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Lactobacillus plantarum CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes

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RESEARCH ARTICLE

Abstract

Hypercholesterolemia drives the development of cardiovascular disease, the leading cause of mortality in western society. Supplementation with probiotics that interfere with cholesterol metabolism may provide a contribution to disease prevention. *Lactobacillus plantarum* CUL66 (NCIMB 30280) has been assessed *in vitro* for its ability to impact cholesterol absorption. *L. plantarum* CUL66 tested positive for bile salt hydrolase activity and the ability to assimilate cholesterol from culture media. RT-qPCR analysis showed that the bacterium significantly decreased the expression of Niemann-Pick C1-like 1 and ATP-binding cassette transporter-1 in polarised Caco-2 cells after 6 h exposure. Conversely, the expression of ATP-binding cassette sub-family G member (ABCG)-5 and ABCG-8, and 3-hydroxy-3-methylglutaryl-CoA reductase were significantly increased. Using a radiolabelled assay, we also observed significant reductions in the uptake and basolateral efflux of cholesterol by Caco-2 cells exposed to *L. plantarum* CUL66. This *in vitro* study identified *L. plantarum* CUL66 as a cholesterol lowering bacteria by highlighting its ability to beneficially regulate multiple *in vitro* events associated with intestinal cholesterol metabolism and provides evidence of efficacy for its inclusion in future *in vivo* studies.

Keywords: hypercholesterolemia, enterocyte, *L. plantarum*, uptake, efflux

1. Introduction

Hypercholesterolemia is one of the major risk factors driving the development of cardiovascular disease (CVD) which accounts for more global mortalities than any other ailment (WHO, 2015) and costs UK healthcare systems more than £ 8.6 billion per annum (Townsend *et al.*, 2012). One of the most effective disease prevention approaches currently available is the routine use of statins which specifically inhibit the *de novo* synthesis of cholesterol (Buckley and Ramji, 2015; McLaren *et al.*, 2011; Michael *et al.*, 2012; Taylor *et al.*, 2013). However, adverse side effects (Banach *et al.*, 2015) and only an overall 25% reduction in fatal and non-fatal CVD-related events (Taylor *et al.*, 2013) indicate the need for additional approaches to reduce the burden of CVD. The lack of evidence correlating cholesterol consumption with serum cholesterol levels (Christie, 2015) has raised questions about the validity of a cholesterol restricted diet as a preventative measure suggesting that lifestyle changes alone cannot reduce susceptibility to the development of CVD. There is now growing evidence that some probiotic organisms (components of the indigenous microbiota) have the capacity to impact multiple aspects of the cholesterol metabolism of the host (Gorenjak *et al.*, 2014; Huang and Zheng, 2010; Huang *et al.*, 2010, 2013; Pereira and Gibson, 2002a,b; Yoon *et al.*, 2011, 2013) resulting in reduced serum cholesterol levels (Fuentes *et al.*, 2013; Mann, 1974; Pereira and Gibson, 2002b; Rerksuppaphol and Rerksuppaphol, 2015) and positive CVD-related outcomes (Sun and Buys, 2015).

A complex symbiotic relationship exists between the host's intestinal epithelium and the resident microbiota. The cholesterol lowering ability of some probiotics, which are defined as 'live organisms which when administered in adequate amounts confer a health benefit to the host' (FAO/

WHO, 2006), can be attributed to bile salt hydrolase (BSH) activity and/or the ability to assimilate cholesterol, that allows them to promote the removal of cholesterol from the intestinal lumen (Kumar *et al.*, 2012). It is also emerging from numerous *in vitro* and *in vivo* studies that probiotics, including strains of *Lactobacillus plantarum*, can impart cholesterol lowering effects by modulating key mechanisms responsible for cholesterol absorption in the intestines of the host (Gorenjak *et al.*, 2014; Huang and Zheng, 2010; Huang *et al.*, 2013; Yoon *et al.*, 2013).

