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Identification of lactic acid bacteria strains modulating incretin hormone secretion and gene expression in enteroendocrine cells

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Short Title: Lactic acid bacteria modulating incretin hormones.
Abstract

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones released from intestinal enteroendocrine (EE) cells and have well-established glucose-lowering actions. Lactic acid bacteria (LAB) colonise the human intestine but it is unknown whether LAB and EE cells interact. Acute co-culture of LAB with EE cells showed that certain LAB strains elicit GLP-1 and GIP secretion (13-194-fold) and upregulate their gene expression. LAB-induced incretin hormone secretion did not appear to involve nutrient mechanisms, nor was there any evidence of cytolysis. Instead PCR array studies implicated signalling agents of the toll-like receptor system, e.g. adaptor protein MyD88 was decreased 23-fold and cell surface antigen CD14 was increased 17-fold.

Mechanistic studies found that blockade of MyD88 triggered significant GLP-1 secretion. Furthermore, blocking of CD14 completely attenuated LAB-induced secretion. A recent clinical trial clearly shows that LAB have potential for alleviating type 2 diabetes and further characterisation of this bioactivity is warranted.

Keywords: probiotic, lactobacilli, diabetes, incretin hormones, enteroendocrine cells
1. Introduction

The incretin hormones are gastrointestinal insulin-releasing peptides involved in the regulation of postprandial nutrient homeostasis. Postprandial release of these hormones forms part of the entero-insular axis which contributes significantly to normal glucose homeostasis, particularly in the period following the consumption of a meal (Flatt & Green, 2006; Baggio & Drucker, 2007; Green et al., 2005). The two established incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) and they are produced by enteroendocrine (EE) cells lining the intestine. GLP-1 is produced by intestinal L-cells which are at the highest densities in the distal small intestine and colon. GIP is produced and secreted by K-cells which are predominantly located in the proximal small intestine (Baggio & Drucker, 2007). It is also evident that EE cells with an L/K phenotype exist and a shift of the intestinal cell population towards this type has been associated with the prevention of beta-cell loss and hyperglycaemia in diabetic animal models (Speck et al., 2011). The incretin hormones have been the basis for a number of clinically approved pharmaceutical compounds with good efficacy for the treatment of human type 2 diabetes and its complications (Neumiller, 2012; Tate et al., 2015]. Importantly their use has been associated with low risk of hypoglycaemia and good tolerability and safety.

A novel and perhaps more radical approach involves the discovery of gut probiotic organisms capable of modulating the incretin hormone system (Yadav et al., 2013; Forssten et al., 2013; Duan, Liu, & March, 2015). Probiotic bacteria routinely come into close proximity with the intestinal lining allowing the possibility that either they or their bacterial metabolites could stimulate the secretion of incretin hormones from enteroendocrine cells. Lactobacilli are present in the small intestine, although cell densities (10^4 to 10^8 cfu/g) are lower than in the large intestine (10^{12-14} cfu/g) (Ley, Peterson, & Gordon, 2006; Walter &
Ley, 2011). Gut microbiota are diverse and abundant constituting approximately $10^{14}$ (100 trillion) cells in an individual person (Ley, Peterson, & Gordon, 2006). They contribute significantly to human nutrition and health (Flint et al., 2012) playing roles in immunity (Hardy et al., 2013; Kelly & Mulder, 2012), the fulfilment of dietary amino acid requirements (Walter & Ley, 2011) and they impact on energy balance (Molinaro et al., 2012; Cani et al., 2012). Besides these physiological effects, interaction with gut epithelial surface elicits several signalling pathways (Audy et al., 2012; Giahi et al., 2012) that are responsible for regulation of the aforementioned functions. Probiotic-based dietary intervention has been proposed for the alleviation of various clinical conditions including gastrointestinal disorders (Horvath & Szajewska, 2013; Hijova & Soltesova, 2013), ulcerative colitis (De Greef et al., 2013; Dylag et al., 2014), necrotizing enterocolitis (Liu et al., 2013), respiratory disorders (Forsythe, 2011) and allergies (Prakash et al., 2013; Castellazzi et al., 2013). The proposed use of probiotics for the alleviation of diabetes and/or obesity is unestablished but is a hotly debated topic (27-29, 9 Sanz, Santacruz, & Gauffin, 2010; Ejtahed et al., 2012; Panwar et al., 2014; Duan, Liu, & March, 2015).

