Improvement of lipid profile by probiotic/protective cultures: study in a non-carcinogenic small intestinal cell model

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SUMMARY

Plasma lipid levels are important risk factors for the development of atherosclerosis and coronary heart disease. Previous findings have shown that probiotic bacteria exert positive effects on hypercholesterolemia by lowering serum cholesterol and improving lipid profile that, in turn, leads to a reduced risk of coronary heart disease and atherosclerosis. Most of these studies were carried out with tumoral cell lines that have a metabolism quite different from that of normal cells and may thus respond differently to various stimuli. Here, we demonstrate the beneficial effects of some probiotics on cholesterol levels and pathways in normal small intestinal foetal epithelial tissue cells. The results show that *Lactobacillus plantarum* strain PCS 26 efficiently removes cholesterol from media, exhibits bile salt hydrolase activity, and up-regulates several genes involved in cholesterol metabolism. This study suggests that *Lactobacillus plantarum* PCS 26 might act as a liver X receptor agonist and help to improve lipid profiles in hypercholesterolemic patients or even dislipidemias in complex diseases such as the metabolic syndrome.

KEY WORDS: Lactobacillus plantarum, Liver X receptors, NPC1L1, ABCG5, ABCG8, LPL.

Received June 19, 2013 *Accepted* November 26, 2013

INTRODUCTION

Hypercholesterolemia is considered one of the risk factors for diseases such as atherosclerosis and coronary heart disease. This hypothesis is supported by the high content of cholesterol in atherosclerotic lesions of cholesterol fed animals and humans (Ignatowski, 1908; Connor, 1961; Armstrong *et al.*, 1970; Jagannathan *et al.*, 1974; NCEP, 1988; Stamler and Shekelle, 1988; LaRosa *et al.*, 1990; Manson *et al.*, 1992). The absorption of dietary cholesterol and reabsorption of biliary cholesterol in the small intestines accompanied by endogenously synthesized cholesterol, contribute to the regulation of plasma cholesterol levels (Wilson and Rudel, 1994). Although only about 40% of dietary cholesterol is absorbed, the feedback inhibition of cholesterol biosynthesis in humans is only partial even with a large dietary cholesterol intake, therefore dietary cholesterol adds considerably to the amount of cholesterol synthesized by the body (Berge *et al.*, 2000). Past studies have shown that probiotic bacteria exert positive effects on hypercholesterolemia by lowering serum cholesterol and improving lipid profile that, in turn, leads to a reduced risk of coronary heart disease and atheroscle-

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rosis (Mann, 1974; Sanders, 2000; Huang and Zheng, 2010). Probiotic/protective cultures can reduce cholesterol levels by binding dietary cholesterol onto protoplast membranes or cell surfaces, by incorporation of cholesterol into cell membranes during growth and by the ability of some probiotic/protective cultures to enzymatically deconjugate bile salts by bile salt hydrolase (Razin, 1975; Tani *et al*., 1993; Noh *et al*., 1997; Liong and Shah, 2005; Kimoto *et al*., 2007; Lye *et al*., 2009; Patel *et al*., 2009; Ooi and Liong, 2010). Deconjugated bile salts are only partially reabsorbed in the intestinal lumen, which results in excretion of free bile salts into feces; in response, cholesterol is used for *de-novo* synthesis of bile acids, thus reducing serum cholesterol level. Free bile salts also inefficiently solubilize lipids in intestines thus further reducing lipid absorption (Begley *et al*., 2006). Past studies have also shown that probiotic bacteria can alter gene expression of Niemann-Pick C-1-like 1 (NPC1L1) and the liver X receptors in carcinogenic CaCo2 cell line (Huang and Zheng, 2010; Yoon *et al*., 2011). NPC1L1 was identified as the major player in cholesterol absorption and is highly expressed in small intestines (Altmann *et al*., 2004; Miura and Saku, 2008). NPC1L1 was also identified as a novel target gene of the liver X receptors (NR1H3 and NR1H2) which are regulators of cholesterol homeostasis and belong to the nuclear receptor super family (Duval *et al*., 2006; Nomiyama and Bruemmer, 2008). The liver X receptor agonists can down-regulate NPC1L1 expression, resulting in decreased cholesterol uptake (Duval *et al*., 2006; Huang and Zheng, 2010). Furthermore, ATP-binding cassette subfamily G (WHITE) member 5 (ABCG5), ATP-binding cassette sub-family G (WHITE) member 8 (ABCG8) and lipoprotein lipase (LPL), were also identified as target genes of liver X receptors (Zhang, 2001; Edwards *et al*., 2002; Repa, 2002). There is further evidence that the ABCG5 and ABCG8 are the major sterol transporters active on the apical side of enterocytes (Hazard and Patel, 2007). Treatment of mice with liver X receptor agonists leads to reduced absorption of dietary cholesterol and increased ABCG5/ABCG8 mediated secretion of cholesterol into bile (Repa *et al*., 2000; Yu *et al*., 2003). In parallel, over-expression of LPL plays a protective role against diet-induced atherosclerosis and suppresses hyperlipidemia (Yagyu *et al*., 1999; Niho *et al*., 2005). Transgenic mice with over-expression of human LPL showed a 75% reduction of plasma triglycerides (Shimada *et al*., 1993). Over-expression of catalytically inactive LPL also seems to improve the high-fat diet-induced systemic insulin resistance and hypertriglyceridemia in these mice (Shibasaki *et al*., 2006).

The aim of this study was to investigate and evaluate the effects of probiotic/protective cultures in relation to *in-vitro* cholesterol removal and modulation of expression of genes involved in cholesterol homeostasis. The study was carried out in a non-carcinogenic functional cell model derived from human small intestine (Gorenjak *et al*., 2012a), and using *Lactobacillus plantarum* PCS 20 and PCS 26 strains isolated from a Slovenian traditional cheese (Nissen *et al*., 2009). *Lactobacillus rhamnosus* LGG (ATCC 53103) was used as reference strain.