Niemann-Pick C1-like 1 (NPC1L1) is a cholesterol transporter critical for the uptake of cholesterol and is highly expressed on the brush border of intestinal epithelial cells (Altmann *et al.*, 2004). Disruption of NPC1L1 expression in genetically modified mice renders them almost completely resistant to diet induced hypercholesterolemia (Davis *et al.*, 2007) and pharmacological inhibition of NPC1L1 function using ezetimibe is an effective strategy for the reduction of serum cholesterol level in humans (Cannon *et al.*, 2015; Sudhop *et al.*, 2002). ATP-binding cassette sub-family G member (ABCG)-5 and ABCG-8 are also key cholesterol transporters that are highly expressed on the apical surface of intestinal epithelial cells and mediate the efflux of intracellular cholesterol to bile salts back into the intestinal lumen (Tachibana *et al.*, 2007; Vrins *et al.*, 2007). Abolishment of ABCG-5/8 gene expression in mice results in the rapid accumulation of plasma cholesterol (Yu *et al.*, 2002) and functional mutations of these genes cause sitosterolemia; a genetic disorder characterised by the accumulation of sterols in blood and tissues (Berge *et al.*, 2000). ATP-binding cassette transporter-1 (ABCA-1) is also involved in the efflux of intracellular cholesterol from intestinal epithelial cells but is predominantly expressed on the basolateral membrane and preferentially utilises apolipoprotein-AI (Apo-AI) as a cholesterol acceptor (Ohama *et al.*, 2002; Tachibana *et al.*, 2007). Genetic deletion of ABCA-1 expression in mice results in reduced absorption of dietary cholesterol from the intestine (Drobnik *et al.*, 2001).

In this study, the cholesterol lowering abilities of the strain *L. plantarum* CUL66 (NCIMB 30280) isolated from a healthy human have been determined and a more detailed assessment of the molecular mechanisms revealed its ability to reduce cholesterol uptake in an *in vitro* model of the intestinal epithelium.

2. Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Bacterial strain and growth condition

Bacterial isolate *L. plantarum* CUL66 (NCIMB 30280) was stored at -20 °C on Cryo-beads (Pro-Lab Diagnostics, Wirral, UK) until use and initial cultures were grown in De Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) for 18 h at 37 °C under anaerobic conditions (10% carbon dioxide, 10% hydrogen, 80% nitrogen).

Bile salt hydrolase activity

BSH activity was tested using a modified plate assay. Briefly, MRS agar plates or MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid (TDCA) were pre-reduced by incubation under anaerobic conditions at 37 °C for 24 h. Filter discs (6 mm) present on the agar surface were then inoculated with approximately 10^7 cfu/ml *L. plantarum* CUL66. BSH activity was indicated by the development of a white precipitate around or on the colonies after 48 h incubation under anaerobic conditions at 37 °C. MRS agar plates lacking TDCA were also inoculated and served as negative controls in each experiment.

Cholesterol assimilation

The ability of *L. plantarum* CUL66 to assimilate cholesterol was assessed according to a modified version of a method described elsewhere (Pereira and Gibson, 2002a). Ten ml aliquots of sterile MRS broth supplemented with 0.3% (w/v) ox-bile and 1 mg/ml polyoxyethanyl-cholesteryl sebacate (delivering approximately 200 µg/ml cholesterol) were left uninoculated (control) or inoculated with approximately 10^8 cfu/ml *L. plantarum* CUL66. All cultures were then incubated for 18 h at 37 °C under anaerobic conditions. Following incubation, bacterial cells were removed by centrifugation (1000×*g*, 20 min) and the culture supernatant was assayed for cholesterol content. In addition, the dry weight of the remaining bacterial pellets were determined following 2 h drying at 100 °C and the amount of cholesterol removed expressed as mg of cholesterol removed (compared to the uninoculated control) per g of bacteria (dry weight).

Cholesterol assay

Total lipid was extracted from the culture supernatant and assayed for cholesterol content according to a modified version of a previously described method by Rudel and Morris (1973). Briefly, 2 ml of pure ethanol and 2 ml of 33% (w/v) potassium hydroxide were added to a 1 ml sample of culture supernatant and mixed thoroughly. Samples were then incubated at 37 °C for 15 min and cooled before 2 ml deionised water and 3 ml n-hexane were added and the mixing repeated. Samples were then incubated at ambient temperature for 15 min to allow phase separation before a 1 ml sample of the upper clear phase was removed into a clean tube for rapid evaporation under nitrogen. Two

ml of *o*-phthaldaldehyde solution (0.5 mg/ml in glacial acetic acid) was then added to each tube and 0.5 ml of concentrated sulphuric acid was then carefully added by slow pipetting down the side of the tube. Each sample was then mixed thoroughly and incubated for a further 10 min at ambient temperature. The absorbance of each sample was read at 550 nm using a UV-V spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) and cholesterol concentration was determined by comparison with a standard curve composed of identically processed samples of known cholesterol concentration (0, 31.25, 62.5, 125, 250 and 500 µg/ml, R^2=0.9975).

