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

Panwar, H., Calderwood, D., Gillespie, A. L., Wylie, A. R., Graham, S. F., Grant, I. R., Grover, S., & Green, B. D. (2016). Identification of lactic acid bacteria strains modulating incretin hormone secretion and gene expression in enteroendocrine cells. *Journal of Functional Foods*, *23*, 348-358. https://doi.org/10.1016/j.jff.2016.02.040

Published in:
Journal of Functional Foods

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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

Harsh Panwar[1,2#], Danielle Calderwood[1], Anna L Gillespie[1], Alastair R Wylie[4], Stewart F Graham[5], Irene R Grant[1], Sunita Grover[3], Brian D Green[1*]

[1]Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Northern Ireland, UK

[2]Department of Dairy Microbiology, College of Dairy Science and Technology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, INDIA

[3]Molecular Biology Unit, Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, INDIA

[4]Agri-Food and Biosciences Institute, Newforge Lane, BT9 5PX, Belfast, Northern Ireland, UK.

[5]Beaumont Research Institute, 3811 W. 13 Road, Royal Oak, MI, 48073, USA.

Commonwealth Scholar, Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, UK

***Corresponding author:** Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast, BT9 5AG, United Kingdom; Email: b.green@qub.ac.uk; Fax: +4428 9097 6543

Short Title: Lactic acid bacteria modulating incretin hormones.

Abbreviations: GLP-1, glucagon like peptide – 1; GIP, glucose dependent insulinotropic peptide; RIA, radioimmunoassay; EE, enteroendocrine; LAB, lactic acid bacteria.

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 10^8cfu/g) are lower than in the large intestine ($10^{12\text{-}14}$ 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^{o}C) in their respective media (10 ml), harvested (12,000g, 15min, 10^{o}C) and washed twice with 1X PBS. Cell pellets were again re-suspended in 1X PBS and diluted to O.D$_{600}$ 1.5 which

corresponded to 1×10^9 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% CO_2 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 $2x10^6$ pGIP/Neo STC-1 cells were seeded into 12-well plates with DMEM and allowed to attach overnight (37°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 $2x10^9$ live bacteria for 3h (37°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°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°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* ($2x10^9$ 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 $2x10^6$ 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 ddH_2O (10ml) for 1 min. Samples were then centrifuged (3,500*g*, 4°C, 25 min) and the supernatant collected. Pellets were re-suspended in ddH_2O (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 3,500*g* 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. RT^2 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 RT^2 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^{TM} 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.

208 *argentorotensis*; KC491380) secreted 86.8±6 pM/10^6 cells/h (Figure 1A). For GIP, secretion
209 of 1.9±0.05 pM/10^6 cells/h occurred with a non-stimulatory vehicle control. Two faecal
210 isolate strains: Lb1 (later identified as being *Lactobacillus plantarum*) and Lb3 stimulated
211 significant GIP secretion (Figure 1B; 100.6±2.9 and 155.8±24.9 pM/10^6 cells/h, respectively.
212 Co-culture with two reference strains *L. johnsonii* (NCIMB8795) and *L. rhamnosus*
213 (NCIMB6375) significantly increased both GLP-1 secretion (Figure 1A; 61.0±8.4 and
214 82.3±26.1 pM/10^6 cells/h, respectively) and GIP secretion (Figure 1B; 369.5±68.9 and
215 285.7±34.7 pM/10^6 cells/h, respectively). The Gram positive (*B. bifidum*) and Gram negative
216 (*E. coli)* control organisms did not stimulate any incretin hormone secretion.
217

218 **3.2 Changes in incretin hormone gene expression following *Lactobacillus* co-culture**