MATERIALS AND METHODS

Growth and maintenance of cell culture

Two intestinal epithelial cell lines were used in this study: non-carcinogenic small intestinal foetal epithelial tissue cells (HUIEC), established at the Department of Biochemistry and Nutrition, University of Maribor (Slovenia), and small intestinal epithelial tissue cells (HIEC) (Beaulieu, 1997, 1999; Belanger and Beaulieu, 2000; Lussier *et al.*, 2000; Pageot *et al.*, 2000). The human small intestinal foetal epithelial cell line (HUIEC) was characterized by confirming the presence of known epithelial markers and trans-epithelial resistance (Carlson *et al.*, 2002; Wood *et al.*, 2003; Geddes and Philpott, 2008; Cencic and Langerholc, 2010). The presence of cytokeratin 18, fatty acid binding protein (FABP) and alkaline phosphatase proved the cells epithelial phenotype and metabolic characteristics. Further, cells were confirmed to develop appropriate trans-epithelial resistance and formation of connectivity. Both cell lines were grown in 25 cm^2 cell culture flasks (Corning Incorporated, Corning, New York) and using Dulbecco's Modified Eagle's Medium (DMEM) advanced medium (Life Tech-

nologies, Carlsbad, California) supplemented with 100 International Units (IU)/mL penicillin (Sigma, Steinheim, Germany), 0.1 mg/mL streptomycin (Fluka, Buchs, Switzerland), 2 mmol/L L-glutamine (Life Technologies) and 5% of foetal bovine serum (FBS) (Life Technologies). Cells were incubated in humidified 5% CO_2 atmosphere at 37°C and culture medium changed routinely until confluent monolayers were obtained. Cell lines were tested for human origin using a polymerase chain reaction method (PCR) recently developed (Gorenjak *et al*., 2012b).

Probiotic strains and growth condition

Probiotic/protective culture strains *Lactobacillus plantarum* PCS 20, *Lactobacillus plantarum* PCS 26, *Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus acidophilus* (ATCC 4356) were stored at -80°C in 20% (v/v) glycerol (Merck, Darmstadt, Germany) with De Man Rogosa, Sharpe (MRS) broth (Merck, Darmstadt, Germany) until use. Prior to assays, the strains were propagated and serially inoculated 3 times in MRS broth for 24h at 37°C under anaerobic conditions using Anaerogen (Oxoid, Hampshire, UK).

Bile tolerance

Bile tolerance was tested using a modified assay for bile tolerance described by Gilliland *et al*. (1985). Overnight probiotic/protective cultures were inoculated at 1% (v/v) into 9.9 mL aliquots of MRS broth supplemented with 0.3% (w/v) bovine bile (Sigma), and MRS broth with no supplements. The inoculated broths were incubated in triplicate for 12h at 37°C under anaerobic conditions using Anaerogen (Oxoid). After incubation, the optical density of inoculated broths was measured at 595 nm using Varioskan Flash spectrophotometer (Thermo Scientific, Florida, USA). Absorbance values of supplemented MRS broth were expressed as percent relative to absorbance values of MRS broth without bovine bile, arbitrarily set to 100%.

Cholesterol removal assay

Cholesterol assimilation assay was performed according to a modified version of the procedure described by Pereira and Gibson (2002). The medium used was modified MRS (mMRS) broth supplemented with 0.3% (w/v) bovine bile (Sigma) and ~200 µg/mL water soluble cholesterol (polyoxyethanyl-cholesteryl sebacate) (Sigma). *Lactobacillus plantarum* PCS 20, *Lactobacillus plantarum* PCS 26 and *Lactobacillus rhamnosus* GG (ATCC 53103) strains were diluted at $1x10^7$ CFU/mL in sterile phosphate buffered saline (PBS) and inoculated at 1% (v/v) into 9.9 mL aliquots of mMRS. The inoculated cultures were incubated in triplicates for 24h at 37°C under anaerobic conditions using Anaerogen (Oxoid). Uninoculated sterile mMRS was also incubated in triplicate under the same conditions and used as a control. After incubation, bacterial cells were removed by centrifugation (2400 rpm for 10 min) and supernatants were measured for cholesterol content using Cobas c111 biochemical analyzer (Roche, Basel, Germany).

Bile salt hydrolase activity assay

Bile salt hydrolase (BSH) activity was tested using the modified plate assay with differential medium (Dashkevicz and Feighner, 1989; Mathara *et al*., 2008). Agar plates were prepared with MRS agar (Merck) supplemented with 0.2% (w/v) taurocholic acid (TCA), 0.2% (w/v) taurodeoxycholic acid (TDCA), 0.05% (w/v) taurochenodeoxycholic acid (TCDCA), 0.2% (w/v) glycocholic acid (GCA) or 0.05% (w/v) glycochenodeoxycholic acid (GCDCA) (Sigma) and 0.37 g/L $CaCl_2$ (Fluka). Plates of MRS agar without conjugated bile acids were used as controls. Plates were placed under anaerobic conditions using Anaerogen (Oxoid) for at least 48 h before use, and then inoculated with overnight cultures of the bacterial strains using a 10 µL loop. Incubation proceeded for a further 72 h under anaerobic conditions. *Lactobacillus acidophilus* (ATCC 4356) was used as a positive control. The BSH activity was determined by examining formation of precipitate halos around the colonies or opaque white colonies due to acidification of the medium.

Viability of cells exposed to probiotic bacteria

Viability of cells exposed to probiotic bacteria was tested using cytotoxicity assay with crystal violet (Saotome *et al*., 1989) and by deter-

mination of metabolic activity of cell cultures (Mosmann, 1983; Bergamini *et al.*, 1992; Ivec *et al.*, 2007). HIEC and HUIEC cells were seeded separately at 10^5 cells/well using flat bottom NUNC™ 96 well plates (Thermo Scientific, New York, USA). Cells were grown as described previously and were incubated in humidified 5% CO_2 atmosphere at 37°C.