Caco-2 cell culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC, Middlesex, UK) and grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 4,500 mg/l glucose, 1% (v/v) non-essential amino acids, 10% (v/v) heat inactivated foetal bovine serum (Labtech, Sussex, UK), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in 5% CO_2 and 95% humidity. Caco-2 cells were seeded at 5×10^5 cells/cm^2 into standard tissue culture coated 24 well plates (Costar, Cambridge, UK) for real-time quantitative polymerase chain reaction (RT-qPCR) analysis and cholesterol uptake assays or polycarbonate semi-permeable transwell membranes (0.4 µM pores; Costar, Cambridge, UK) for cholesterol efflux assays. Caco-2 cells were maintained for 18 to 21 days to allow complete polarisation (Natoli *et al.*, 2012) and used when the trans-epithelial electrical resistance exceeded 900 Ω/cm^2 (Supplementary Figure S1A).

Caco-2/*Lactobacillus plantarum* CUL66 co-incubation

Overnight cultures of *L. plantarum* CUL66 were centrifuged (1000×*g*, 10 min) and the resultant pellet washed, by centrifugation and re-suspension, with antibiotic free DMEM supplemented with 4,500 mg/l glucose, 1% (v/v) non-essential amino acids and 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) and adjusted to 1×10^7 or 1×10^8 cfu/ml before application to Caco-2 cells. For gene expression studies, 70 µg/ml cholesterol was added to all cells (including controls). Prior to co-incubation with *L. plantarum* CUL66, Caco-2 monolayers were washed three times with warm phosphate buffered saline (PBS) to remove all traces of antibiotic.

Real-time quantitative polymerase chain reaction

Caco-2 monolayers were washed three time with warm PBS and total RNA was isolated using Ribozol (Amresco LLC, Solon, OH, USA) and was reverse transcribed into cDNA using the High Capacity cDNA reverse transcription Kit (Life Technologies, Paisley, UK) in accordance with manufacturer protocols. RT-qPCR was performed on 10 ng cDNA using the iTag Universal SYBR Green SuperMix (Bio-Rad, Hemel Hempstead, UK) in combination with 50 nM each of the following gene specific primers (Eurofins Genomics, Ebersberg, Germany): 5'-TCTTCCCCTTCCTTGCCATT-3' and 5'-CGGCAGGGTAATTGTTGAGG-3' for NPC1L1; 5'-CCCAAGGGACTCCGGGGTCA-3' and 5'-GACCC-ATGGACCCTCCGGGG-3' for ABCG-5 (Alemany *et al.*, 2013); 5'-GCCGCCCTCTTGTTCATG-3' and 5'-TAACA-TTTGGAGATGACATCCAGAA-3' for ABCG-8 (Kim *et al.*, 2013); 5'-TTTCTCAGACAACACTTGACCAAGTA-3' and 5'-GGTTTTTGTGTAATGAGAGGTCTTTTAA-3' for ABCA-1 (Kim *et al.*, 2013); 5'-GACC-TTTCCAGAGCAAGCAC-3' and 5'-TCAACAAGAGCATCGAGGGT-3' for 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and 5'-ACTCTTCCAGCCTTCCTTCC-3' and 5'-CGT-ACAGGTCTTTGCGGATG-3' for β-actin. Initial melting (95 °C for 5 min) followed by 40 cycles of melting (94 °C for 15 s), annealing (60 °C for 15 s) and extension (72 °C for 30 s) was performed using a CFX Connect™ Real-Time Instrument (Bio-Rad) and fold changes in transcript level were determined using $2\text{-}(^{\Delta Ct1 - \Delta Ct2})$, where Δ*Ct* represents the difference between the threshold cycle (*CT*) for each target gene and β-Actin mRNA transcript levels. In order to eliminate amplification from contaminating genomic DNA all primer sets spanned an exon boundary and reverse transcriptase negative controls were included in each experiment. The amplicon sizes of each primer set were confirmed by agarose gel electrophoresis.

Cholesterol uptake assay

Cholesterol uptake was measured using a previously described method with minor adaptations (Huang *et al.*, 2013). Polarised Caco-2 cells were left untreated or incubated with *L. plantarum* CUL66 (1×10^8 cfu/ml) for 6 h. One hour before the end of incubation, 0.5 µCi [4-^{14}C]-cholesterol (Amersham Plc, Amersham, UK) was added to each well. At the end of the incubation, the cells were washed three times with cold DMEM to remove unincorporated radiolabelled cholesterol. Intracellular lipids were extracted by incubation with 1.0 ml of ice cold hexane:isopropyl alcohol:water (3:2:0.1, v/v/v) for 30 min and radioactivity (disintegrations per min (DPM)) was measured using a liquid scintillation counter. Meanwhile, the remaining Caco-2 cell fraction was solubilised by incubation with 1 ml of 0.2 M NaOH for 30 min at 37 °C and total cellular protein levels determined using the BCA protein assay kit (Life Technologies, Paisley, UK) in accordance with the manufacturer's instructions. DPM were normalised to protein levels and cholesterol uptake expressed as a percentage of the untreated control that has been arbitrarily set as 100%.

