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Published in:
Molecular Nutrition & Food Research

Link to article, DOI:
10.1002/mnfr.201700638

Publication date:
2018

Document Version
Peer reviewed version

Journal: Molecular Nutrition and Food Research

Plant polyphenols stimulate adhesion to intestinal mucosa and induce proteome changes in the probiotic *Lactobacillus acidophilus* NCFM

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Abbreviations: 5(6)-CFDA, 5(6)-carboxyfluorescein diacetate; GAPDH, glyceraldehyde-3-p dehydrogenase; GIT, gastrointestinal tract; LABSEM, semisynthetic lactic acid bacteria medium; NCFM, *Lactobacillus acidophilus* NCFM

Received: 24-Jul-2017; Revised: 20-Nov-2017; Accepted: 23-Nov-2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201700638.

Key words: ferulic acid, probiotics, resveratrol, surface proteome, whole-cell proteome

Abstract

Scope: Plant phenolics, known to exert beneficial effects on human health, were supplemented to cultures of the probiotic bacterium *Lactobacillus acidophilus* NCFM (NCFM) to assess their effect on its adhesive capacity and the abundancy of individual proteins.

Methods and Results: The presence of resveratrol and ferulic acid during bacterial growth stimulated adhesion of NCFM to mucin and human intestinal HT-29 cells, while tannic acid improved adhesion only to HT-29 cells and caffeic acid had very modest effect overall. Some dosage dependence was found for the four phenolics supplemented at 100, 250 or 500 μg/mL to the cultures. Notably, 500 μg/mL ferulic acid only stimulated adhesion to mucin. Analyses of differential whole-cell as well as surface proteomes revealed relative abundancy changes for a total of 27 and 22 NCFM proteins, respectively. These changes include enzymes acting in metabolic pathways, such as glycolysis, nucleotide metabolism and stress response as well as being known moonlighting or surface-associated proteins.

Conclusion: The five plant phenolics found in various foods stimulate the adhesive capacity of NCFM in diverse ways and elicited relative abundancy changes of specific proteins providing molecular level insight into the mechanism of the putative beneficial effects of the polyphenols.

1. Introduction

Plant phenolics are secondary metabolites abundant in foods and beverages where they confer bitterness, astringency, color, flavor, odor and oxidative stability [1]. Polyphenols can interact with molecular targets in various organisms and compete with pathogenic microorganisms

and insects in plant defense [2]. We selected five common plant phenolics to explore their impact on the widely used probiotic bacterium *Lactobacillus acidophilus* NCFM (NCFM). Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is found in grapes, wine, peanuts, pistachios, and berries and has been reported to have a large number of health-promoting effects as an antioxidant, anti-inflammatory, antitumor, anti-platelet aggregation, cardioprotective, and longevity agent [3]. Tannic acid belongs to tannins, widely distributed in plants of the human diet, and possesses radical scavenging, antimicrobial and anti-carcinogenic properties [4]. Caffeic acid (3,4-dihydroxycinnamic) found in cereals, berries, herbs and spices is produced in the shikimate pathway [5] and can be esterified by quinic acid to chlorogenic acid in fruits, vegetables, coffee and tobacco [6]. The closely related ferulic acid (4-hydroxy-3-methoxycinnamic acid) together with dihydroferulic acid is abundant in plant cell-walls as a constituent of lignocellulose [7]. Ferulic acid is identified as monomer, dimer, free oligomer or in polymers esterifying polysaccharides, polyamines and glycoproteins [8] and high amounts are found in common foods such as bran, vegetables, fruits and herbs [9]. Finally salicin, a phenolic glycoside from willow bark that possesses analgesic and anti-rheumatic activities [10] can be carbon source for NCFM.

NCFM is a Gram-positive, homofermentative, rod shaped lactic acid bacterium (LAB) residing in the gastrointestinal tract [11] and used in dairy products and dietary supplements [12]. NCFM has excellent ability to adapt its metabolism in response to gut nutrients by negative transcriptional regulation enabling survival in nutrient scarce competitive environments [13]. Its adaptation to the gastrointestinal tract (GIT) involves mucus-binding proteins belonging to the surface layer, as previously identified [14,15]. NCFM shows excellent stability in dairy and fermented products [16].

Phenolics and probiotics are very important food components residing in the same environment (the GIT) and providing health benefits to humans. The present study describes

effects of typical food phenolics on the adhesive capacity of NCFM towards mucin and intestinal HT-29 cells and gains insights into the molecular mechanisms involved in adhesion by analysis of whole cell and surface-associated proteome changes.

2. Materials and methods

2.1 Growth conditions

L. acidophilus NCFM (1.50×10^{10} CFU/g, DuPont) was grown aerobically without shaking at 37 °C in 50 mL batch culture on semisynthetic lactic acid bacteria medium (LABSEM) [17] containing 1% glucose as carbon source. Stock solutions of phenolics were prepared in ethanol (50 mg/mL), except tannic acid (prepared in distilled water). Different cultures from the same bacterial stock were supplemented with final concentrations of 100 or 250 µg/mL resveratrol (Veri-te™ resveratrol; kind gift of Evolva, Denmark) or tannic acid (Sigma); 100, 250, or 500 µg/mL caffeic or ferulic acids (both Sigma) and were sub-cultured for three cycles. NCFM release glucose from salicin (1%) that was used as sole carbon source and compared to glucose-grown bacteria. Cells for proteome analyses were grown in the presence of 100 µg/mL resveratrol or tannic acid and 500 µg/mL caffeic or ferulic acids and were sub-cultured for three cycles. Cultures (glucose-grown) without phenolics served as control.