After confluent monolayers were obtained, cells were washed twice with warm sterile PBS and exposed to 100 µL of probiotic suspension with concentration 10^9 CFU/mL in DMEM advanced medium without phenol red, supplemented with L-Glutamine and without antibiotics and FBS. Each suspension of probiotic strains PCS 20, PCS 26 and LGG was added in triplicate to separate wells, and plates were incubated for 90 min in humidified 5% CO_2 atmosphere at 37°C, to allow bacteria to attach to cell monolayers. Non-exposed cells were used as a control. After incubation, cells were washed 3 times with warm sterile PBS to remove non-attached bacteria. After washing, 100 µL of fresh medium were added to the cells and plates were incubated for 24h in humidified 5% CO_2 atmosphere at 37°C. In parallel, the same procedure was done on separate plates without the washing procedure to obtain the non-attached bacteria in the wells.

Cytotoxicity assay

After incubation, the medium was discarded, cells were washed and 100 µL of 0.1% crystal violet (Fluka) in 2% (v/v) ethanol (Sigma) was added to each well with cell monolayer. After 5 min, the wells were washed with warm sterile deionized water and plates were left to dry at room temperature over night. After the plates were dry, 100 µL of 10% acetic acid (Sigma) was added to each well and plates were placed on a horizontal orbital shaker for 60 min. Detection of crystal violet previously retained in living cells was measured spectrophotometrically at 595 nm with Varioskan Flash spectrophotometer (Thermo Scientific).

Metabolic activity

Metabolic activity was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) activity assay where active dehydrogenase enzymes reduce the tetrazolium yellow substrate into formazan dark blue crystals and the reduction serves as an index of living, metabolically active cells (Mosmann, 1983). After incubation, the medium was discarded, the wells with cell monolayer were washed once with warm sterile PBS and 200 µL of DMEM advanced medium without phenol red, supplemented with L-Glutamine and without antibiotics and FBS, and 20 µL of MTT (Sigma) solution (5mg/ml in PBS) was added to each well.

After the addition of MTT solution the plates were placed on a horizontal orbital shaker for 5 min and incubated for 5h in humidified 5% CO_2 atmosphere at 37°C. After incubation, the medium with MTT solution was discarded and plates were left to dry at room temperature overnight. After the plates were dry, 100 µL of 0.04 HCl (Sigma) in isopropanol (Sigma) were added to each well to solubilize fromazan crystals and plates were placed on a horizontal orbital shaker for 5 min followed by incubation for 20 min at room temperature. After incubation, absorbance was measured spectrophotometrically at 570 nm with Varioskan Flash spectrophotometer (Thermo Scientific). In both experiments, absorbance values of treated wells were expressed as percent relative to absorbance values of non-treated wells, arbitrarily set to 100%.

Gene expression assay and RNA extraction

Once confluent, cell monolayers were washed twice with warm sterile PBS and seeded with the probiotic strains PCS 20, PCS 26 and LGG that were resuspended at $1x10^7$ CFU/mL in DMEM advanced medium supplemented with 200 µg/mL water soluble cholesterol and L-Glutamine and without antibiotics and FBS. Each strain was seeded in triplicate to separate HIEC and HUIEC cells, and culture flasks were incubated for 6h in humidified 5% CO_2 atmosphere at 37°C. Three separate HIEC and HUIEC cell cultures were manipulated and incubated in parallel with no probiotic strain and used as control. After incubation, cells were washed with ice cold sterile PBS to stop transcriptional processes, detached from flasks with 2 mL of 0.25% trypsine-EDTA solution, collected into a centrifuge tube, diluted in ice cold sterile PBS, and centrifuged at 120 × g for 5 minutes. Af-

ter centrifugation, the supernatants were discarded, and cell pellets resuspended in ice cold sterile PBS and centrifuged again at 320 × g for 15 minutes. Cell pellets were then immediately processed for RNA extraction using the TRI reagent (Sigma) according to the manufacturer's instructions. Cellular RNA was transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quality and purity of extracted RNA

Concentration of extracted RNA was measured with optical density readings at 260 nm and purity calculated from 260/280 nm ration using a photometer (Eppendorf, Hamburg, Germany).

Selection of target and reference genes

Target and reference genes were selected according to the literature cited in Table 1.

Retrieving sequences and primer design

The mRNA sequences of cellular genes used for primer design are listed in Table 1 and were retrieved from PubMed Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) and AceView database (Mieg and Mieg, 2006). Primers for the target genes were designed using a primer web tool IDT oligo analyzer (http://eu.idtdna.com/analyzer/Applications/), web sequence alignment software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Roche Universal Probe Library Assay Design Center (http://www.roche-applied-science.

TABLE 1 - *Target and reference genes.*

Gene	*Gene names*	*NCBI references*	*References*	*Primer Sequence 5' → 3'*
LPL	Lipoprotein lipase	NM_000237.2	(Zhang, 2001; Zabaneh and Balding, 2010)	CATTGCAGGAAGTCTGACCA GGACCCTCTGGTGAATGTGT
NR1H3	nuclear receptor subfamily 1, group H, member 3	NM_001130102.1 NM_005693.2 NM_001130101.1	(Edwards *et al.*, 2002; Nomiyama and Bruemmer, 2008; Huang and Zheng, 2010)	TCAGAGAGGAAGCCAGGATG ATAGCTCGTTCCCCAGCAT
NR1H2	nuclear receptor subfamily 1, group H, member 2	NM_007121.4	(Edwards *et al.*, 2002; Nomiyama and Bruemmer, 2008; Huang and Zheng, 2010)	TACAACCACGAGACAGAGTG GCCGAGAAGATGTTGATG
NPC1L1	Niemann-Pick C1-like protein 1	NM_001101648.1 NM_013389.2	(Altmann *et al.*, 2004; Huang and Zheng, 2010)	ACATCAGCGTGGGACTGG AGTCAAGCAGGTACGAGTCCTT
ABCG5	ATP-binding cassette, subfamily G (WHITE), member 5	NM_022436.2	(Lee *et al.*, 2001; Repa, 2002; Yu, 2002, 2004)	ACCCAAAGCAAGGAACGGGAA CAGCGTTCAGCATGCCTGTGT
ABCG8	ATP-binding cassette, subfamily G (WHITE), member 8	NM_022437.2	(Repa, 2002; Yu, 2002, 2004)	GGGTGAGCGCAGGAGAGTCAG TCACGCTGCTTTCCACACAGG
ACTB	Beta actine	NM_001101.3	(Mehta *et al.*, 2010)	CTCTTCCAGCCTTCCTTCCT AGCACTGTGTTGGCGTACAG
RPII	polymerase (RNA) II (DNA directed) polypeptide A	NM_000937.4	(Radonić *et al.*, 2004; Mehta *et al.*, 2010)	CTTCACGGTGCTGGGCATT GTGCGGCTGCTTCCATAA