219 A number of *Lactobacillus* strains affected the levels of gene expression of
220 proglucagon and GIP in pGIP/Neo STC-1cells. Two *Lactobacillus* isolates Lb4 and Lb6
221 (both identified as *Lactobacillus plantarum)* upregulated proglucagon gene expression 3.6-
222 and 2.5-fold, respectively (Figure 2A). Four reference strains *L. acidophilus*
223 (NCIMB701748), *L. casei* (NCIMB4114), *L. plantarum* (NCIMB1406) and *L. rhamnosus*
224 (NCIMB6375) significantly increased proglucagon gene expression (Figure 2A; 2.9-, 1.8-,
225 1.9- and 2.9-fold, respectively). Interestingly, *B. bifidum* up-regulated GLP-1 proglucagon
226 gene expression 2.1-fold. Four *Lactobacillus* isolates Lb4, Lb6, Lb8 and Lb9 (all
227 *Lactobacillus plantarum)* along with three reference cultures (*L. casei*, *L. plantarum* and *L.*
228 *rhamnosus*) significantly up-regulated GIP gene expression (Figure 2B; 2.5-, 2.7-, 2.3, 2.2-,
229 2.4-, 3.2- and 5.4-fold, respectively). The Gram-negative bacterium *E. coli* did not affect
230 either proglucagon or GIP gene expression.

231 **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 secrete 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. *argentorotensis* (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 (Ref 1) 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, &

328 Tobias, 1998). It could be postulated that CD14 is a surface antigen which facilitates
329 adhesion of *L. rhamnosus* peptidoglycans to the EE cell surface, but the exact signalling role
330 (if any) for eliciting incretin hormone secretion clearly requires further investigation. CD14 is
331 best characterised as a feature of monocytes and macrophages with most subpopulations of
332 these cells expressing CD14. The interaction of commensal bacteria with the gut lining is an
333 incredibly understudied area and there is presently very little scientific literature elucidating
334 the role of CD14 in the intestine. It ha s been shown however, that an *E. coli* probiotic
335 organisms used in the treatment of inflammatory bowel disorders stimulated the gene
336 expression of CD14 in the Caco-2 intestinal epithelial cell line (Hafez et al., 2010). Although
337 this study did also report that the expression of the adaptor molecules MyD88 and Ticam1
338 (TRIF) was up-regulated which differs from the present study (Hafez et al., 2010).

339 Currently, the investigation of the functional and physiological actions of probiotics is
340 an extremely active research field, and many health benefits are proposed including the
341 improvement of gastrointestinal function and lowering of blood cholesterol levels
342 (Macfarlane & Cummings, 1999). Their incorporation into fermented and non-fermented
343 dairy products is well accepted, and their inclusion in functional foods such as e.g. fruit
344 juices, breakfast cereals, cereal bars, etc. has also been investigated. There is growing support
345 for the concept of utilising probiotic organisms as a dietary prophylactic or therapeutic
346 strategy for type 2 diabetes mellitus (Yadav *et al.* 2013*;* Panwar *et al.,* 2014; Panwar *et al.,*
347 2016). The concept has been demonstrated in a recent clinical trial which found that
348 administration of the probiotic *L. reuteri* increased insulin secretion and incretin release in
349 humans (Simon et al., 2015). This group concluded that since *L. reuteri* did not modulate
350 faecal microbiota it is likely that *Lactobacillus* spp. have a direct effect on host physiology –
351 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.

Acknowledgements

Authors would like to acknowledge funding from Commonwealth Scholarship Commission, UK as part of PhD studentship to Harsh Panwar tenable at Queen's University Belfast (UK).

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.

References

Audy, J., Mathieu, O., Belvis, J., & Tompkins, T. A. (2012). Transcriptomic response of immune signalling pathways in intestinal epithelial cells exposed to lipopolysaccharides, Gram-negative bacteria or potentially probiotic microbes. Beneficial Microbes, 3(4), 273-86.

Baggio, L. L., & Drucker, D. J. (2007). Biology of incretins: GLP-1 and GIP. Gastroenterology, 132(6), 2131-2157.