2.2 *In vitro* adhesion to mucin and HT-29 cells

Adhesion was measured as previously [18] with some modification [19,20]. Briefly, freshly late-log phase grown NCFM (20 h, OD 0.5 for tannic acid; 24 h, OD 1.0 – 1.1 for control and resveratrol; 24 h, OD 1.4 – 1.5 for caffeic and ferulic acids) was labeled with 100 µM 5(6)-carboxyfluorescein diacetate (Sigma-Aldrich) in PBS (37°C, 30 min), washed twice and re-suspended in PBS to OD_{600} 0.5±0.05. A 96-well microtiter plate (Greiner Bioone) was coated with porcine mucin (1 mg/mL, Sigma-Aldrich) in PBS (200 µL/well; 4°C, overnight). After decanting the mucin solution, wells were washed with PBS, added labeled NCFM (200 µL, OD_{600} 0.5), incubated (2 h, 37°C), followed by decanting the bacterial suspension and

washing of wells thrice with PBS. Adhered bacteria were lysed by 1% (w/v) SDS in 0.1 M NaOH (200 µL; 1 h, 37°C) and quantified by fluorescence measurements (Cytation5 Cell Imaging Multi-Mode Reader, BioTek) using 485 nm and 538 nm as excitation and emission wavelength, respectively. Adhesion was expressed as percentage of fluorescence recovered from the lysed bacteria that were bound versus the fluorescence of the total bacterial suspension added to the wells. Three independent experiments were conducted, each in quadruplicate, and data were subjected to one-way ANOVA using OriginPro ver. 9. Human colonic HT-29 cell line (American Type Culture Collection, ATCC® HTB38™) was cultured and maintained according to the supplier's instructions. HT-29 cells in complete growth medium containing McCoy's 5a medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Lonza) were seeded in 24-well plates and cultivated until a confluent state, followed by removing the medium and washing with PBS to remove remaining antibiotics. Labeled NCFM (500 µL, OD_{600} 0.5) was added to the wells (2 h) and adhesion analyzed as above.

2.3 Sample preparation for proteome analyses

NCFM in late-log phase (OD_{600} 0.5-1.6, Supporting Figure S1) was harvested by centrifugation (3200 x *g*, 10 min) and washed with 0.9% NaCl. Extracts were prepared by mechanical grinding (5 x 1-min vortex at maximum speed) with a small amount of acid washed glass beads (<100 µm diameter; Sigma) in sample buffer (28 mM Tris-HCl, 22 mM Tris-base pH 8.5, 100 mM DTT) containing protease inhibitors (complete, MiniProtease Inhibitor Tablets, Roche). Following heating (100°C, 2 min) and addition of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT), mixtures were vortexed, centrifuged (10000 x *g*, 10 min), and supernatants were collected. Surface proteomes were prepared by incubating cell pellets with 5 M lithium chloride (Sigma-Aldrich) (30 min, R.T.) and collecting supernatants after centrifugation (15000 x *g*, 15 min) [21]. Proteins were

precipitated by TCA/acetone [22], washed with acetone, dissolved in rehydration buffer and concentrations were determined by using 2-D Quant Kit (GE Life Sciences).

2.4 CyDye labeling and differential gel electrophoresis (DIGE)

Proteins in whole cell extracts were CyDye minimal-labeled for DIGE analysis using a dye-swapping approach [17,23]. Briefly, protein aliquots (50 µg) from four biological replicates were labeled interchangeably with 250 pmol of either Cy5 or Cy3, vortexed, and left in the dark (30 min, 4 ºC). For internal standard aliquots from both samples (25 µg protein of each) were combined and labeled with 250 pmol Cy2. Labeling was quenched by 1 µL 10 mM lysine in the dark (10 min). Labeled internal standard and samples were mixed and adjusted to 450 µL with rehydration buffer (8 M urea, 2 M thiourea, 33 mM CHAPS, 195 mM DTT, 1% pharmalyte pH 4−7; GE Life Sciences). Separation in the first dimension (IEF) using IPG strips (pH 3−10; 18 cm Ettan™ IPGphor; GE Lifesciences) was performed after rehydration (20 ºC, 12 h, 30 V) at a total of 65 kVh. Subsequently, the strips were equilibrated 2 x 15 min in 5 mL equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 0.01% bromophenol blue) supplemented with 65 mM DTT and 135 mM iodoacetamide in first and second step, respectively. The second dimension (12.5% SDS-PAGE) was run overnight (Ettan™ DALT*six* Electrophoresis unit; GE Lifesciences) at 1 W/gel until the dye front reached the gel bottom. The gels were image analyzed immediately after the second dimension, using excitation/emission wavelengths of Cy2 (488/520 nm), Cy3 (532/580 nm) and Cy5 (633/670 nm), respectively (100 µm resolution; Typhoon 9410 Variable Mode Imager; GE Lifesciences).

2.5 2-DE PAGE

First dimension separation was performed using IPG strips (pH 3–10, 11 or 18 cm; GE Healthcare) on Ettan™ IPGphor (GE Healthcare) to a total of 65 kVh and subsequently the strips were treated as above first with DTT and then with iodoacetamide. Second dimension

(SDS-PAGE) was run with 12.5% Tris-HCl gels for 45 min at 2 W/gel and 4 h at 12 W/gel. The gels were stained by colloidal CBB [24] and scanned (Microtek Scan maker 9800 XL; Microtek).

2.6 Image analysis

Gel images were aligned by automated calculation of manually assigned landmark vectors (Progenesis SameSpots version 3.3). Scanned gels were analyzed by intra-gel (difference in-gel) and inter-gel (biological variance) analysis. Spot volume ratio change of $\geq$ 1.3 fold and ANOVA $p \leq 0.05$ were chosen as criteria for identification of proteins showing abundancy differences.