com). ABCG5 and ABCG8 primer sequences were from Yoon and colleagues (Yoon *et al.*, 2011), ACTB and RPII primer sequences from Mehta and colleagues (Mehta *et al.*, 2010) (Table 1). All primers were synthesized by Sigma.

Quantitative real time PCR

Quantitative real time PCR was carried out with 2x Maxima SYBR Green qPCR master mix (Fermentas, Maryland, USA), according to the manufacturer's instructions, and using LightCycler 480 (Roche, Basel, Germany). After each PCR process, a melting curve was performed to confirm specificity of amplification. Normalization of real time PCR data was performed by geometric averaging of reference genes (ACTB and RPII) (Vandesompele *et al.*, 2002) and calculated with $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).

Statistical analysis

Data were analyzed with SPSS Statistics software 18 (IBM Inc., Armonk, NY, USA) using parametrical ANOVA with post hoc Dunnett's T-test and Bonferroni, and after a Shapiro-Wilk test of normality. Gene expression data was analyzed as normalized linear form using $2^{-\Delta Ct}$ calculation. Correlation between expression profiles was determined using Spearman correlation tests. *P* value ≤0.05 or *P* value ≤0.01 in correlation tests were considered statistically significant.

RESULTS

Analysis of bile tolerance, capacity of cholesterol removal, and activity of bile salt hydrolase

The bacterial strains were first tested for their capacity to grow in the presence of 0.3% bovine bile, which approximates the bile concentrations found in the gastrointestinal tract (Gilliland *et al.*, 1984). After the incubation period all strains showed a significantly lower ($P<0.01$) percent of absorbance in supplemented MRS broth (Figure 1a). The most resistant strain was *Lactobacillus plantarum* PCS 26 (56%) followed by *Lactobacillus plantarum* PCS 20 (40%) and *Lactobacillus rhamnosus* GG (8%), which was the most susceptible strain to the presence of the bile.

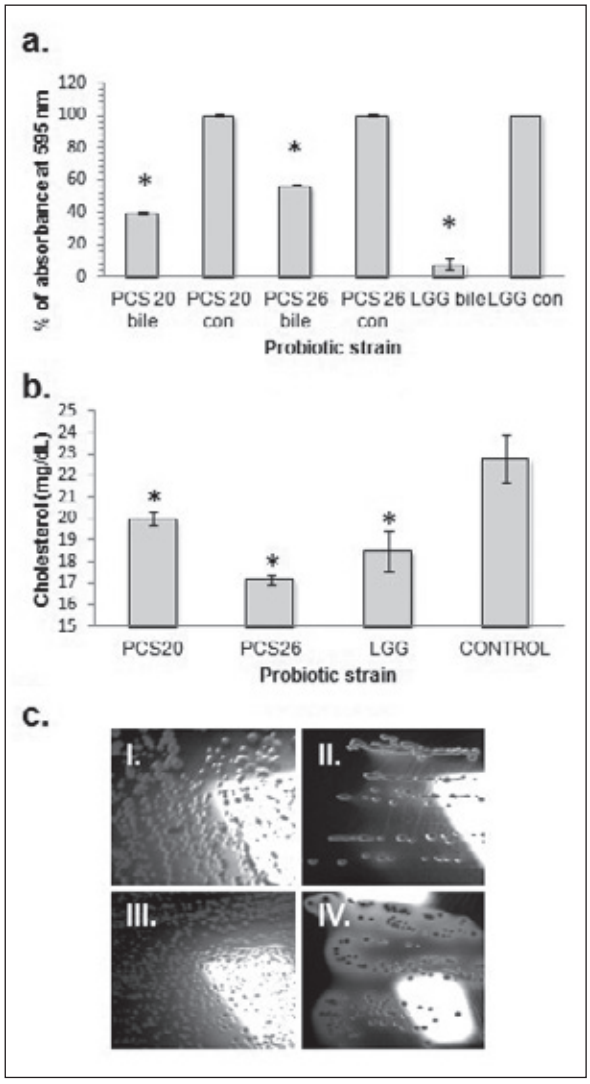

FIGURE 1 - *Analysis of probiotic/protective strains for their ability to tolerate bile salts, capacity to remove cholesterol, and activity of bile salt hydrolase. a. Ability to survive and grow in media supplemented with 0.3% bovine bile and control media. Average of 3 independent experiments with 3 replicates each ± standard deviation expressed as percent; b. Reduction of cholesterol concentration in culture media inoculated with probiotic/protective strains. Mean values of three independent experiments with 3 replicates each ± standard deviation. Statistically significant differences compared to uninoculated control media were determined using ANOVA post hoc Dunnett's T-test (*P≤0.05); c. Precipitation halos and cloudiness around the bile salt hydrolase active colonies. (I.) Lactobacillus plantarum PCS 20 on MRS agar containing 0.2% GCA; (II.) plantarum PCS 20 on MRS agar containing 0.05% GCDCA; (III.) Lactobacillus plantarum PCS 26 on MRS agar containing 0.2% GCA; (IV.) plantarum PCS 26 on MRS agar containing 0.05% GCDCA.*

TABLE 2 - *Results of the hydrolaze bile salt activity assay of the bacterial strains plated on MRS agar supplemented as indicated.*

	0.2% TCA	*0.2% TDCA*	*0.05% TCDCA*	*0.2% GCA*	*0.05% GCDCA*
Lactobacillus plantarum PCS 20	-	-	-	+	+
Lactobacillus plantarum PCS 26	-	-	-	+	+
Lactobacillus rhamnosus LGG ATCC 53103	-	-	-	-	-
Lactobacillus acidophilus ATCC 4356	+	+	+	+	NA

NA: Not applicable.