Brown, A. J., Goldsworthy, S. M., Barnes, A. A., Eilert, M. M., Tcheang, L., Daniels, D., Muir, A. I., Wigglesworth, M. J., Kinghorn, I., Fraser, N. J., Pike, N. B., Strum, J. C., Steplewski, K. M., Murdock, P. R., Holder, J. C., Marshall, F. H., Szekeres, P. G., Wilson, S., Ignar, D. M., Foord, S. M., Wise, A., Dowell, S. J. (2003). The Orphan G Protein-coupled Receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. The Journal of Biological Chemistry, 278(13), 11312–11319.

Cani, P. D., Osto, M., Geurts, L., & Everard, A. (2012). Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut Microbes, 3(4), 279-288.

Castellazzi, A. M., Valsecchi, C., Caimmi, S., Licari, A., Marseglia, A., Leoni, M. C., Caimmi, D., Miraglia del Giudice, M., Leonardi, S., La Rosa, M., & Marseglia, G. L. (2013). Probiotics and food allergy. Italian Journal of Pediatrics, 39(47), 1-10.

De Greef, E., Vandenplas, Y., Hauser, B., Devreker, T., & Veereman-Wauters, G. (2013). Probiotics and IBD. Acta Gastro-Enterologica Belgica, 76, 15-19.

Duan, F. F., Liu, J. H., & March, J. C. (2015). Engineered commensal bacteria reprogram intestinal cells into glucose-responsive insulin-secreting cells for the treatment of diabetes. Diabetes, 1-10. Doi:10.2337/db14-0635.

Dubernet, S., Desmasures, N., & Gueguen, M. (2002). A PCR based method for identification of lactobacilli at the genus level. FEMS Microbiology Letters, 214(2), 271-275.

Dylag, K., Hubalewska-Mazgaj, M., Surmiak, M., Szmyd, J., & Brzozowski, T. (2014). Probiotics in the mechanism of protection against gut inflammation and therapy of gastrointestinal disorders. Current Pharmaceutical Design, 20(18), 1149-1155.

Dziarski, R., Tapping, R. I., & Tobias, P. S. (1998). Binding of bacterial peptidoglycan to CD14. The Journal of Biological Chemistry 273(15), 8680-8690.

Ejtahed, H. S., Mohtadi-Nia, J., Homayouni-Rad, A., Niafar, M., Asghari-Jafarabadi, M., & Mofid, V. (2012). Probiotic yogurt improves antioxidant status in type 2 diabetic patients. Nutrition, 28(5), 539-543.

Flatt, P. R., & Green, B. D. (2006). Nutrient regulation of pancreatic beta cell function in diabetes problems and potential solutions. Biochemical Society Transactions, 34(5), 774-778.

Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. Nat. Rev. Journal of Gastroenterology and Hepatology, 9(10), 577-589.

Forssten, S. D., Korczynska, M. Z., Zwijsen, R. M. L., Noordman, W. H., Madetoja, M., & Ouwehand, A.C. (2013). Changes in satiety hormone concentrations and feed intake in rats in response to lactic acid bacteria. Appetite, 71, 16-21.

Forsythe, P. (2011). Probiotics and lung diseases. Chest, 139(4), 901-908.

Giahi, L., Aumueller, E., Elmadfa, I., & Haslberger, A. G. (2012). Regulation of TLR4, p38 MAP kinase, IκB and miRNAs by inactivated strains of lactobacilli in human dendritic cells. Beneficial Microbes, 3(2), 91-98.

Gillespie, A.L., Calderwood, D., Hobson, L., Green, B. D. (2015). Whey proteins have beneficial effects on intestinal enteroendocrine cells stimulating cell growth and increasing the production and secretion of incretin hormones. Food Chemistry 189:120-8.

Green, B. D., Gault, V. A., O'Harte, F. P. M., & Flatt, P. R. (2005). Development and therapeutic potential of incretin hormone analogues for type 2 diabetes. The British Journal of Diabetes & Vascular Disease, 5, 134-140.