2.7 In-gel digestion and protein identification by MS

Differentially abundant spots were excised manually from gels, subjected to in-gel digestion by trypsin and MS identification [17]. Briefly, gel pieces were washed in 40% ethanol until colorless, followed by 100% ACN, incubated with 5 µL 12.5 ng/mL trypsin (Promega) in 10 mM ammonium bicarbonate (45 min, on ice), added 10 mM ammonium bicarbonate for rehydration and kept at 37°C overnight. Supernatant (1 µL) was applied onto an Anchor Chip target (Bruker-Daltonics), added 1 µL matrix (0.5 mg/mL α-cyano-4-hydroxycinnamic acid in 90% ACN, 0.1% TFA) and washed with 2 µL 0.02% TFA. MALDI-TOF MS spectra were obtained (Ultraflex II; Bruker-Daltonics) in auto-mode using Flex Control v3.0 and processed by Flex Analysis v3.0 (both Bruker-Daltonics). Spectra were externally calibrated using a trypsin digest of β-lactoglobulin (5 nM). MS spectra were searched against the NCBI database for bacteria using the MASCOT 2.0 software (http://www.matrixscience.com) integrated with BioTools v3.1 (Bruker-Daltonics). Protein identifications by PMF were confirmed with a MASCOT score of 80, $p \leq 0.05$ and a minimum of six matched peptides.

3. Results

The effects of five plant phenolics on the probiotic *Lactobacillus acidophilus* NCFM were monitored by determining adhesive capacity onto a mucin coating and an HT-29 cell layer as well as by differential whole cell and surface proteome analyses.

3.1 Effects of phenolics on adhesion

The adhesive ability of NCFM varied with the different phenolics and their concentrations supplemented during growth. Resveratrol (100 μg/mL) significantly increased ($p<0.05$) adhesion to mucin and HT-29 cells by +2.4 and +1.4 fold, respectively, and tannic acid (100 μg/mL) increased the adhesion to HT-29 cells by +5.1 fold, compared to a control grown on glucose alone (Fig. 1). By contrast, ferulic acid (100 μg/mL) and tannic acid (250 μg/mL) reduced ($p<0.05$) adhesion to mucin by –1.4 and –3.2 fold, respectively, whereas caffeic and ferulic acids (250 μg/mL) increased ($p<0.05$) adhesion to mucin by +1.3 and +2.0 fold, respectively. Notably, adhesion to HT-29 cells increased significantly after growth in the presence of 250 μg/mL resveratrol (+2.3 fold) and ferulic acid (+1.6 fold). At the highest phenolic concentration (500 μg/mL) only ferulic acid stimulated adhesion and only to mucin (+1.3 fold). Although salicin was used previously as carbon source for selective enumeration of NCFM [25], growth in 1% salicin did not modulate adhesion (Fig. 2).

3.2 Effects of phenolics on the whole-cell proteome

Phenolic-treated bacteria and controls were analyzed by comparative proteomics. Growth at the presence of 100 μg/mL resveratrol (Supporting Figure S1) altered relative abundancies of 12 protein spots in the whole-cell proteome (Fig. 2, Table 1, Supporting Table S1, Supporting File S1a), 11 showing +1.3 – +2.0 fold increase (preprotein translocase subunit SecA, multiple sugar-binding ABC-transporter ATPase, tRNA N6-adenosine(37)-threonylcarbamoyl transferase complex transferase subunit TsaD (two spots), oxalyl-CoA decarboxylase, ribokinase, molecular chaperone Hsp33, and alanyl-tRNA synthetase),

whereas a spot containing one of several glyceraldehyde-3-p dehydrogenase (GADPH) forms present in the proteome showed –1.5 fold lower relative abundance compared to the control. Tannic acid (100 μg/mL, Supporting Figure S1) similarly altered abundancy for 11 protein spots (Fig. 2, Table 1, Supporting Table S2, Supporting File S1b), ten increased +1.4 – +2.1 fold (DNA-binding response regulator, prolyl-tRNA synthetase, pyruvate kinase, GADPH (two spots), glutamyl-tRNA synthetase, oligoendopeptidase F, and adenylosuccinate lyase), whereas tRNA (guanine-N(7)-)-methyltransferase was –3.0 fold less abundant.

Growth in the presence of caffeic acid (500 μg/mL, Supporting Figure S1) slightly increased abundancy for D-lactate dehydrogenase (+1.4 fold) and elongation factor Ts (+1.3 fold), while abundancy decreased of GAPDH (–2.3 fold), 50S ribosomal protein L1 (–1.5 fold), and heat shock protein Hsp33 (–1.3 fold) (Fig. 3, Table 1, Supporting Table S3, Supporting File S1c). Ferulic acid (500 μg/mL, Supporting Figure S1) increased transcriptional regulator LBA0733 (+1.9 fold), while purine trans deoxyribosylase, 50S ribosomal protein L1, fructokinase, ribose-p-pyrophosphokinase, and phosphomethylpyrimidine kinase decreased in abundancy (–1.4 – –1.7 fold) (Fig. 3, Table 1, Supporting Table S4, Supporting File S1d).