Evaluation of the ability to remove cholesterol demonstrated that all three tested probiotic/protective strains significantly lowered cholesterol levels in comparison with the uninoculated control mMRS. The highest removal of cholesterol was observed in mMRS inoculated with PCS 26 ($P<0.01$), followed by LGG ($P<0.01$) and PCS 20 ($P=0.005$). The mean values of cholesterol concentrations from triplicate experiments in mMRS after incubation were 17.18 mg/dL for media inoculated with PCS 26, 18.5 mg/dL for media inoculated with LGG, 20.01 mg/dL for media inoculated with PCS 20 and 22.8 mg/dL for uninoculated control media (Figure 1b).

Both probiotic strains *Lactobacillus plantarum* PCS 20 and PCS 26 showed bile salt hydrolase activity on agar plates supplemented with GCA or GCDCA, but not on MRS agar plates supplemented with TCA, TDCA or TCDCA. Enzyme activity of both *Lactobacillus plantarum* strains was demonstrated by an intense cloudiness surrounding or under bacterial colonies plated on GCA supplemented MRS agar and opaque precipitation halos around the colonies on MRS agar supplemented with GCDCA (Figure 1c). In contrast, the LGG strain showed no bile salt hydrolase activity whereas the control strain *Lactobacillus acidophilus* showed some activity in MRS agar plates supplemented with TCA, TDCA, TCDCA and GCA, but activity in 0.05% GCDCA could not be determined due to the strain's inability to grow in such conditions as reported previously (McAuliffe *et al*., 2005). The complete results of the hydrolase bile salt activity assay are summarized in Table 2.

Viability of cells exposed to probiotic bacteria

Before the gene expression modulation assays, the viability of cell lines exposed to probiotic bacteria was tested. Data obtained from cytotoxicity assay did not show any toxic effects in HIEC and HUIEC cell lines exposed to the probiotic strains *Lactobacillus plantarum* PCS 20, *Lactobacillus plantarum* PCS26 and *Lactobacillus rhamnosus* LGG. Cytotoxicity assay with washing procedure did not show any significant reduction of crystal violet retention in comparison with the control (Figure 2a). Parallel cytotoxicity assay without the washing procedure showed that all three probiotic strains elevated the retention of crystal violet in both cell lines (Figure 2b). In HIEC cells, PCS 20 elevated the retention of crystal violet up to 8% ($P=0.007$), PCS 26 up to 9% ($P=0.005$) and LGG up to 6% ($P=0.043$) in comparison with the control. In HUIEC cells, the retention of crystal violet was also elevated, but to a lesser non-significant extent. Probiotic strain PCS 20 elevated the retention up to 4% ($P=0.890$), PCS 26 up to 7% ($P=0.165$) and LGG up to 4% ($P=1.000$). MTT activity assay demonstrated that probiotic strains PCS 20, PCS 26 and LGG did not significantly influence the metabolic activity in exposed HIEC and HUIEC cell lines with and without the washing procedure in comparison with the control (Figure 2c, 2d).

Gene expression

These experiments aimed to investigate the modulation of the expression of genes involved in lipid homeostasis in HIEC and HUIEC cells