Cholesterol efflux assay

Cholesterol efflux was assessed using a method adapted from a previous study (Tachibana *et al.*, 2007). Briefly, 21-day polarised Caco-2 cell monolayers grown on semi-permeable transwell inserts were incubated with [4-^{14}C]-cholesterol (0.5 µCi/well) in supplemented DMEM for 24 h. Excess cholesterol was then removed with three rounds of washing with warm PBS. Apical intracellular cholesterol efflux was initiated by the addition of 1 mM TDCA micelles alone or in combination with *L. plantarum* CUL66 (1×10^8 cfu/ml) to the apical compartment while basolateral cholesterol efflux was simultaneously initiated by the addition of 10 µg/ml Apo-AI to the basolateral compartment. After 6 h incubation the media in both the apical and basolateral compartments was removed and retained for analysis. The remaining Caco-2 cells were washed once with warm PBS and solubilised by incubation with 1 ml 0.2 M NaOH for 30 min at 37 °C. DPM was measured in the apical, basolateral and cell fractions using a liquid scintillation counter and the percentage of intracellular cholesterol effluxed from the cells was determined by dividing the radioactivity of the apical media or basolateral media by the combined radioactivity in the apical media, basolateral media and cell fraction.

Preparation of taurodeoxycholic acid micelles

TDCA micelles were prepared as described elsewhere (Ikeda *et al.*, 2002). Briefly, 0.6 mM phosphatidylcholine, 1.0 mM of oleic acid and 6.6 mM of TDCA were thoroughly mixed in a glass vial and solvents evaporated under a flow of nitrogen. The resultant lipid film was then resolved in DMEM supplemented with 4,500 mg/l glucose, 1% (v/v) non-essential amino acids and 10 mM HEPES and filtered through a 0.4 µM acetate filter before use.

Statistical analysis

All data are presented as the mean ± standard deviation (SD) of the assigned number of independent experiments. Prior to significance testing, the normality of the data and the equality of group variance was confirmed using the Shapiro-Wilk and Levene's tests respectively. All data transformations are outlined in the figure legends. For single comparisons, values of *P* were determined using Student's t-test. For multiple comparisons, values of *P* were determined using one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. All statistical tests were performed using SPSS statistical software package version 22 (IBM, New York, NY, USA). Significance was defined when $P<0.05$.

3. Results

BSH and cholesterol removal activity of *Lactobacillus plantarum* CUL66

In three independent experiments a white precipitate was observed in the presence of 0.5% TDCA (Figure 1B), that was absent from the control (Figure 1A), indicating that *L. plantarum* CUL66 has BSH activity and this observation was confirmed on streak plates (Figures 1C and 1D). *L. plantarum* CUL66 also removed 28.3±15.2% of available cholesterol from MRS broth when compared to uninoculated control cholesterol levels ($P=0.009$, Figure 1E) that equates to 3.35±1.98 mg cholesterol per g of dry weight bacteria. The average pH of 18 h broths of *L. plantarum* CUL66 was 4.1.