3.3 Effects of phenolics on the surface proteome

Differential abundancies in the surface proteome were previously reported for NCFM to accompany increased adhesion when using plant-derived oligosaccharides as carbon sources [17, 23]. Addition of resveratrol (100 μg/mL) during growth altered nine spots in the surface proteome. Five increased in abundance by +1.4 – +2.1 fold (pyruvate kinase, 50S ribosomal protein L7/L12, elongation factor P, 50S ribosomal protein L22 and hypothetical protein LBA1769) and four decreased –2.0 ––1.4 fold (GAPDH (two spots), adenylosuccinate synthetase, and 6-phosphofructokinase) (Fig. 4A, Table 2, Supporting Table S5, Supporting File S2a). Tannic acid (100 μg/mL) slightly increased relative abundancy of aminopeptidase

and glycoprotein endopeptidase by +1.4 and +1.3 fold, while elongation factor G and manganese-dependent inorganic pyrophosphatase decreased by –2.0 and –1.5 fold, respectively (Fig. 4A, Table 2, Supporting Table S6, Supporting File S2b). Caffeic acid (500 µg/mL) affected nine surface protein spots, of which Glutamate tRNA ligase increased +1.5 fold, whereas eight decreased in abundancy from –1.5 to –2.2 fold (D-lactate dehydrogenase, elongation factor Tu, triosephosphate isomerase, 30S ribosomal protein S1, adenylosuccinate synthetase, lysine tRNA ligase, elongation factor P, aspartate tRNA ligase (Fig. 4B, Table 2, Supporting Table S7, Supporting File S2c). Ferulic acid (500 µg/mL) only caused seven protein spots to reduce in abundancy including L-lactate dehydrogenase (–1.6 fold), oligoribonuclease (–1.8 fold), and pyruvate kinase (–1.6 fold) (Fig. 4B, Table 2, Supporting Table S8, Supporting File S2d).

4. Discussion

The daily intake of polyphenols varies a lot, typically ranging from <100 mg to >2 g. Over 95% of the phenolics in the diet supposedly reach the colon and become metabolized by the gut microbiota [26]. Although phenolics are generally known to exert positive human health effects, their efficacy depends on the bioavailability of different forms - esters, glycosides, polymers - which must be hydrolyzed by intestinal enzymes or the microbiota prior to absorption [27]. Phenolics and probiotic bacteria have been shown to interact with each other when coexisting in food products, dietary supplements or in the gastrointestinal tract [28]. The presence of plant phenolics did not inhibit the growth of NCFM cells (Supporting Figure S1). However, tannic acid had some growth-reducing effects on NCFM. In general, to survive in tannic acid organisms express tannase but this is not the case of NCFM that did not display tannase activity. On the other hand, NCFM cells can protect themselves by producing exopolysaccharides, which can help the cells growing in the presence of tannic acid [29,30]. This is probably the reason why the growth is decreased but not fully inhibited.

Adhesion of microorganisms to the intestinal mucosa is important for GIT residence time and correlated to the ability of the strains to beneficially influence host health including immune modulation and competitive exclusion of pathogens [27,28,31,32]. Favourable effects of polyphenols were reported for the probiotic *Lactobacillus rhamnosus* 299 enhancing proliferation and adhesion simultaneously with inhibition of growth and adhesion of pathogens [3]. The impact largely depended on the phenolic structure and its dosage [34], as also found in our study to represent important factors in conferring beneficial effects.

To gain deeper insight into modulation of epithelial adhesion and changes in relative protein abundancies in the bacterial whole cell and surface-associated proteomes, four phenolics were individually added to NCFM growing with glucose as carbon source (Supporting Figure S1), while salicin was used as carbon source. The mechanism of bacterial adhesion to the gastrointestinal mucosa is complex and includes nonspecific electrostatic and hydrophobic interactions along with specific phenomena sustained at the molecular level by bacterial adhesins and mucosal receptors [35,36]. Indeed, the most important bacterial determinants for mucosa adherence are cell-wall components and adhesins. Both specific and nonspecific mechanisms apply to the interaction of NCFM with the intestinal mucosa involving molecules of different nature, including proteins, lipids and carbohydrates [15]. Whole-cell proteome comparison indicated that plant polyphenols can alter abundance of NCFM proteins involved in energy metabolism, general and oxidative stress responses, transcription and translation processes, as also observed in previous proteomics studies on phenolics and LAB [37–40].

Preprotein translocase subunit SecA is coupled with the SecYEG channel to transport polypeptides from inside to outside of the cell playing a central role in bacterial protein secretion [41]. The mechanism by which SecA cooperates in this function includes two phases: i) SecA (alone or together with chaperones) aids the targeting of extracellular-located

proteins from the ribosome to the membrane and then ii) uses ATP for translocation of the preproteins through the SecYEG channel [41]. Increased abundancy of SecA induced by resveratrol may be a first step in adjustment of NCFM to environmental changes as extracellular proteins are crucial for this adaptation [42].

tRNA N6-adenosine(37)-threonylcarbamoyltransferase complex transferase subunit TsaD (TsaD), also annotated as endopeptidase [43] and DNA-binding/iron metalloprotein/AP endonuclease (NCBI Reference Sequence: YP_193312.1) is universally occurring. Its exact function is unknown, but it is thought to participate in modification of adenosine in tRNAs reading codons beginning with adenine [44], as well as in modification of cell wall peptidoglycan connected with cell division [45]. Resveratrol is known to reduce negative effects of oxidative stress on DNA and RNA and also interacts with tRNA synthetase [46] and tRNAs [47] and the increased abundancy of TsaD may protect RNA against oxidative stress. Secondly, cell wall stability may be maintained during environmental changes through increased abundancy of TsaD.

Oxalyl-CoA decarboxylase together with formyl-CoA transferase is responsible for catabolism of oxalate, a toxic compound in normal human diet [48]. Oxalate is a strong chelator of cations, especially Ca^{2+}, and can lead to severe pathologies such as hyperoxaluria, urolithiasis, and renal failure. Oxalate is primarily absorbed in the colon and the gut microbiota has evolved to degrade it [48]. Even though the main organism for this degradation is *Oxalobacter formigenes*, also probiotic bacteria belonging to *Bifidobacteria* and *Lactobacilli* genera encode genes responsible for removal of oxalate [49,50]. NCFM is known for this activity [48] and the increase in oxalyl-CoA decarboxylase concurs with the health promoting potential of resveratrol.