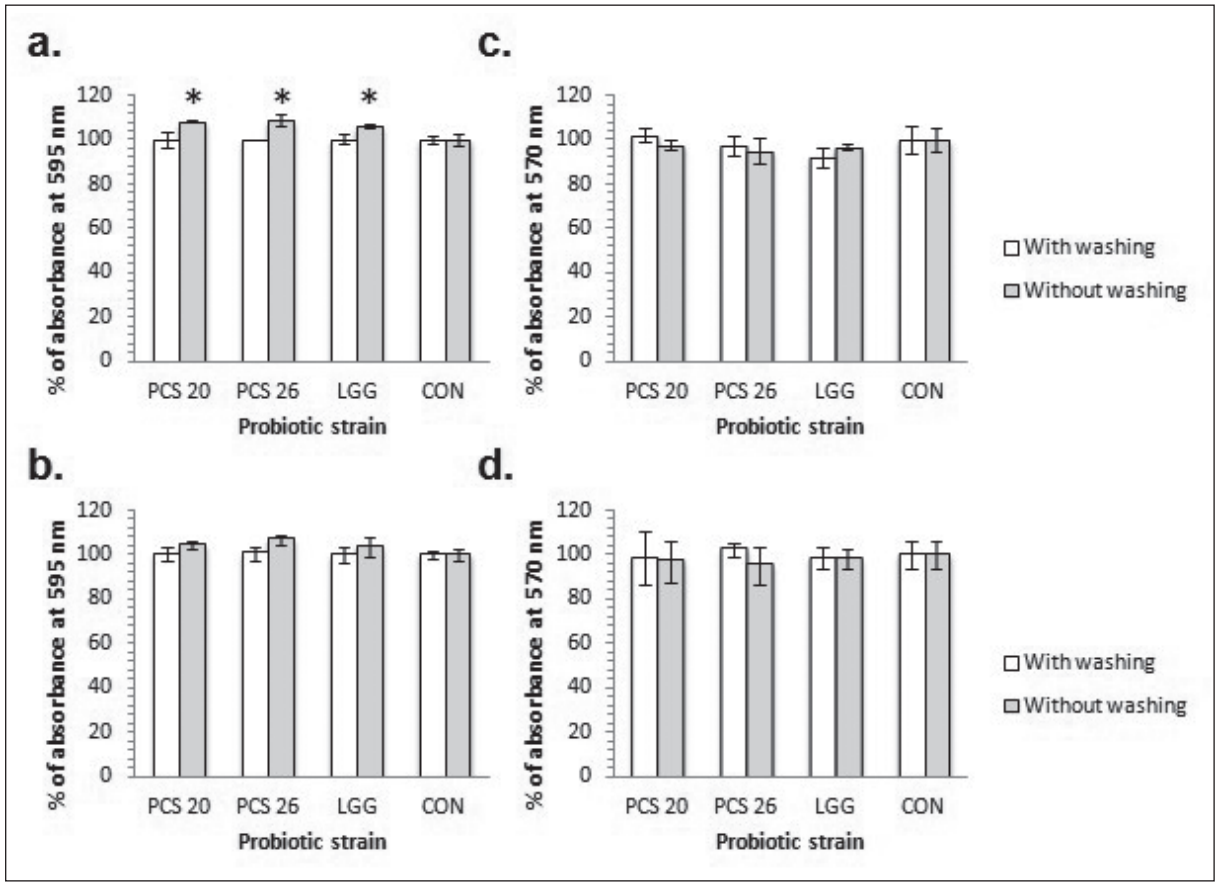

FIGURE 2 - *Viability of HIEC and HUIEC cells exposed to probiotic/protective strains for 24 hours. a. Cytotoxicity with crystal violet in HIEC cells; b. Cytotoxicity with crystal violet in HUIEC cells; c. Metabolic activity using MTT assay in HIEC cells; d. Metabolic activity using MTT assay in HUIEC cells. Results are presented as mean values of 3 independent experiments with 3 replicates each ± standard deviation. Statistically significant differences in comparison to control were determined using ANOVA post hoc Bonferroni test (*P≤0.05).*

exposed to the probiotic strains. As described before, liver X receptors (NR1H3 and NR1H2) regulate genes controling cholesterol homeostasis through absorption and secretion of cholesterol in the intestines.

To this purpose, the cell line cultures were incubated with the probiotic strains PCS 20, PCS 26 and LGG for 6h and then tested. Untreated cells were used as controls. Before proceeding to examining the selected genes, the reference genes ACTB and RPII were assessed for their stability in HIEC and HUIEC cell lines with and without exposure to the tested probiotic strains. Stability of the refernece genes may further enhance the robustness of quantitative real time PCR (Mehta *et al*., 2010). Both reference genes proved stably expressed and with no statistically significant differences in either cell line indicating that these genes are suited for use for target gene expression normalization in the present study (data not shown).

Addition of probiotic strains in cell cultures deeply impacted the level of expression of the target genes involved in cholesterol metabolism. The *Lactobacillus plantarum* PCS 26 significantly up-regulated NR1H3 in HIEC and HUIEC cells of 49% ($P<0.01$) and 44% ($P<0.01$), respectively, compared to control cells. This effect was negligible with PCS 20 and LGG (Figure 3a).

Expression of NR1H2 was significantly up-regulated by *Lactobacillus plantarum* PCS 26 and PCS 20 in both cell lines (Figure 3b). Once again, however, *Lactobacillus plantarum* PCS

26 was the most effective and increased NR1H2 expression up to 71% (P<0.01) and 91% in HIEC and HUIEC cells, respectively. The difference reached statistical significance in both cases (P<0.01) compared to the control cells. *Lactobacillus plantarum* PCS 20 up-regulated NR1H2 expression of about 47%, while LGG had no effect in both cell lines (Figure 3b).
LPL was also up-regulated by *Lactobacillus plantarum* PCS 20 and PCS 26 in both cell lines (Figure 3c). *Lactobacillus plantarum* PCS 26 was again the most effective as it up-regulated LPL by 76% and 139% in HIEC and HUIEC cells, respectively and the increment was higly significant (P<0.01) compared to respective controls. Albeit less pronounced, the up-regulation triggered by *Lactobacillus plantarum* PCS 20 (33% and 45% in HIEC and HUIEC cells, respectively) also reached high statistical significance (P<0.01) compared to controls. Conversely, incubation with LGG strain, did not significantly impact LPL expression (Figure 3c).

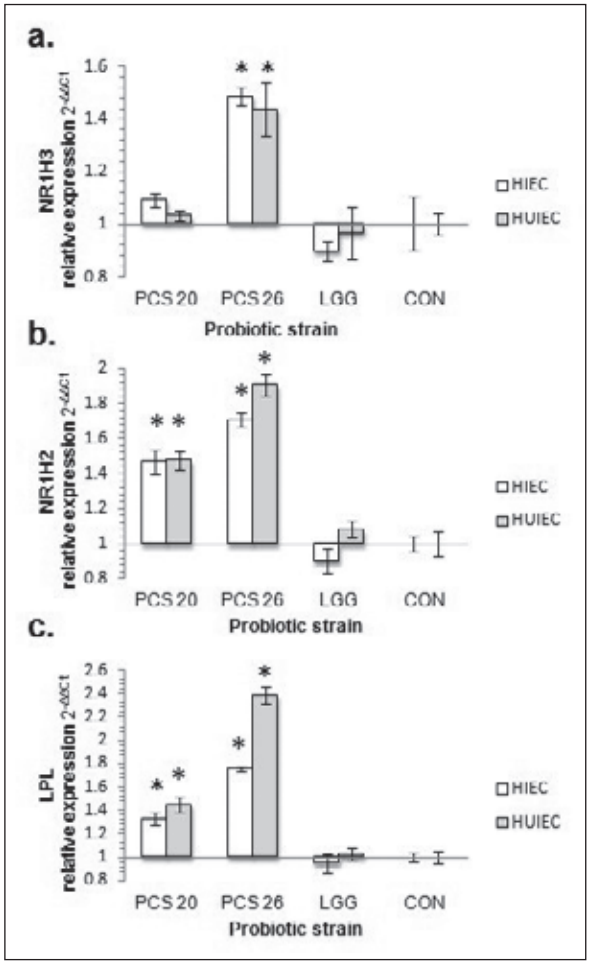

FIGURE 3 - *Analysis of NR1H3, NR1H2 and LPL mRNA expression level in HIEC and HUIEC cells co-cultured with the probiotic/protective strains for 6 hours. a. Expression of NR1H3; b. Expression of NR1H2; c. Expression of LPL. Results are presented as mean 2-ΔΔCt expression relative to the control of 3 independent experiments with 3 replicates each ± error calculated from standard deviations. Statistically significant differences in comparison to control were determined using ANOVA post hoc Bonferroni test (*P≤0.05).*