The aim of this study was to probe the ability of one genus of lactic acid bacteria (LAB) to modulate the secretion and gene expression of the incretin hormones in EE cells. The strains investigated included *Lactobacillus* isolates originating from human infant faeces and a number of *Lactobacillus* reference cultures. For each strain we examined how co-culture with pGIP/Neo STC-1 cells affected GLP-1 secretion, GIP secretion, as well as, changes in the expression of proglucagon (the precursor of GLP-1) and GIP genes. The most promising *Lactobacillus* organism was then used to investigate possible mechanisms through which it exerted effects on EE cells.

2. Materials and methods
2.1 Chemicals and reagents

De Man, Rogosa and Sharpe (MRS) broth (M369) was obtained from HiMedia Laboratories (Mumbai, India). Mueller-Hinton broth (CM0405) from Oxoid (Hampshire, UK). Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.5 g/l D-glucose, without sodium pyruvate (GlutaMAX) was obtained from GIBCO, Paisley, UK. Penicillin, streptomycin and geneticin (G418) were purchased from Sigma (Poole, Dorset, UK). Radioiodinated GLP-1 was obtained from Perkin Elmer (Waltham, MA, USA). GIP ELISA kits were purchased from Millipore (Billerica, MA, USA). Cytotoxicity Detection Kit PLUS (LDH) kits were purchased from Roche Diagnostics Ltd (West Sussex, UK).

2.2 Isolation, culture and Identification of Lactobacillus strains

Faecal samples were collected from five healthy breast-fed infants <9 months in age living in Shamli, Uttar Pradesh, India. In each case parental consent was obtained. Lactobacillus cultures were isolated from faecal samples of healthy human infants (Lb1-15; Table 1). Lactobacillus reference strains (Ref1-7; Table 1) and a Gram positive control (Bifidobacterium bifidum; Ctrl1; Table 1) were obtained from the National Collection of Industrial, Food and Marine Bacteria (Aberdeen, UK). E. coli K12 (Ctrl 2; Table 1) was procured from National Collection of Type Cultures (NCTC) (Colindale, London). Identity of Lactobacillus isolates was determined to genus level by PCR using a genus-specific primer pair (Table 2). Amplified products (Table 2; 1400bp for 16SrRNA and 600bp for Phe) were sequenced using an external DNA sequencing service (DNA Sequencing and Services, University of Dundee, UK).

In preparation for experiments bacterial cultures were grown overnight (37°C) in their respective media (10 ml), harvested (12,000g, 15min, 10°C) and washed twice with 1X PBS. Cell pellets were again re-suspended in 1X PBS and diluted to O.D600 1.5 which
corresponded to $1 \times 10^9$ cfu/mL of viable cells as determined by standard viable count method (Wehr & Frank, 2004). One millilitre of bacterial culture at $O.D_{600} 1.5$ was pelleted down and re-suspended in 600 µl of freshly prepared HEPES buffer (pH7.4) for co-culture with pGIP/Neo STC-1 cells.

### 2.3 Cell Culture

pGIP/Neo STC-1 cells were a gift from Dr. B. Wice (Washington University of St. Louis) (Ramshur, Rull, & Wice, 2002) with permission from Dr D. Hanahan (University of California, San Francisco, CA). pGIP/Neo STC-1 cells are a GIP enriched sub-clone of heterogeneous pluripotent murine STC-1 cells. The cell line secretes measurable amounts of GLP-1 and GIP, retains secretory function and is responsive to various stimuli (Gillespie et al. 2015; Jafri et al. 2016). Cells were cultured as previously described (Hand, Giblin, & Green, 2012; Rafferty et al., 2011). Briefly, they were maintained in a humidified incubator at 37°C and 5% CO₂ DMEM containing 4.5 g/L with L-glutamine, without sodium pyruvate (Life Technologies, Paisley, UK) and supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 mg/L streptomycin and geneticin - G418, 400 µg/mL. Cells were trypsinised at 70-80% confluency and seeded in flasks or plates as required, and only used between 20-50 passages.