DNA-binding response regulator is a component of bacterial two-component signal transduction pathway involved in sensing and responding to environmental changes [51]. It is

coupled with histidine kinase, a transmembrane protein responsible for recognition of signals from the extracellular environment by autophosphorylation. This phosphoryl group is transferred to the response regulator for activation, which once phosphorylated initiates differential gene transcription to trigger metabolic reactions [51]. NCFM responds to tannic acid by increasing abundance of this protein.

The glycolytic enzymes pyruvate kinase (PK) and GAPDH both increased in tannic acid-treated NCFM, indicating elevated ATP production necessary for dealing with environmental stress, as observed in other phenolic-LAB interactions [38,40,52]. By contrast, abundancy of GAPDH was lower when resveratrol or caffeic acid were present during the NCFM culture. As it has been hypothesized that multiple GAPDH forms have different functions [53] these results suggest that the individual plant phenolics influence occurrence of distinct GADPH forms and hence different cellular functions. Different GAPDH forms of altered abundancy were derived from the same gene and appeared in several spots probably reflecting various posttranslational modifications.

Elongation factor Ts during translation escorts aminoacyl tRNAs to the ribosome as it proceeds along the mRNA. Studies on LAB showed that acid stress and also tannic acid induce elongation factor proteins [37,38,54] and the increased abundancy of elongation factor Ts by caffeic acid may be a defensive response. Furthermore, 50S ribosomal protein L1 plays a role in structure and activity of the ribosome and can participate in the mechanism of stress adaptation as shown in *L. plantarum* [55].

The chaperone Hsp33, which was +1.4 fold increased in resveratrol- and −1.3 fold decreased in caffeic acid-treated bacteria, deals with misfolded proteins and provides an immediate response to oxidative stress. Under oxidative conditions, Hsp33 is activated by disulfide bond formation, while under non-stressed conditions, it is deactivated by elimination of disulfide bonds with reversal of conformational changes [56].

Remarkably, proteins changing in abundancy differ for resveratrol and tannic acid, suggesting that these polyphenols have different roles in molecular reactions eliciting beneficial effects and thus act in a cooperative manner.

Regarding the surface protein profiles, some interesting observations emerge. Actually, several moonlighting or putative moonlighting proteins known to play a role in adhesion [57] undergo abundancy changes in phenolics-stimulated NCFM. Surface proteomes after resveratrol treatment revealed increase in PK that may be responsible for adhesion, being identified as a moonlighting protein in adhesion to mucin, HT-29 cells, and yeast mannan [58,59]. Other higher-abundant proteins, such as elongation factor P (EF-P) and ribosomal proteins are putative moonlighting proteins previously found on bacterial surfaces [60]. Elongation factors besides participating in protein synthesis are often described as moonlighting, especially elongation factor Tu (EF-Tu) that promoted adhesion of *L. johnsonii* to human tissues [61], thus contributing to its health-promoting effect. Similarly, EF-Ts and EF-G, seen in the exoproteome of *Bacillus anthracis* [62] were referred to as signal peptide-lacking exoproteins in *Staphylococcus aureus* [63]. The very interesting protein trigger factor behaves in *Lactobacillus reuteri* NCIB11951 as a collagen I binding protein [64] and cooperates in *Streptococcus mutans* with surface adhesin P1 [65]. Notably NCFM EF-P is more abundant after resveratrol treatment, stimulating adhesion to both mucin and HT-29 cells, and less abundant in caffeic acid-treated NCFM not showing improved adhesion. It is tempting to hypothesize that different plant phenolics influence synthesis of EF-P in NCFM in different ways or regulate secretion of EF-P to the outside of the cell, thus controlling adhesion to host and tissue components such as mucin and collagen. The lack of effect of caffeic acid on adhesion at 500 μg/mL agrees with low abundancy of EF-Tu [61].

Remarkably, improved adhesion by resveratrol-treatment does not fit with the well-known

moonlighting adhesive protein GAPDH being less abundant [60]. Probably several proteins including EF-P and PK support the adhesive effect.

Apart from the EF-P and EF-Tu discussed aboveconnected with the good (resveratrol) or poor (caffeic acid) adhesive capabilities of stimulated NCFM, it can be underlined that other elongation (EF-G) and trigger factors are of low abundancy in the surface proteome of tannic and ferulic acid-stimulated bacteria, respectively. In the case of ferulic acid (500 μg/mL) reduced abundancy of PK, is also consistent with poor adhesion to HT-29 at this concentration. By contrast, improved binding to HT-29 resulting for tannic acid, probably is due to other components, maybe exopolysaccharides often reported as involved in cell adhesion [66]. This hypothesis is supported by that cells stimulated with tannic acid did not adhere to mucin and possibly factors stimulating adhesion to HT-29 are unfavorable for binding to mucin.

Finally, it is worth considering that both ferulic acid and resveratrol seem to preferentially influence L-lactate dehydrogenase (L-LDH) synthesis, whereas caffeic acid seems to specifically control D-lactate dehydrogenase (D-LDH) abundancy. Curiously, caffeic acid causes enhancement of intracellular D-LDH and decreases abundance of surface D-LDH, suggesting that it directs the cellular location of this enzyme rather than stimulating the synthesis.

In conclusion, the present study has brought new knowledge to elucidate complex interactions occurring in the human gut between health-promoting bacteria and diet components. It has been demonstrated that some plant phenolics (but not all) can improve the adhesive capabilities to mucin and HT-29 cells of NCFM probably by inducing biosynthesis or secretion of moonlighting proteins engaged in adhesion. Among these compounds, resveratrol proved most effective.

Author contributions

HUC, SB, EP, and BS conceived and designed the work. HUC and MD performed experimental work, data analysis and interpretation and drafted the paper. SB, EP, and BS helped writing and revising the paper. All authors read and approved the final version.

