefiri exposure, such as histone H2A type 2-A (HIST2H2AA3, − 21.11 fold), a replication-dependent histone which is down-regulated in colon cancer cells after DNA damage [32]; proteasome activator complex subunit 1 (PSME1, − 12.06 fold), a proteasome immunosubunit [33] induced during the inflammatory process of the colonic mucosa [34]; mitochondrial tricarboxylate transport protein (SLC25A1, − 8.75 fold), a transcriptional target of mutant p53 which promotes tumorigenesis and cancer cell proliferation [35, 36] and plays a key role in cytokine-induced inflammation [37]; and eukaryotic initiation factor 5A (EIF5A, − 5.23 fold), which is important for cell survival and proliferation of colon cancer cells [38] and has pro-inflammatory role in the release of cytokines [39].

To verify if SGL 13 reduces the viability of HT-29 cells through an induction of apoptosis, the expression of Bax, caspase 3, and p53 was assessed at the protein level using
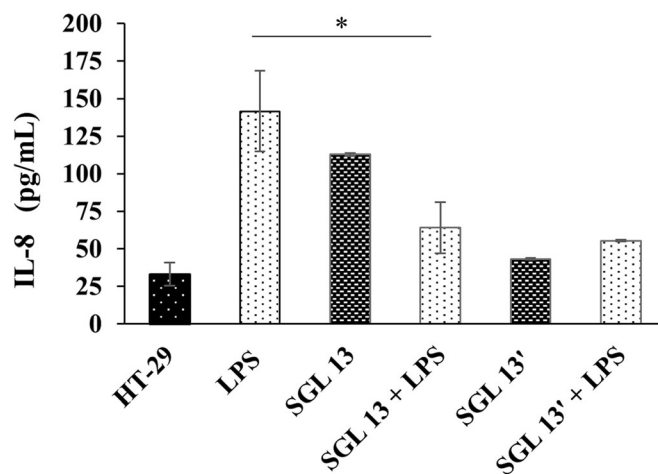
Figure 6. IL-8 ELISA assay of HT-29 supernatant. ELISA assay on IL-8 production after pre-incubation of HT-29 cells with live (SGL 13) and inactivated (SGL 13') *L. kefiri* (5×10^8 CFU/ml) for 2 h and subsequent treatment with LPS (10 ng/ml) for 22 h. Data are represented as mean ± standard deviation of two independent experiments

Western blotting in HT-29 cells (Figure 5). Consistent with the induction of apoptosis, we observed an increase in Bax and a decrease in caspase 3 upon SGL 13 treatment. Furthermore, results showed a decrease in the expression of p53 in kefiri-treated cells. HT-29 cells are characterized by mutant p53-R273H, an oncoprotein which mediates cancer cell survival and anoikis resistance [40]. Therefore, a reduced expression of this oncoprotein following treatment with SGL 13 further confirms the anti-tumor effect of kefiri.

Moreover, to assess the anti-inflammatory properties of kefiri, IL-8 production was evaluated by incubating HT-29 cells with LPS in the presence of SGL 13. The data obtained by ELISA assay demonstrated that live and inactivated SGL 13 inhibited LPS induced up-regulation of IL-8 (Figure 6). The ability of kefiri to inhibit IL-8 secretion from HT-29 cells stimulated with LPS was statistically significant for live SGL 13.

Conclusions

In this work, SWATH-MS quantitative method was applied to investigate the changes in the proteome of colon cancer cells exposed to *L. kefiri* SGL 13. Through different experiments, we have first demonstrated the probiotic characteristics of SGL 13 and then established that this specific strain of kefiri exhibits pro-apoptotic and anti-proliferative properties on colorectal adenocarcinoma cells, HT-29, *in vitro*.

This study is the first to elucidate a molecular mechanism for kefiri's effect on colon cancer cells from a proteomic point of view. The data obtained in the present work and, in particular, the identified deregulated proteins of HT-29 cells, are helpful for further understanding the anti-tumoral and immunomodulatory potential of kefiri. Further experiments on different human colon cancer lines and on *in vivo* models would further validate our results and improve our understanding of the mechanisms involved in the *L. kefiri*–intestinal cell interactions.

Compliance with Ethical Standards

Conflict of Interest   The authors declared the following potential conflicts of interest with respect of the research, authorship, and/or publication of this article: FF, ER, and LM are employees of Sintal Dietetics s.r.l., Italy, performing all research and development activities for Sintal Dietetics. Sintal Dietetics s.r.l. deposited *Lactobacillus kefiri* SGL 13 for purposes of European patent.

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