Light microscopy of *L. rhamnosus* and pGIP/Neo STC-1 cells was carried out by fixing with methanol (10 min at room temperature), removing methanol, staining with crystal violet for 30s and washing twice immediately with PBS buffer. Plates were allowed to air dry and viewing using a confocal light microscope (Nikon, Surrey, UK).

### 2.4 GLP-1 and GIP secretion studies
For hormone secretion and gene expression studies approximately $2 \times 10^6$ pGIP/Neo STC-1 cells were seeded into 12-well plates with DMEM and allowed to attach overnight ($37^\circ C; 5\% CO_2$), media was removed and cells were washed (3 times; HEPES buffer) 

(McCarthy et al., 2015). Cells were pre-incubated in 1 ml of HEPES buffer for 1h. Buffer was removed and cells were co-cultured with $2 \times 10^9$ live bacteria for 3h ($37^\circ C; 5\% CO_2$). Cell supernatant (HEPES Buffer) was aspirated and collected in a fresh tube, placed on ice and centrifuged (5000g, 5 min) to remove any cellular debris. Supernatant was collected and stored at -70$^\circ$C prior to GLP-1 and GIP immunoassays. mRNA was isolated from cells using a commercial RNeasy Mini Kit (Quigen, Manchester, UK). Additional GLP-1 secretion studies (3h; $37^\circ C; 5\% CO_2$) were performed with a mixture of L-alanine (20 nmol/L), L-histidine (20 nmol/L) and L-proline (10 nmol/L). Studies were also conducted with L. rhamnosus ($2 \times 10^9$ CFU/mL) alone or in combination with either a Myd88 blocking peptide (50µM; Pepinh-MYD, Invivogen, Toulouse, France), or an anti-CD14 antibody (anti-mouse IgG, Cambridge Biosciences, Cambridge, UK). To ensure that hormone measurements were not the result of cytolysis the release of lactate dehydrogenase (LDH) was measured in a series of experiments where, $10^{10}$, $10^9$, $10^8$, $10^7$ or $10^6$ LAB were co-cultured with $2 \times 10^6$ pGIP/Neo STC-1 cells for 3h. No cytolysis was detected. GIP concentrations were determined by commercial competitive ELISA kit (Phoenix pharmaceuticals, Inc. California, USA) by following the manufacturer’s instructions. GLP-1 concentrations were measured using an in-house fully optimised radioimmunoassay which used anti-rabbit IgG Sac-Cel (IDS, Boldon, UK) and had zero cross-reactivity with glucagon or GIP. GLP-1 and GIP secretion studies were performed in triplicate.

2.5 Amino acid analysis
Samples of test buffer (3 ml) were spiked with 0.3 ml Norleucine (1.5 mg/ml; internal standard) and mixed in ddH₂O (10ml) for 1 min. Samples were then centrifuged (3,500g, 4°C, 25 min) and the supernatant collected. Pellets were re-suspended in ddH₂O (5 ml), centrifugation was repeated and both supernatants were combined. The supernatant (500 µl) was filtered through a molecular weight cut off filter (Vivaspin, MWCDO 3000, Sigma) with centrifugation at 3,500g for 90 min at 4°C. The filtered sample (100 µl) was analysed using an Agilent GC (model 7890, Delaware, USA) coupled to an MS detector (Agilent model 5975C, Delaware, USA) in combination with an amino acid analysis kit (EZ:faast; Phenomenex, Cheshire, UK).