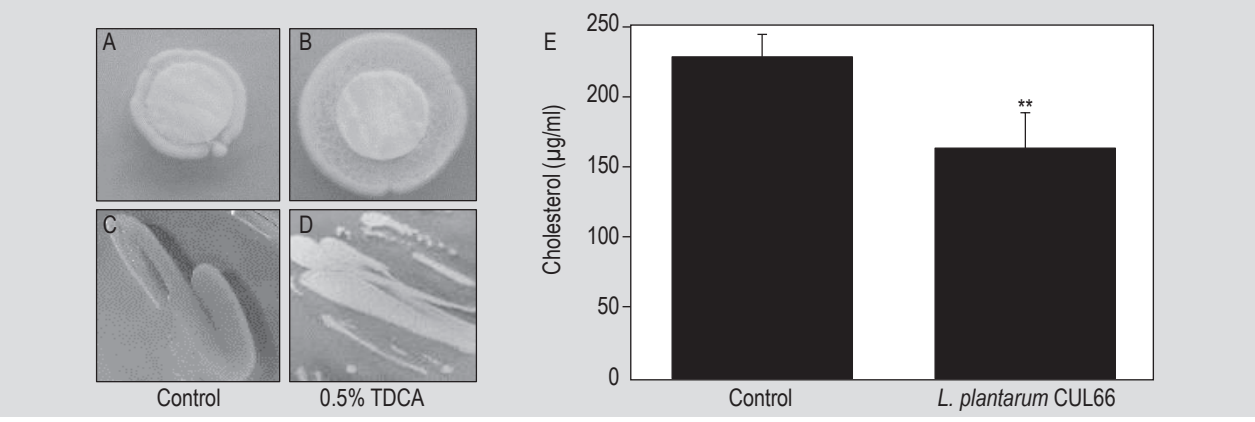

Figure 1. Bile salt hydrolase activity and ability to remove cholesterol from culture media by *Lactobacillus plantarum* CUL66. (A-D) MRS agar plates (control, A, C) or MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA) (B and D) were inoculated with *L. plantarum* CUL66 on filter discs (A and B, n=3) or as bacterial streaks (C and D, n=1) for 48 h under anaerobic conditions and assessed for the development of a white precipitate. (E) Cholesterol concentration in MRS broth containing 0.3% (w/v) ox-bile and 200 µg/ml cholesterol (control) or in MRS broth containing 0.3% (w/v) ox-bile and 200 µg/ml cholesterol inoculated with *L. plantarum* CUL66 for 18 h under anaerobic conditions. Data are presented as the mean ± standard deviation from three independent experiments. Statistical analysis was performed using Student's t-test, where ** *P*<0.001.

Expression of genes involved in Caco-2 cell cholesterol transport and metabolism

As shown in Figure 2A, co-incubation of polarised Caco-2 cells with cholesterol (70 µg/ml) and *L. plantarum* CUL66 (1×10^8 cfu/ml) resulted in a 19% (P=0.015) reduction in the expression of NPC1L1 when compared to cells treated with cholesterol alone (Control). Under the same experimental conditions the expression of ABCG-5 (Figure 2B) and ABCG-8 (Figure 2C) were significantly increased by 3.19-fold (P<0.001) and 2.18-fold (P=0.004), respectively, while ABCA-1 transcript levels (Figure 2 D) were significantly reduced (45%; P<0.001). Analysis of HMGCR transcript levels (Figure 2E) revealed a 63% (P=0.039) induction in response to 1×10^8 cfu/ml *L. plantarum* CUL66 when compared to the control. No significant changes in expression were observed for any of the genes tested in response to 1×10^7 cfu/ml *L. plantarum* CUL66 suggesting that a threshold number of viable organisms is required. There were indications of a dose-response in expression for most of the genes at the two doses tested. The viability of both Caco-2 cells and *L. plantarum* CUL66 were retained throughout the duration of the experiment (Supplementary Figures S1B and S1C, respectively). The average pH of the 6 h co-culture supernatants was 6.3.

Cholesterol uptake by Caco-2 cells inhibition by *Lactobacillus plantarum* CUL66

Reduced transcript levels of NPC1L1 (Figure 2A) suggest that *L. plantarum* CUL66 may have the ability to inhibit the uptake of cholesterol by intestinal epithelial cells. Polarised