Acknowledgements

Karina Jensen, Anne Blicher, and Lisbeth Buus Rosholm are thanked for technical assistance. The authors are grateful to Prof. Ayse Mine Gencler-Ozkan, Ankara University, for very stimulating discussions about plants and plant phenolics. This work was supported by the Danish Strategic Research Council's Program Committee on Health, Food and Welfare (FøSu), the Danish Council for Independent Research | Natural Sciences and the Danish Center for Advanced Food Studies (LMC). HUC is grateful to the Republic of Turkey, Ministry of National Education for a PhD scholarship. MD benefitted from an Erasmus agreement to perform her master project at DTU.

Conflict of interest statement

The authors have no conflict of interest.

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Figure legends

Figure 1 *In vitro* adhesion of *Lactobacillus acidophilus* NCFM. **A.** Adhesion to mucin coating by bacteria grown with glucose as carbon source in the presence of resveratrol, tannic acid, caffeic acid, ferulic acid or with salicin as carbon source. **B.** Adhesion to HT-29 cells by bacteria exposed during growth to the phenolics as above. Asterisks indicate statistically significant differences (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) as compared to control.

Figure1

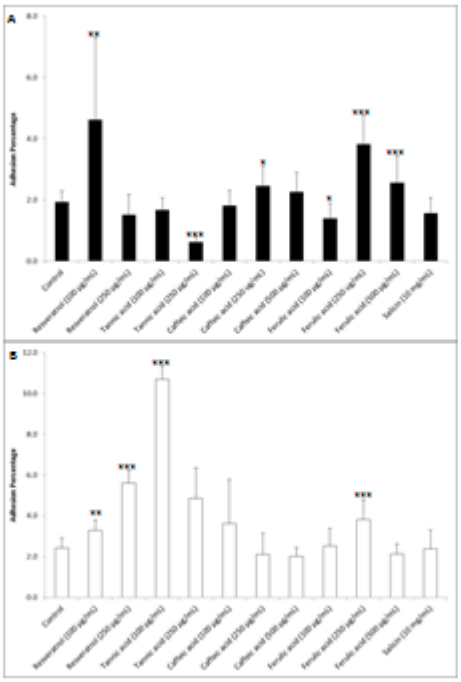


Figure 2 Representative whole cell 2D proteome map of *Lactobacillus acidophilus* NCFM grown with glucose as carbon source in the presence of resveratrol or tannic acid. Numbers indicate differentially abundant spots (ANOVA $p \leq 0.05$), compared to control (non-treated), identified by in-gel digestion and mass spectrometry. NCFM was grown in the presence of 100 µg/mL resveratrol (Res-treated) and 100 µg/mL tannic acid (TA-treated). Selected spots are shown for relative abundancy.

Figure 2

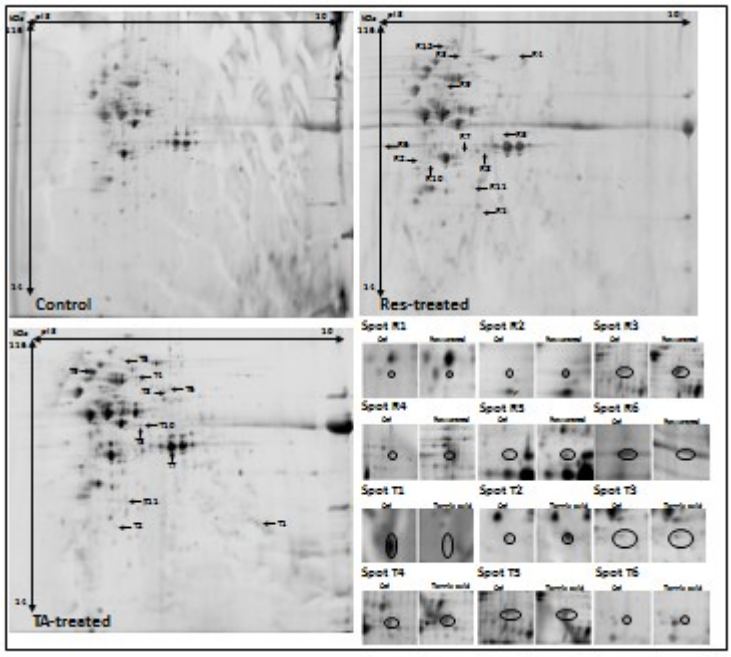


Figure 3 Representative whole cell 2D proteome map of *Lactobacillus acidophilus* NCFM grown with glucose as carbon source in the presence of caffeic acid or ferulic acid. Numbers indicate differentially abundant spots (ANOVA $p \leq 0.05$), compared to control (non-treated), identified by in-gel digestion and mass spectrometry. NCFM was grown in the presence of 500 µg/mL caffeic acid (CA-treated) and 500 µg/mL ferulic acid (FA-treated). Selected spots are shown for relative abundancy.