2.6 Gene expression studies and real time PCR array

SYBR green Quantitative real-time PCR was used to determine changes in gene expression with β-actin used as a reference gene to normalise data. RNA quality and quantity were checked by nanodrop/spectrophotometric (260/280) analysis and gel electrophoresis (1% agarose), respectively. RNA (1µg) was converted to cDNA using commercial QuantiTect Reverse Transcription Kit (Qiagen) and was quantified using nanodrop. cDNA was diluted to working dilution of 30ng/µl by dissolving in nuclease free water. Primer sequences for proglucagon (GLP-1), GIP, β-actin, GPR40, GPR 41 and GPR 120 can be found in Table 2. RT² Profiler PCR arrays were used to detect the expression of 84 genes implicated in regulating TLR pathways. For PCR array, RNA was further purified using SABiosciences RT² qPCR-Grade RNA Isolation Kit according to the manufacturer’s protocol. RNA quality was analysed and met the required criteria for Real-time PCR arrays. Mouse TLR PCR array kits were purchased from Qiagen (RT Profiler™ PCR Array Mouse Toll-Like Receptor Signalling Pathway [PAMM-018A-2]). The kit profiles the expression of 84 genes (n=2 biological replicates) related to TLR-mediated signal transduction and five
housekeeping genes (GUSB, HPRT1, HSP90ab1, GADPH and ACTB). A negative control for genomic DNA and contaminating RNA was also conducted in each sample. Amplification, data acquisition, and the melting curve were carried out using a Mastercycler ep Realplex (Eppendorf, Stevenage, UK). The PCR cycling program was set as follows: stage 1: 95°C for 10 min, stage 2: 95°C for 15 sec followed by 60°C for 1 minute repeated for 40 cycles, and stage 3: 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The cycle threshold (Ct) and melting curve of each gene were established and recorded by the software. The delta Ct (ΔCt) method was used for PCR array data analysis. The normalized ΔCt for each gene of interest (GOI) was calculated by deducting the average Ct of the 5 housekeeping genes (HKG) from the Ct of each gene of interest. Then the double delta Ct (ΔΔCt) for each gene of interest was calculated by deducting the average ΔCt in the control group from the ΔCt of each gene of interest. The fold-change of each GOI compared to the sham group was calculated as $2^{-\Delta\Delta Ct}$.

2.7 Data analysis

Graphs were produced and statistically analysed using Graph pad Prism (Version 6, La Jolla, CA, USA). Bar graphs display mean ± SEM. A heat map of PCR array data was generated (MetATT) which employed mean centred data normalisation.

3. Results

3.1 GLP-1 and GIP secretion following Lactobacillus co-culture

Co-culture of a number of Lactobacillus strains with pGIP/Neo STC-1 cells elicited significant GLP-1 secretion which was not associated with cytotoxicity or cytolysis. Cells incubated in a non-stimulatory vehicle control secreted 4.5±0.5 pM/10^6 cells/h whereas 3h co-culture with faecal isolate, Lb3 (later identified as Lactobacillus plantarum subsp.
argentorotensis; KC491380) secreted 86.8±6 pM/10⁶ cells/h (Figure 1A). For GIP, secretion
of 1.9±0.05 pM/10⁶ cells/h occurred with a non-stimulatory vehicle control. Two faecal
isolate strains: Lb1 (later identified as being Lactobacillus plantarum) and Lb3 stimulated
significant GIP secretion (Figure 1B; 100.6±2.9 and 155.8±24.9 pM/10⁶ cells/h, respectively.
Co-culture with two reference strains L. johnsonii (NCIMB8795) and L. rhamnosus
(37x704)(NCIMB6375) significantly increased both GLP-1 secretion (Figure 1A; 61.0±8.4 and
82.3±26.1 pM/10⁶ cells/h, respectively) and GIP secretion (Figure 1B; 369.5±68.9 and
285.7±34.7 pM/10⁶ cells/h, respectively). The Gram positive (B. bifidum) and Gram negative
(E. coli) control organisms did not stimulate any incretin hormone secretion.

3.2 Changes in incretin hormone gene expression following Lactobacillus co-culture

A number of Lactobacillus strains affected the levels of gene expression of
proglucagon and GIP in pGIP/Neo STC-1 cells. Two Lactobacillus isolates Lb4 and Lb6
(both identified as Lactobacillus plantarum) upregulated proglucagon gene expression 3.6-
and 2.5-fold, respectively (Figure 2A). Four reference strains L. acidophilus
(NCIMB701748), L. casei (NCIMB4114), L. plantarum (NCIMB1406) and L. rhamnosus
(NCIMB6375) significantly increased proglucagon gene expression (Figure 2A; 2.9-, 1.8-, 1.9- and 2.9-fold, respectively). Interestingly, B. bifidum up-regulated GLP-1 proglucagon
gene expression 2.1-fold. Four Lactobacillus isolates Lb4, Lb6, Lb8 and Lb9 (all
Lactobacillus plantarum) along with three reference cultures (L. casei, L. plantarum and L.
rhamnosus) significantly up-regulated GIP gene expression (Figure 2B; 2.5-, 2.7-, 2.3, 2.2-, 2.4-, 3.2- and 5.4-fold, respectively). The Gram-negative bacterium E. coli did not affect
either proglucagon or GIP gene expression.