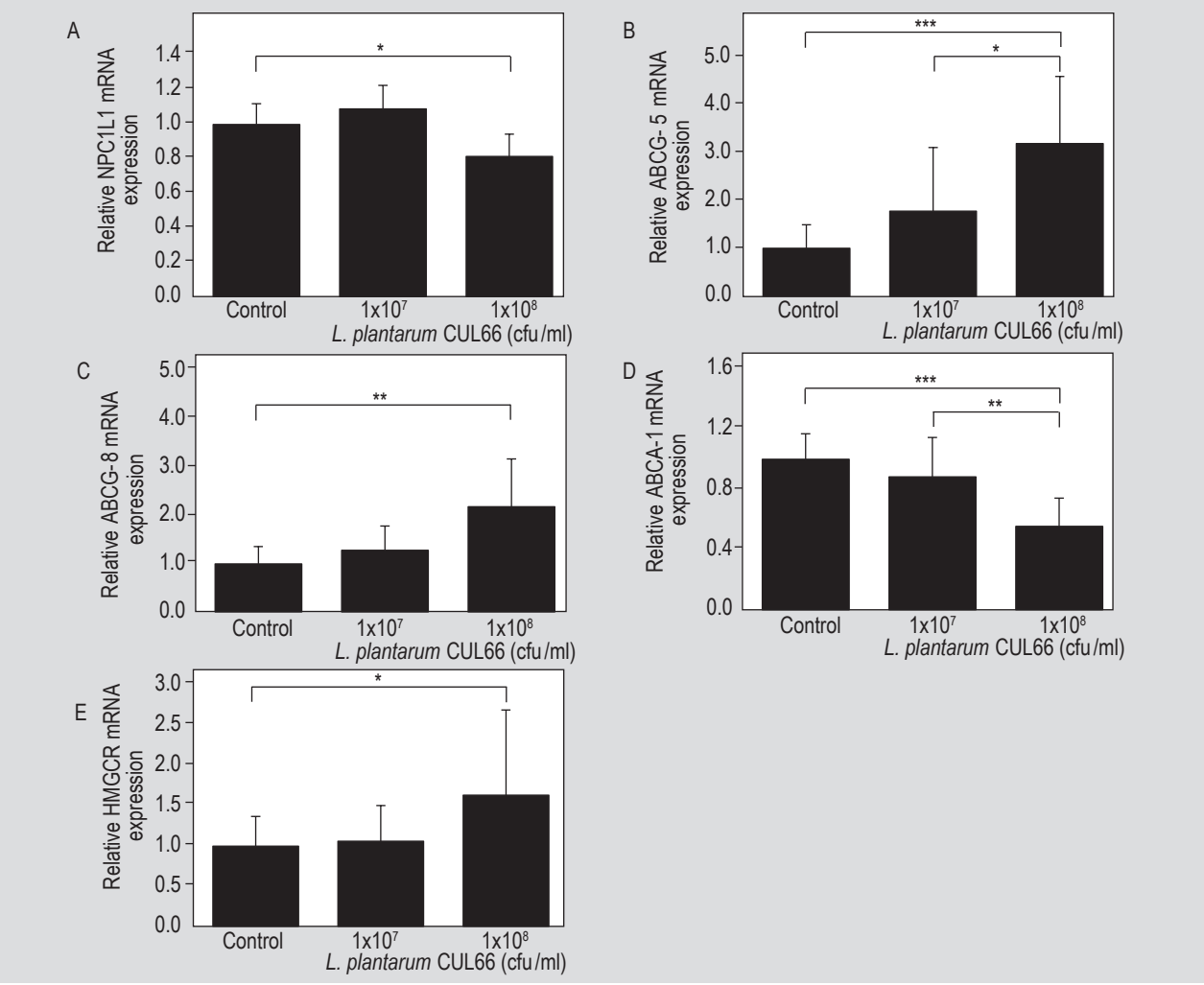

Figure 2. Gene transcript levels of (A) Niemann-Pick C1-like 1 (NPC1L1), (B) ATP-binding cassette sub-family G member (ABCG)-5, (C) ABCG-8, (D) ATP-binding cassette transporter-1 (ABCA-1) and (E) 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in polarised Caco-2 cells treated with 70 µg/ml cholesterol (Control) or cholesterol (70 µg/ml) and *Lactobacillus plantarum* CUL66 (1×10^7 or 1×10^8 cfu/ml) for 6 h. Data are presented as the mean ± standard deviation from at least four independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-hoc analysis (on log-transformed data for ABCG-5, ABCG-8 and HMGCR), where * P<0.05, ** P<0.01 and *** P<0.001.

Caco-2 cells were incubated with *L. plantarum* CUL66 for 5 h before radiolabelled cholesterol was added for 1 h. As shown in Figure 3, incubation with *L. plantarum* CUL66 significantly reduced the uptake of radiolabelled cholesterol by Caco-2 cells by 16.52% ($P<0.001$) when compared to the untreated control. The magnitude of reduction of uptake (16%) is in line with the reduced gene expression of NPC1L1 (19%).

Inhibition of cholesterol efflux into Caco-2 basolateral compartments by *Lactobacillus plantarum* CUL66

Caco-2 cells were grown on semi-permeable transwell inserts that allowed assessment of intracellular cholesterol efflux into the apical or basolateral compartments. The inoculation of polarised Caco-2 cells with *L. plantarum* CUL66 (1×10^8 cfu/ml) had no significant effect on the apical efflux of cholesterol (Figure 4A), while a significant 51.97% ($P<0.001$) reduction in the proportion of cholesterol moved to the basolateral compartment was observed in response to *L. plantarum* CUL66 when compared to untreated cells (Figure 4B). This is in line with the observed reduction in gene expression of ABCA-1 (45%).

4. Discussion

These studies have focused on the impact of *L. plantarum* CUL66 (NCIMB 30280) on cholesterol uptake/metabolism by Caco-2 epithelial cells. *L. plantarum* CUL66 was shown to remove nearly 30% of the cholesterol component from culture medium over a 24 h incubation period and was found to possess significant BSH activity. After 5 h incubation with *L. plantarum* CUL66, uptake of radiolabelled cholesterol by Caco-2 cells was significantly lower than in cells exposed to radiolabelled cholesterol without prior exposure to *L. plantarum* CUL66. *L. plantarum* CUL66 caused a significant decrease in NPC1L1 mRNA expression by the epithelial cells which is likely to contribute to these observations. The expression of both ABCG-5 and ABCG-8 was increased in Caco-2 cells in the presence of *L. plantarum* CUL66 which would favour the apical efflux of cholesterol from the intestinal epithelium into the intestinal lumen, however, no differences in the amount of radiolabelled cholesterol were observed in the apical compartment of the transwell model after 6 h exposure to *L. plantarum* CUL66. In contrast, a clear reduction in the amount of cholesterol efflux from Caco-2 cells into the basolateral compartment was observed after 6 h exposure to *L. plantarum* CUL66 and occurred alongside reduced expression of cholesterol transporter ABCA-1. Significantly elevated transcript levels of HMGCR; the rate limiting enzyme during the *de novo* synthesis of cholesterol, were also observed in Caco-2 cells exposed to *L. plantarum* CUL66.