Compared to the above genes, the presence of probiotic/protective strains had an inverse effect on NPC1L1 mRNA expression. Both, *Lactobacillus plantarum* strains significantly down-regulated NPC1L1 expression in HIEC and HUIEC cells, while LGG did not cause overt effects (Figure 4a). In particular, *Lactobacillus plantarum* PCS 26 down-regulated NPC1L1 expression up to 55% (P=0.002) in HIEC and up to 65% (P<0.01) in HUIEC cells. Down-regulation of NPC1L1 expression was less pronounced in cells exposed to *Lactobacillus plantarum* PCS 20 (Figure 4a).
ABCG5 and ABCG8 expression was also significantly up-regulated in both cell lines co-cultured with *Lactobacillus plantarum* PCS 20 and PCS 26, while no variations were observed in cells co-cultured with *Lactobacillus rhamnosus* LGG. Again, *Lactobacillus plantarum* PCS 26 had a stronger impact on ABCG5 expression that increased up to 165% and 110% in HIEC and HUIEC cells, respectively (P<0.01). *Lactobacillus plantarum* PCS 20 up-regulated ABCG5 expression up to 114% (P<0.01) and 56% (P=0.009) in HIEC and HUIEC cells, respectively. No influence on expression was observed with the LGG strain (Figure 4b).
Similarly to ABCG5, ABCG8 expression was also strongly up-regulated. Exposure to *Lactobacillus plantarum* PCS 26 increased expression up to 242% and 268% in HIEC and HUIEC cell lines, respectively (P<0.01), while exposure to *Lactobacillus plantarum* PCS 20 up-regulated ABCG8 expression up to 185% and 235% in HIEC and HUIEC cells, respectively (P<0.01). Minimal effects, if any, were observed in cells incubated with the LGG strain (Figure 4c).
To understand whether modulation of gene expression was casual or followed a specific

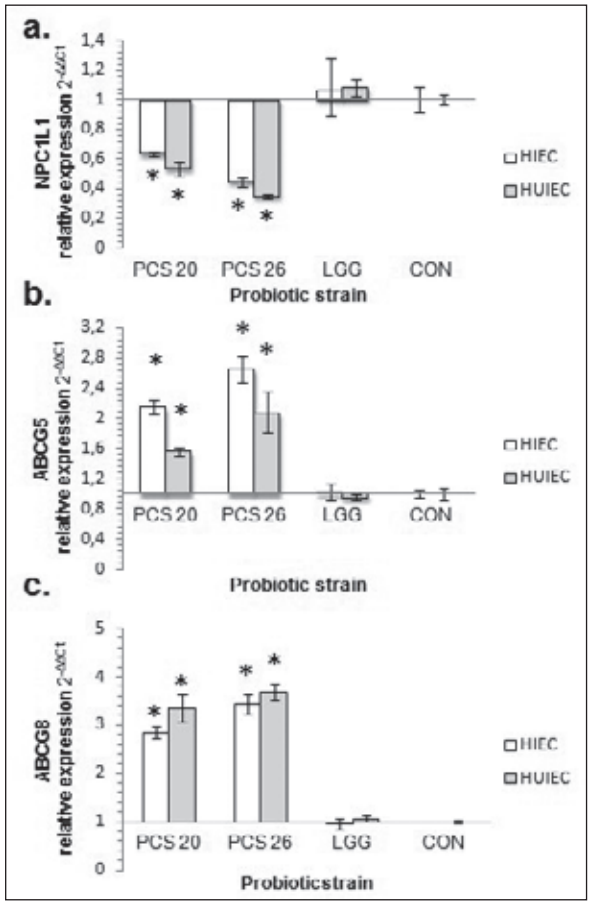

FIGURE 4 - *Analysis of NPC1L1, ABCG5 and ABCG8 mRNA expression level in HIEC and HUIEC cells co-cultured with the probiotic/protective strains for 6 hours. a. Expression of NPC1L1; b. Expression of ABCG5; c. Expression of ABCG8. Results are presented as mean 2-ΔΔCt expression relative to the control of 3 independent experiments with 3 replicates each ± error calculated from standard deviations. Statistically significant differences in comparison to control were determined using ANOVA post hoc Bonferroni test (*P≤0.05).*

pattern, we performed correlation analyses with the mRNA expression data. We found strong correlations between the liver X receptors (NR1H3, NR1H2) and the target genes (NPC1L1, ABCG5, ABCG8 and LPL). These findings suggest that the liver X receptors had a direct impact on the target genes (Zhang, 2001; Edwards *et al.*, 2002; Repa, 2002). Results of correlation analyses are shown in Table 3.

DISCUSSION

It has long been known that cancerous epithelial cell lines have different sugar composition on the cell surfaces, than do normal ones (Hakomori, 1996; Brooks *et al.*, 2008). In all, tumoral cells have quite different metabolic pathways and functions compared to normal cells and may thus respond differently to various stimuli, including probiotic strains.

This is one of the first studies to examine probiotic/protective cultures with normal epithelial cells, and in particular using the non-tumor-derived cell lines HIEC and HUIEC that retain many properties of the intestinal tissue from which they were originated (Gorenjak *et al.*, 2012a) and should thus provide more reliable results.