Figure 3

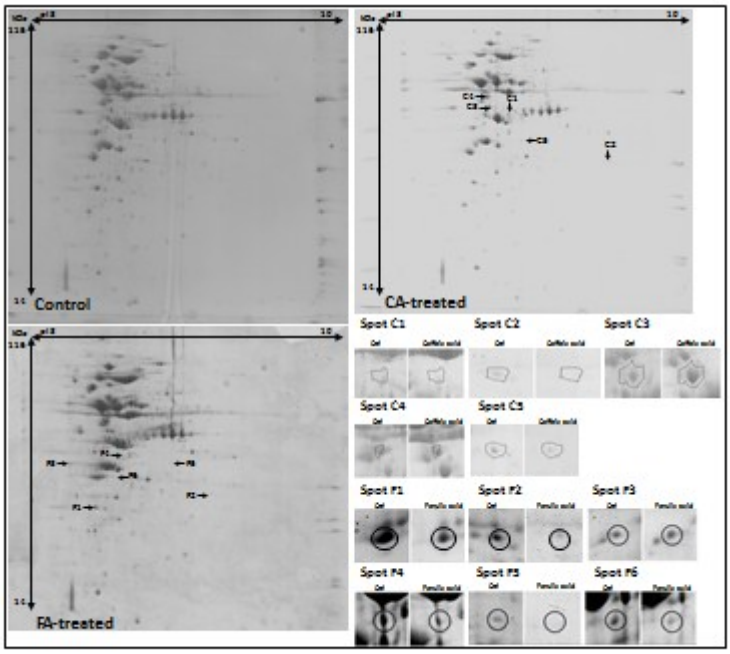
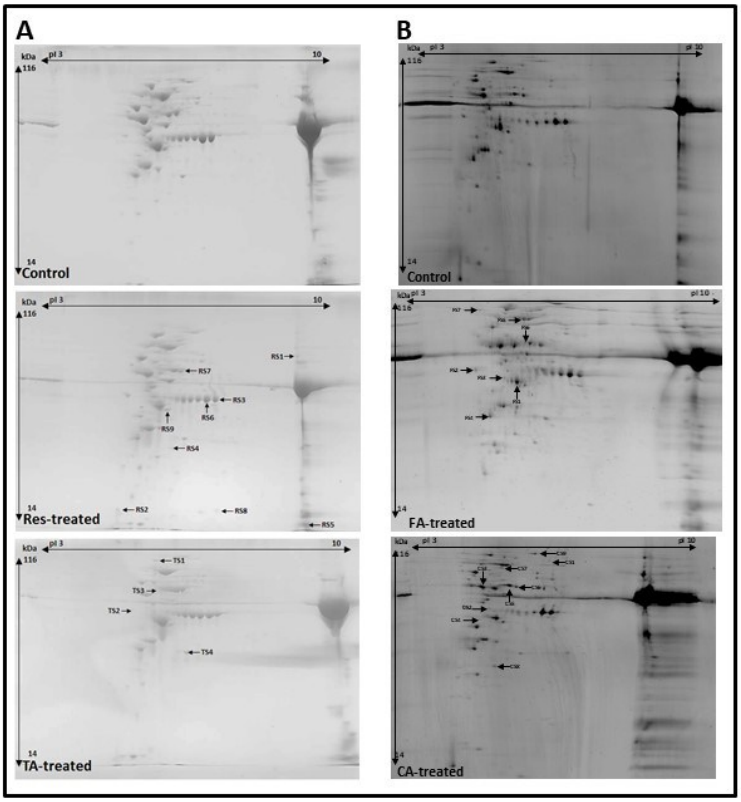


Figure 4 Surface proteomes of *Lactobacillus acidophilus* NCFM grown with glucose as carbon source in the presence of phenolics. Numbers indicate differentially abundant spots (ANOVA $p \leq 0.05$), compared to control (non-treated), which were identified by in-gel digestion and mass spectrometry. NCFM was grown in the presence of 100 µg/mL resveratrol (Res-treated) and tannic acid (TA-treated) (A), or 500 µg/mL ferulic acid (FA-treated) and caffeic acid (CA-treated) (B).

Figure 4



Graphical abstract description:

Plants contain polyphenols which when consumed through the diet are beneficial to human health. Microbes in the gut can interact with these plant ingredients to increase their health benefits. The present study investigates interactions between the probiotic bacterium *Lactobacillus acidophilus* NCFM and common plant polyphenols at the molecular level by analyzing effects on the bacterium whole-cell and surface proteomes and cellular and mucin adhesion capacity.

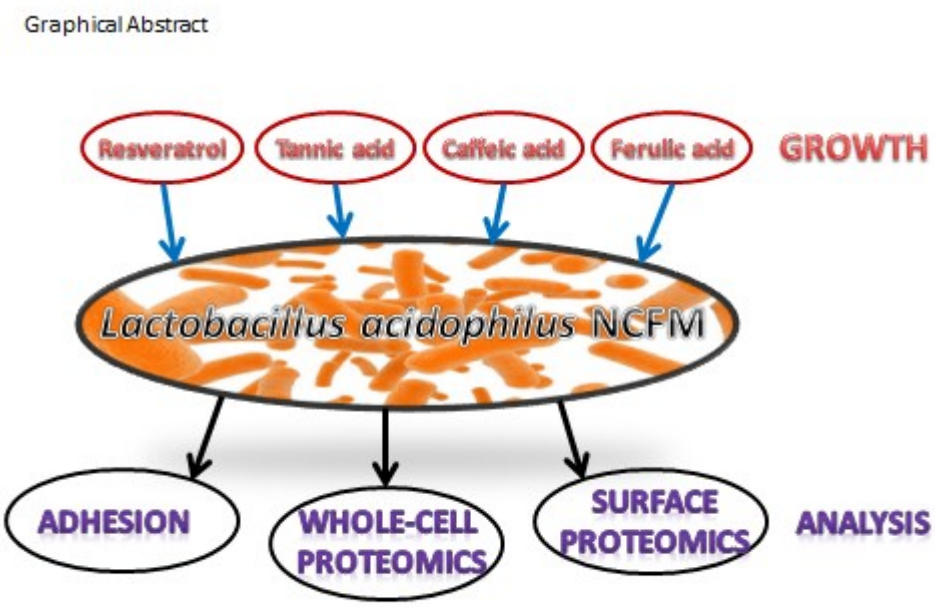


Table 1 Protein identifications of differentially abundant spots (ANOVA $p \leq 0.05$) of whole-cell proteins of *Lactobacillus acidophilus* NCFM grown in the presence of resveratrol (100 μg/mL), tannic acid (100 μg/mL), caffeic acid (500 μg/mL), or ferulic acid (500 μg/mL) compared to non-treated control. Protein identifications were confirmed with MASCOT score of 80 for peptide mass fingerprint, ANOVA $p \leq 0.05$, and a minimum of six matched peptides.