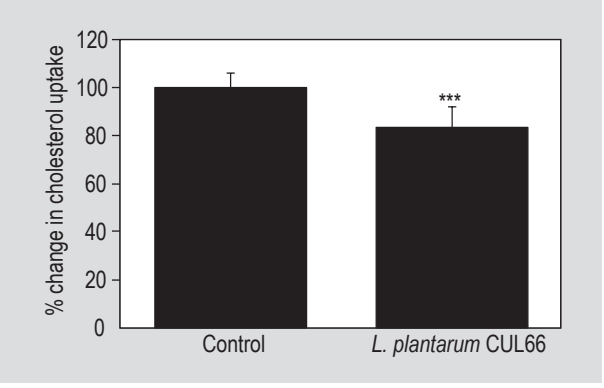

Figure 3. *Lactobacillus plantarum* CUL66 inhibits the uptake of cholesterol by polarised Caco-2 cells. Polarised Caco-2 cells were untreated (Control) or incubated with *L. plantarum* CUL66 (1×10^8 cfu/ml). Intracellular radioactivity was normalised to total protein content and presented as a percentage of the control that has been arbitrarily assigned as 100%. Data are presented as the mean ± standard deviation from three independent experiments. Statistical analysis was performed using Student's t-test, where *** $P<0.001$.

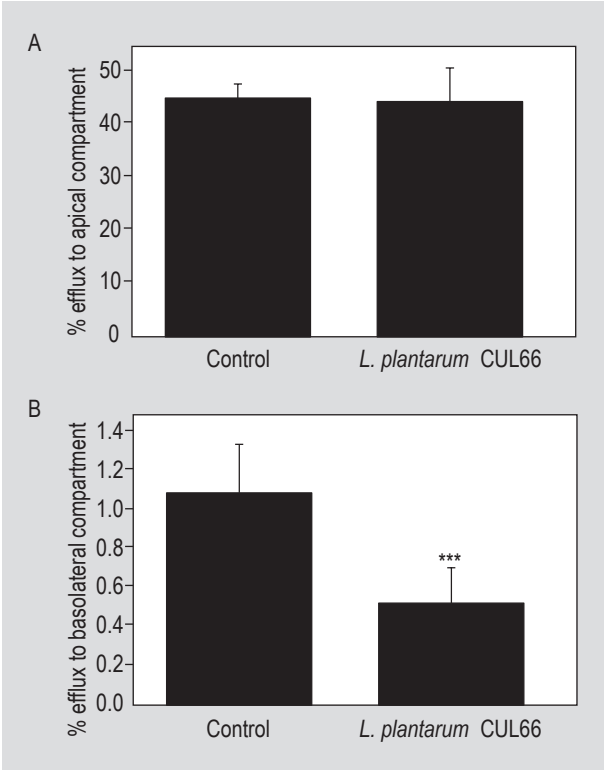

Figure 4. Influence of *Lactobacillus plantarum* CUL66 on the efflux of (A) intracellular radiolabelled cholesterol to taurodeoxycholic acid micelles in the apical compartment and (B) apolipoprotein-AI in the basolateral compartment of polarised Caco-2 cells. Polarised Caco-2 cells were untreated (Control) or incubated with *L. plantarum* CUL66 (1×10^8 cfu/ml). Data are presented as mean ± standard deviation from at least three independent experiments. Statistical analysis was performed using Student's t-test, where *** $P<0.001$.