Here we demonstrated that the probiotic/protective cultures helped improve the lipid profile by lowering cholesterol concentration and by regulating the expression of genes involved in cholesterol homeostasis. In this regard, the most effective strain was *Lactobacillus plantarum* PCS 26 that was isolated from a Slovenian traditional cheese. In a previous study, Nissen and colleagues showed that *Lactobacillus plan-*

TABLE 3 - *Spearman correlation coefficient analysis of expression of studied genes.*

	LPL	NR1H3	NR1H2	NPC1L1	ABCG5	ABCG8
HUIEC cells						
NR1H3	0.573*	1	0.587*	-0.727**	0.776**	0.685*
NR1H2	0.888**	0.587*	1	-0.727**	0.783**	0.839**
HIEC cells						
NR1H3	0.909**	1	0.839**	-0.790**	0.846**	0.804**
NR1H2	0.902**	0.839**	1	-0.888**	0.839**	0.853**

*Correlation significant at the 0.05 level; ** Correlation significant at the 0.01 level.

tarum PCS 26 and PCS 20 strains are resistant to lower pH and bile (Nissen *et al*., 2009), which results in successful gastric transit in the host (Mathara *et al*., 2008). In this study, *Lactobacillus plantarum* PCS 26 outperformed *Lactobacillus plantarum* PCS 20 and LGG strains for higher bile tolerance, suggesting that the PCS 26 strain is the most resistant and can survive better in the gastric tract. The capacity to reduce cholesterol was assessed by examining the ability to remove cholesterol from the media and deconjugate glycine-conjugated bile salts, which could also help to improve the lipid levels in the host. Reduction of soluble cholesterol in the media also appears to be related to bile salt deconjugation as deconjugation reduces solubility and causes co-precipitation of cholesterol with free bile salts (Klaver and van der Meer, 1993). *Lactobacillus rhamnosus* LGG reference strain was also able to lower cholesterol concentration in media, but had no bile salt deconjugation activity.

In addition, probiotic strains PCS 20, PCS 26 and LGG were assessed for cytotoxic effects on HIEC and HUIEC cell lines. Neither cytotoxicity crystal violet or MTT activity assays performed for determination of cell viability showed any toxic effects of all three probiotic strains on both cell lines in both experimental procedures. In experiments without the washing procedure, the presence of probiotic strains PCS 20, PCS 26 and LGG elevated the crystal violet retention in both cell lines. Since crystal violet is retained by living cells and the unattached probiotic bacteria weren't washed off, the elevated crystal violet retention could be explained by the large number of bacteria in this experimental procedure.

As judged by the modulation of gene expression, both *Lactobacillus plantarum* PCS 26 and PCS 20 strains have the potential to promote biliary cholesterol efflux or inhibit intestinal absorption through up-regulation of liver X receptors (NR1H3 and NR1H2) and subsequent regulation of their target genes. In the presence of both *Lactobacillus plantarum* PCS 26 and PCS 20 strains ABCG5 and ABCG8 expression was significantly up-regulated in HIEC and HUIEC cells. The increased expression of ABCG5 and ABCG8 reduces intestinal absorption of cholesterol and promotes biliary cholesterol secretion, thus further controlling cholesterol levels (Yu, 2002). In contrast to ABCG5/ABCG8 up-regulated expression, NPC1L1 mRNA expression was significantly down-regulated.

Previous studies have shown that NPC1L1-null mice were resistant to diet-induced hypercholesterolemia (Davis *et al.*, 2004; Davis *et al.*, 2007). In addition, *Lactobacillus plantarum* PCS 26 and PCS 20 strains also up-regulated LPL expression in HIEC and HUIEC cells. Reduction of LPL expression may result in lower plasma triglycerides as inferred from previous studies showing that high expression of LPL plays a protective role against diet-induced hyperlipidemia and atherosclerosis in mice (Yagyu *et al.*, 1999; Niho *et al*., 2005). A few studies performed by Niho and colleagues (2005) showed that the decrease in serum triglycerides, very low density lipoprotein, and low density lipoprotein, and the increment of high density lipoprotein correlated with elevated levels of LPL expression in Apc-deficient mice.

Contrary to our findings, Huang and Zheng (2010) reported a significant down-regulation of NPC1L1 mRNA levels in CaCo2 cells in the presence of *Lactobacillus rhamnosus GG*. This discrepancy could be due to two possible factors. Firstly, different cell lines were used in the two studies. HIEC and HUIEC cell lines are normal cells from human small intestinal epithelium and CaCo2 cell line is a tumor-derived cell line from human epithelial colorectal adenocarcinoma (Sambuy *et al*., 2005). The second factor is the qPCR primer design. Primer pairs used by Huang and Zheng (2010) both target the same exon (exon 2) in NPC1L1 transcripts and in present study, the primers for targeting NPC1L1 transcripts were designed to span the introns to avoid amplification of DNA in the total RNA preparation (Gibson *et al.*, 1996). Based on the results obtained by both studies, differences between cell models, established from normal and tumor-derived cell lines should be investigated further to assure appropriate cell models.

In conclusion, we found that the probiotic/protective culture *Lactobacillus plantarum* PCS 26 may act as liver X receptor agonist and thus improve the lipid profiles in hypercholesterolemic patients or even dislipidemias in complex diseases such as the metabolic syndrome. The

ability of *Lactobacillus plantarum* PCS 26 to up-regulate the intestinal LPL expression may exert a direct beneficial effect on the reduction of triglycerides and elevation of high density lipoprotein in plasma. An *in-vivo* clinical trial is warranted to assess whether the same positive outcome also occurs *in vivo* and investigate interaction and potential synergistic effects of *Lactobacillus plantarum* PCS 26 with the intestinal microbiota.

ACKNOWLEDGEMENT

The authors would like to dedicate this study to Professor Dr Avrelija Cencič, former head and founder of the Dept. of Biochemistry and Nutrition, Faculty of Medicine, University of Maribor, Slovenia. The authors have no conflicts of interest to declare. This study was supported by the Slovenian Research Agency, and Faculty of Agriculture and Life Sciences, University of Maribor, research group for food safety and health assurance (P1-0164).

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