3.3 Nutrient-related mechanisms involved in Lactobacilli-stimulated GLP-1 secretion
As a particularly potent enhancer of GLP-1/GIP secretion and gene expression \( L. \)\( rhamnosus \) was selected for further studies. Changes in the amino acid composition of the test buffer were examined by GC-MS (Figure 3A) which indicated that there was a significant increase in the levels of L-alanine, L-proline and L-Histidine. However, a combination of these three amino acids failed to stimulate GLP-1 secretion in STC-1pGIP/Neo cells (Figure 3B). The effects of \( L. \)\( rhamnosus \) co-culture on the expression of free fatty acid (FFA) receptors (GPR40, 41 and 120) were examined. These were compared against \( L. \)\( casei \), which did not stimulate incretin hormone secretion but did alter incretin gene expression. \( L. \)\( rhamnosus \) modestly increased the expression of GPR40 (2.4±1.4-fold) and decreased GPR120 (0.4±0.01-fold) and had no effect on GPR41. By comparison \( L. \)\( casei \) upregulated GPR-40 by 6.7±1 and GPR-41 by 28.0±4 fold and left GPR120 unchanged. The isolate Lb-3 was also examined (data not shown) and it did not affect the expression of any of the three FFA receptors.

3.4 Molecular mechanisms involved in \( Lactobacilli \)-stimulated GLP-1 secretion

Confocal light microscopy (Figure 4A) demonstrated that \( L. \)\( rhamnosus \) cells (purple) are closely localised to pGIP/Neo STC-1 cells (blue), perhaps even adhering to the cell surface. A mouse PCR array examined the effect of \( L. \)\( rhamnosus \) co-culture on the expression of 84 genes related to Toll-like receptor signalling pathways (Figure 4B). A full list of the genes affected can be found in Supplementary Table 1. Whilst up-regulation in the expression of some genes was evident the majority were down-regulated following \( L. \)\( rhamnosus \) co-culture (Figure 4B). Some of the most profound changes in expression occurred in genes identified as “Adaptors & Interacting Proteins” (Supplementary Table 1). Most notably CD14 expression was up-regulated most (17.5-fold) and Myd88 was down-regulated greatest (23.4-fold). The application of the MyD88 blocking peptide (Pepinh-
MYD) alone evoked a significant GLP-1 secretory response (Figure 4C; 2.3-fold; P<0.001), but Pepinh-MYD did not significantly affect *L. rhamnosus*-stimulated GLP-1 secretion. No GLP-1 secretory responses were evident when an antibody directed against the cell surface antigen CD14 (anti-CD14) was applied alone or in combination with *L. rhamnosus* (Figure 4C).

4. Discussion

This study is the first to demonstrate that lactic acid bacteria can interact with physiologically important intestinal cells. The EE cells collectively constitute the largest endocrine system in the body, producing and secreting a range of different gastrointestinal hormones. Co-culturing of EE cells with various *Lactobacillus* strains/isolates clearly affected the extent to which the cells secrete and express the incretin hormones. We have identified novel bacterial isolates which modulate the secretion and expression of both GLP-1 and GIP. For example *L. plantarum* subsp. *argenterotensis* (Lb3) which triggered potent GLP-1 and GIP secretion in pGIP/Neo STC-1 cells. Various isolates identified as strains of *L. plantarum* (i.e. Lb1, 4, 6, 8, and 9), along with the corresponding reference culture (Ref6), positively influenced either incretin hormone secretion or incretin gene expression (but not both simultaneously). 16S rRNA sequencing revealed none of the *L. plantarum* isolates to be genetically identical, which may explain why their effects on EE cells were inconsistent. Indeed there were some *L. plantarum* isolates (e.g. Lb2, Lb5) which had no impact on incretin secretion or expression.