The cholesterol lowering activities of probiotic bacteria have been demonstrated and a diversity of bacterial strains have been identified (Fuentes *et al.*, 2013; Guo *et al.*, 2011; Sun and Buys, 2015). BSH activity has been observed in various strains of *L. plantarum* and cholesterol lowering activity has been identified in a diversity of probiotic organisms at levels comparable with *L. plantarum* CUL66 (Gorenjak *et al.*, 2014; Pereira and Gibson, 2002a; Tanaka *et al.*, 1999; Tomaro-Duchesneau *et al.*, 2014). Variations in the levels of cholesterol uptake were observed with *L. plantarum* CUL66 as was seen by Pereira and Gibson for a range of bacterial strains (Pereira and Gibson, 2002a). In our *L. plantarum* CUL66 study the pH was not controlled (final pH of 4.1) and it has been found that cholesterol can precipitate out of culture media with conjugated bile salts when the pH drops below 5.5 (Klaver and Van der Meer, 1993) which could have potentially made some contribution to the outcome. It is generally accepted that *in vitro* cholesterol assimilation can be used as an important predictor of *in vivo* cholesterol lowering ability although Madani *et al.* (2013) found little correlation to support this assumption. However, work by Mahenthiralingam *et al.* (2009) has clearly demonstrated the ability of lactic acid bacteria to survive transit through the human gut suggesting that *L. plantarum* CUL66 could be expected to be viable in the intestine, and non-viable bacteria have been shown to take up cholesterol (Liong and Shah, 2005; Wang *et al.*, 2014; Zeng *et al.*, 2010) which may suggest the involvement of other probiotic-mediated cholesterol lowering mechanisms.

To this end, we have shown that *L. plantarum* CUL66 can inhibit NPC1L1 gene expression and reduce cholesterol uptake by polarised Caco-2 cells. These observations are corroborated by *in vitro* and *in vivo* studies that report similar changes in NPC1L1 gene expression in response to *L. plantarum* strains PCS20, PCS26, NR74 or Lp27 (Gorenjak *et al.*, 2014; Huang *et al.*, 2013; Yoon *et al.*, 2013) that manifest as reduced cholesterol uptake by NR74 treated Caco-2 cells (Yoon *et al.*, 2013) and reduced plasma cholesterol levels in Lp24 fed hypercholesteremic rats (Huang *et al.*, 2013). The ability of *L. plantarum* CUL66 to regulate cholesterol transport by Caco-2 cells may also extend to the regulation of cholesterol efflux in light of the increased levels of ABCG-5 and ABCG-8 transcripts observed in our system. Similar observations have been made in response to *L. plantarum* strains PCS20, PSC26 or NR74 in numerous intestinal epithelial cell types (Gorenjak *et al.*, 2014; Yoon *et al.*, 2011) that, in the case of *L. plantarum* NR74, correlated with a clear increase in apical cholesterol excretion from non-polarised Caco-2 cells (Yoon *et al.*, 2011). While we did not observe similar changes in cholesterol efflux into the apical compartment of our transwell model we did observe clear reductions in ABCA-1 expression and cholesterol efflux from Caco-2 cells to the basolateral compartment in response to *L. plantarum* CUL66 and are therefore the first to suggest that the ABCA-1/ApoA-I pathway can be regulated by such bacteria in enterocytes.

In addition, we also observed an increase in the expression of HMGCR in response to *L. plantarum* CUL66 that contrast the findings of another study showing reduced HMGCR expression in Caco-2 cells exposed to a mixed culture of *Escherichia coli* 6-1 and *L. plantarum* (ATCC 202195) (Panigrahi *et al.*, 2007). Interestingly, increased intestinal expression of HMGCR has also been observed in numerous *in vivo* experiments examining the molecular event associated with ezetimibe-mediated inhibition of cholesterol absorption (Catry *et al.*, 2015; Engelking *et al.*, 2012; Repa *et al.*, 2005; Telford *et al.*, 2007; Valasek *et al.*, 2008) and is thought to form part of a sterol regulatory element-binding protein-dependent compensatory mechanism by the host to maintain cholesterol levels (Catry *et al.*, 2015; Engelking *et al.*, 2012; Telford *et al.*, 2007).

In summary, this study reports the ability of *L. plantarum* CUL66 to beneficially regulate multiple *in vitro* models associated with intestinal cholesterol absorption and therefore implicates it as a holistic approach to reduce serum cholesterol levels via the intestine. To date, no other studies have reported the ability of *L. plantarum* CUL66 to deconjugate bile salts and assimilate cholesterol *in vitro*, nor its ability to beneficially regulate cholesterol transport across polarised Caco-2 cells through the coordinated inhibition of intestinal cholesterol uptake and basolateral cholesterol efflux. This study provides adequate evidence of efficacy for the inclusion of *L. plantarum* CUL66 in future *in vivo* studies.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2015.0146.

Figure S1. Trans-epithelial electrical resistance of cultured Caco-2 cells and the cell viability under experimental conditions.

Conflict of interest

This study was supported by Cultech Ltd, Port Talbot, UK. DRM, DLC, IG and SFP are employees of Cultech Ltd. JWEM is a PhD student funded by a joint studentship from the School of Biosciences, Cardiff University and Cultech Ltd.

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