Protein name	Gene	Fold change in resveratrol (spot no)	Fold change in tannic acid (spot no)	Fold change in caffeic acid (spot no)	Fold change in ferulic acid (spot no)
L-lactate dehydrogenase	*lba0271*	**+2.0 (R2)**			
Preprotein translocase subunit SecA	*lba0673*	**+1.7 (R3)**			
Ribonucleoside triphosphate reductase	*lba0041*	**+1.6 (R4)**			
Multiple sugar-binding ABC-transporter ATPase	*lba1645*	**+1.5 (R5)**			
GAPDH	*lba0698*	**–1.5 (R6)**	**+1.5 (T6-T7)**	**–2.3 (C1)**	
TsaD	*lba0390*	**+1.4 (R7-R8)**			
Oxalyl-CoA decarboxylase	*lba0396*	**+1.4 (R9)**			
Ribokinase	*lba0587*	**+1.4 (R10)**			
Molecular chaperone Hsp33	*lba0279*	**+1.4 (R11)**		**–1.3 (C5)**	
Alanyl-tRNAsynthetase	*lba0417*	**+1.3 (R12)**			
tRNA (guanine-N(7)-)-methyltransferase	*lba1582*		**–3.0 (T1)**		
DNA-binding response regulator	*lba1820*		**+2.1 (T2)**		
tRNA-specific 2-thiouridylase MnmA	*lba0822*		**+1.7 (T3)**		
Prolyl-tRNA synthetase	*lba1262*		**+1.6 (T4)**		
Pyruvate kinase	*lba0957*		**+1.6 (T5)**		
Glutamyl-tRNA synthetase	*lba0347*		**+1.5 (T8)**		
Oligoendopeptidase F	*lba1763*		**+1.5 (T9)**		
Adenylosuccinate lyase	*lba1891*		**+1.4 (T10)**		
Phosphoglycerate kinase	*lba0699*		**+1.4 (T11)**		
50S ribosomal protein L1	*lba0360*			**–1.5 (C2)**	**–1.7 (F2)**
D-Lactate dehydrogenase	*lba0055*			**+1.4 (C3)**	
Elongation factor Ts	*lba1269*			**+1.3 (C4)**	
Purine trans deoxyribosylase	*lba0145*				**–1.4 (F1)**
Transcriptional regulator	*lba0733*				**+1.9 (F3)**

LBA0733					
Fructokinase	*lba0016*				**–1.4 (F4)**
Ribose-p-pyrophosphokinase	*lba0224*				**–1.6 (F5)**
Phosphomethylpyrimidine kinase	*lba1879*				**–1.4 (F6)**

Table 2 Protein identifications of differentially abundant spots (ANOVA $p \leq 0.05$) of surface proteins of *Lactobacillus acidophilus* NCFM treated with resveratrol (100 µg/mL), tannic acid (100 µg/mL), caffeic acid (500 µg/mL), or ferulic acid (500 µg/mL), compared to non-treated control. Protein identifications were confirmed with MASCOT score of 80 for peptide mass fingerprint, ANOVA $p \leq 0.05$, and a minimum of six matched peptides.

Protein name	Gene	Fold change in resveratrol (spot no)	Fold change in tannic acid (spot no)	Fold change in caffeic acid (spot no)	Fold change in ferulic acid (spot no)
Pyruvate kinase	*lba0957*	**+2.1 (RS1)**			**–1.6 (FS4)**
50S ribosomal protein L7/L12	*lba0370*	**+2.0 (RS2)**			
GAPDH	*lba0698*	**–2.0 (RS3), –1.6 (RS6)**			
Elongation factor P	*lba1668*	**+1.7 (RS4)**		**–1.7 (CS8)**	
50S ribosomal protein L22	*lba0296*	**+1.6 (RS5)**			
Adenylosuccinate synthetase	*lba1892*	**–1.5 (RS7)**		**–1.5 (CS6)**	
Hypothetical protein LBA1769	*lba1769*	**+1.4 (RS8)**			
6-phosphofructokinase	*lba0956*	**–1.4 (RS9)**			
Elongation factor G	*lba0289*		**–2.0 (TS1)**		
Mn-dependent inorganic pyrophosphatase	*lba1125*		**–1.5 (TS2)**		**–1.5 (FS2)**
Aminopeptidase	*lba1849*		**+1.4 (TS3)**		
Glycoprotein endopeptidase	*lba0388*		**+1.3 (TS4)**		
Glutamyl-tRNA synthetase	*lba0347*			**+1.5 (CS1)**	
D-Lactate dehydrogenase	*lba0055*			**–1.5 (CS2)**	
Elongation Factor Tu	*lba0845*			**–1.8 (CS3)**	
Triosephosphate isomerase	*lba0700*			**–2.0 (CS4)**	**–1.7 (FS5)**
30S ribosomal protein S1	*lba0968*			**–1.8 (CS5)**	**–1.7 (FS6)**
Lysine tRNA ligase	*lba0281*			**–1.9 (CS7)**	
Aspartate tRNA ligase	*lba0936*			**–2.2 (CS9)**	

L-Lactate dehydrogenase	*lba0271*				**–1.6 (FS1)**
Oligoribonuclease	*lba0415*				**–1.8 (FS3)**
Trigger factor	*lba0846*				**–1.7 (FS7)**