There were similar observations with *L. acidophilus* where the isolate Lb15 had no appreciable effects, yet the corresponding reference culture (Ref1) significantly up-regulated proglucagon gene expression. The reference culture of *L. johnsonii* showed particularly promising effects on incretin hormone secretion but did not affect gene expression. Only the
reference culture of *L. rhamnosus* positively influenced all four incretin hormone parameters which prompted us to select it for further investigation. It is well established that incretin hormone secretion can be stimulated by the presence of either amino acids or fatty acids in the lumen of the intestine (Baggio & Drucker, 2007). Therefore, we examined whether *L. rhamnosus* was influencing GLP-1 secretion through a nutrient-based mechanism. The bacterial metabolism of *L. rhamnosus* appeared to elevate the levels of three amino acids (L-alanine, L-proline and L-histidine) in the test buffer, yet when tested these amino acids did not stimulate GLP-1 secretion. It is well known that *Lactobacillus* spp. (including *L. rhamnosus*) can produce various fatty acids, most notably short-chain fatty acids (SCFAs) such as butyrate (Umeki et al., 2004; Licciardi et al., 2010). Fatty acids are known to be secretagogues of GLP-1 and GIP. We found that FFA receptor expression (GPR40, 41 and 120) in EE cells was affected by co-culture with lactobacilli. *L. rhamnosus* modestly increased mRNA transcripts of one medium/long chain FFA receptor (GPR40) but reduced that of another medium/long chain FFA receptor (GPR120). However, it was clear that *L. rhamnosus* did not affect the expression of the SCFA receptor GPR41 and when measured the levels of butyrate in the culture medium were unaffected (data not shown) indicating that production of SCFAs was unlikely to be responsible for observed effects on EE cells. There is a potential limitation in this study - that changes in other FA receptors expressed on enteroendocrine cells (such as GPR119 for example) were not examined. A role for medium/long chain fatty acids cannot be completely ruled out, however, it is clear that *L. casei* (which is devoid of incretin secretory activity) had more profound effects than *L. rhamnosus* on FFA receptor expression (i.e. GPR40 and 41).

In a separate phase of studies we attempted to ascertain whether *L. rhamnosus* could be influencing GLP-1 secretion through its direct interaction with the EE cell surface. This
was prompted by the observation that in co-culture the majority of *L. rhamnosus* cells closely co-localise with pGIP/Neo STC-1 cells, even when cells were seeded at lower densities. We thought that the most logical mechanism for a bacterial-mammalian cell interaction was through the toll-like receptor (TLR) family of pattern recognition receptors which detect a wide range of exogenous factors including bacteria, viruses, fungi and parasites (Kamdar, Nguyen, & DePaolo, 2013). A qPCR array measuring the expression of 84 TLR-related genes (See Supplementary Table 1) was performed. This produced quite startling results – there was a broad (but not exclusive) down-regulation of the genes in the TLR family, some of which were reduced by more than 20-fold. TLR2 and TLR4 receptors were significantly down-regulated, but some of the biggest changes were in the expression of adaptor proteins involved in TLR signalling. These included the cell surface antigen CD14 which was increased almost 18-fold and the adapter protein MyD88 which was decreased 23-fold.

These two proteins were tentatively investigated for their potential involvement in *L. rhamnosus*-induced stimulation of GLP-1 secretion. Interestingly, we found that the addition of pepinh-MYD (which blocks the homo-dimerisation of MyD88) alone caused significant GLP-1 secretion. Importantly pepinh-MYD did not have an additive effect on *L. rhamnosus*-induced GLP-1 secretion. This finding suggests that down-regulation of MyD88 expression/activity leads to higher levels of GLP-1 secretion, although it cannot be definitively stated that this is the precise mechanism for *L. rhamnosus*-stimulated GLP-1 secretion. We also found that the application of an antibody directed against murine CD14 alone had no effect on GLP-1 secretion but it significantly attenuated *L. rhamnosus*-stimulated secretion. CD14 plays a key role in initiating cell activation by a range of bacterially-derived molecules, such as the lipopolysaccharides from Gram-negative bacteria and peptidoglycans from Gram-positive and Gram-negative bacteria (Dziarski, Tapping,
Tobias, 1998). It could be postulated that CD14 is a surface antigen which facilitates adhesion of *L. rhamnosus* peptidoglycans to the EE cell surface, but the exact signalling role (if any) for eliciting incretin hormone secretion clearly requires further investigation. CD14 is best characterised as a feature of monocytes and macrophages with most subpopulations of these cells expressing CD14. The interaction of commensal bacteria with the gut lining is an incredibly understudied area and there is presently very little scientific literature elucidating the role of CD14 in the intestine. It has been shown however, that an *E. coli* probiotic organisms used in the treatment of inflammatory bowel disorders stimulated the gene expression of CD14 in the Caco-2 intestinal epithelial cell line (Hafez et al., 2010). Although this study did also report that the expression of the adaptor molecules MyD88 and Ticam1 (TRIF) was up-regulated which differs from the present study (Hafez et al., 2010).

Currently, the investigation of the functional and physiological actions of probiotics is an extremely active research field, and many health benefits are proposed including the improvement of gastrointestinal function and lowering of blood cholesterol levels (Macfarlane & Cummings, 1999). Their incorporation into fermented and non-fermented dairy products is well accepted, and their inclusion in functional foods such as e.g. fruit juices, breakfast cereals, cereal bars, etc. has also been investigated. There is growing support for the concept of utilising probiotic organisms as a dietary prophylactic or therapeutic strategy for type 2 diabetes mellitus (Yadav et al. 2013; Panwar et al., 2014; Panwar et al., 2016). The concept has been demonstrated in a recent clinical trial which found that administration of the probiotic *L. reuteri* increased insulin secretion and incretin release in humans (Simon et al., 2015). This group concluded that since *L. reuteri* did not modulate faecal microbiota it is likely that *Lactobacillus* spp. have a direct effect on host physiology – a proposition which this study supports.
5. Conclusion

In conclusion, this study has shown that there is considerable potential to increase endogenous GLP-1 and GIP secretion using naturally-occurring commensal bacteria. Our findings demonstrate that there are cell-to-cell interactions between human commensal bacterial and EE cells, and that the adaptor proteins of the TLR system are one plausible signalling mechanism. The underlying protein interactions of specific Lactobacilli with EE cells should be investigated including the profound changes observed in MyD88 and CD14 expression. The precise role of these proteins in triggering LAB-induced secretion needs to be elucidated. A limitation of the study is that it focused only on *Lactobacillus* spp. and the contribution of the many other species of commensal bacteria needs to be investigated. Probiotic organisms represent a novel therapeutic strategy for type 2 diabetes but it will be necessary to undertake dietary intervention studies involving safe, well-characterised and commercially available probiotic stains.

Conflict of interest

The authors have no conflicts of interest to declare.

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Author Contributions: Harsh Panwar designed the experiments, analysed and interpreted the data and drafted the manuscript. Danielle Calderwood, Alastair Wylie, Sunita Grover and Stewart Graham designed experiments, analysed and interpreted the data. Brian Green and Irene Grant conceived the study, designed the experiments, analysed and interpreted the data,
and drafted the manuscript. All authors approved the final version of the manuscript to be published.


Investigating the effects of physiological bile acids on GLP-1 secretion and glucose tolerance in normal and GLP-1 R(−/−) mice. The Journal of Biological Chemistry, 392(6), 539-546.


Engineering an L-cell line that expresses insulin under the control of the glucagon-like peptide-1 promoter for diabetes treatment. BMC Biotechnology, 11(99), 1-8.


Intake of *Lactobacillus reuteri* Improves incretin and insulin secretion in glucose
tolerant humans: a proof of concept. Diabetes Care, pii: dc142690. [Epub ahead of print]


Figures Legends

**Figure 1** Co-culture of enteroendocrine cells with *Lactobacillus* strains stimulates incretin hormone secretion. Graphs show effects of 15 *Lactobacillus* isolates (Lb1-15), 7 *Lactobacillus* reference cultures, a Gram positive control (*B. bifidum*) and a Gram negative control (*E. coli*) on the secretion of (A) GLP-1 and (B) GIP in pGIP/Neo STC-1 cells following 3h co-culture. Data represent means \(\pm\) SEM (n=6) and statistical significance is indicated (*P <0.05 and **P<0.001 compared with control; One-way ANOVA).

**Figure 2** Co-culture of enteroendocrine cells with *Lactobacillus* strains upregulates incretin hormone gene expression. Graphs show effects of 15 *Lactobacillus* isolates (Lb1-15), 7 *Lactobacillus* reference cultures, a Gram positive control (*B. bifidum*) and a Gram negative control (*E. coli*) on the gene expression of (A) proglucagon (the precursor for GLP-1) and (B) GIP in pGIP/Neo STC-1 cells following 3h co-culture. Data represent means \(\pm\) SEM (n=6) and statistical significance is indicated (**P <0.01 and ***P<0.001 compared with control; One-way ANOVA).

**Figure 3** Possible metabolite-based mechanisms responsible for *Lactobacillus*-stimulated incretin hormone secretion. *L. rhamnosus* was selected for further studies due to its ability to potently stimulate both GLP-1 and GIP secretion. (A) Changes in amino acid composition of buffer were identified by GC-MS. (B) Exposure of pGIP/Neo STC-1 cells with the 3 elevated amino acids (alanine, histidine and proline) did not influence GLP-1 secretion. (C) Changes in free fatty acid receptor gene expression were examined following *L. rhamnosus*
co-culture and compared against vehicle control and *L. casei* (a *Lactobacillus* strain which did not stimulate incretin hormone secretion but did influence incretin gene expression. Data represent means ± SEM (n=3) and statistical significance is indicated (*P <0.05 and ***P<0.001 compared with control; ns- not significant; One-way ANOVA).

**Figure 4** Other molecular mechanisms possibly involved *Lactobacillus*-stimulated incretin hormone secretion. (A) Confocal light microscopy (x400) indicated that many *L.rhamnosus* organisms (black) are closely localised to pGIP/Neo STC-1 cells (blue), perhaps adhering to the cell surface. (B) Toll-like receptor signalling pathways were probed using a mouse TLR PCR array which demonstrated that a large number of these genes were downregulated following *L rhamnosus* co-culture (also see Supplementary Table 1). CD14 expression was up-regulated most (17.5-fold) and Myd88 was down-regulated most (23.4-fold). (C) Application of a My88 blocking peptide or an antibody directed against the cell surface antigen CD14 significantly affected GLP-1 secretion. Data represent means ± SEM (n=3) with *P <0.05 and ***P<0.001 compared with control; ∆∆P<0.01; ∆∆∆P<0.001 compared with *L.rhamnosus*; One-way ANOVA).
Figure 1

A  
GLP-1 secretion

B  
GIP secretion
Figure 2

A

Proglucagon gene expression

B

GIP gene expression
Figure 3

A

Concentration in test buffer (nmol/l) for control and L. rhamnosus.

Free amino acids

B

Comparison of GLP-1 secretion (pmol GLP-1/10^6 cells/hour) between control and L. rhamnosus.

C D E

Legend:

- Control
- L. rhamnosus

Statistical significance:
- * p < 0.05
- ** p < 0.001
- *** p < 0.0001

ns: not significant
**Figure C**

A bar graph showing the secretion of GLP-1 (per million GLP-1 per 10^6 cells/hour) across different conditions:

- **L. rhamnosus**
- **Pepinh-MYD**
- **Anti-CD14**

The graph indicates significant differences in GLP-1 secretion levels among the conditions tested. The bars are labeled with symbols indicating statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.
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<th>Strain code</th>
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<th>% Sequence Similarity</th>
<th>Accession or culture collection no.</th>
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<tr>
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**Table 1.** List of bacterial strains examined in the study. Bacterial strains Lb-1 to Lb15 were isolated from faeces from healthy human infants. Reference strains (Ref1-7) were obtained from NCIMB. n/a- not applicable.
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<td>5’- gaagacctgctcttgtgtgtggt -3’</td>
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### Supplementary Table 1: TLR Gene Array: changes in gene expression in pGIP/Neo STC-1 cells following co-culture with *L. rhamnosus*.

Note: Positive fold change indicates up-regulation. Negative fold change indicates down-regulation. *P<0.05.

<table>
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<th>Genes</th>
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