Hafez, M., Hayes, K., Goldrick, M., Grencis, R. K., & Roberts, I. S. (2010). The K5 capsule of *Escherichia coli* strain Nissle 1917 is important in stimulating expression of Toll like receptor 5, CD14, MyD88, and TRIF together with the induction of interleukin-8 expression via the mitogen-activated protein kinase pathway in epithelial cells. Infection and Immunity 78, 2153-2162.

Hand, K. V., Giblin, L. & Green, B. D. (2012). Hormone profiling in a novel enteroendocrine cell line pGIP/neo: STC-1. Metabolism, 61(12), 1683–1686.

Hardy, H., Harris, J., Lyon, E., Beal, J., & Foey, A. D. (2013). Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. Nutrients, 5(6), 1869-1912.

Hijova, E., & Soltesova, A. (2013). Effects of probiotics and prebiotics in ulcerative colitis. Bratislava Medical Journal, 114(9), 540-543.

446 Horvath, A., & Szajewska, H. (2013). Probiotics, prebiotics, and dietary fiber in the
447 management of functional gastrointestinal disorders. World Review of Nutrition and
448 Dietetics, 108, 40-48.

449 Jepeal, L. I., Boylan, M. O., & Wolfe, M. M. (2008). GATA-4 upregulates glucose
450 dependent insulinotropic polypeptide expression in cells of pancreatic and intestinal
451 lineage. Molecular and Cellular Endocrinology, 287(1-2), 20–29.

452 Kamdar, K., Nguyen, V., & DePaolo, R. W. (2013). Toll-like receptor signaling and
453 regulation of intestinal immunity. Virulence, 4(3), 207-212.

454 Katsuma, S., Hatae, N., Yano, T., Ruike, Y., Kimura, M., Hirasawa, A., & Tsugimoto, G.
455 (2005). Free fatty acids inhibit serum deprivation-induced apoptosis through
456 GPR120 in a murine enteroendocrine cell line STC-1. The Journal of Biological
457 Chemistry, 280, 19507-19515.

458 Kelly, D., & Mulder, I. E. (2012). Microbiome and immunological interactions. Nutrition
459 Reviews, 70, S18-830.

460 Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces
461 shaping microbial diversity in the human intestine. Cell, 124(4), 837-848.

462 Licciardi, P. V., Wong, S. S., Tang, M. L., & Karagiannis, T. C. (2010). Epigenome targeting
463 by probiotic metabolites. Gut Pathogens, 2(1), 24.

464 Liu, Y., Fatheree, N. Y., Dingle, B. M., Tran, D. Q., & Rhoads, J. M. (2013). *Lactobacillus*
465 *reuteri* DSM 17938 changes the frequency of Foxp3+ regulatory T cells in the
466 intestine and mesenteric lymph node in experimental necrotizing enterocolitis. PLoS
467 One. 8(12), e56547.

Mccarthy, T., Green, B. D., Calderwood, D., Gillespie, A., Cryan, J. F., Giblin, L. (2015). STC-1 cells in Verhoeckx, K., Cotter, P., Lopez-Exposito, I., Kleiveland, C., Lea, T. (Ed.), The Impact of Food Bioactives on Health In Vitro and Ex Vivo Models: 211-220.

Macfarlane G.T., Cummings J.H. (1999). Probiotics and prebiotics: Can regulating the activities of intestinal bacteria benefit health? British Medical Journal. 318(7189), 999–1003.

Molinaro, F., Paschetta, E., Cassader, M., Gambino, R., & Musso, G. (2012). Probiotics, prebiotics, energy balance, and obesity: mechanistic insights and therapeutic implications. Gastroenterology Clinics of North America, 41(4), 843-854.

Naser, S. M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M., Swings, J. (2007). Identification of lactobacilli by pheS and rpoA gene sequence analyses. International Journal of Systematic and Evolutionary Microbiology, 57(12), 2777-2789.

Neumiller, J. J. (2012). Incretin pharmacology: a review of the incretin effect and current incretin-based therapies. Cardiovascular & Hematological Agents in Medicinal chemistry, 10(4), 276-288.

Panwar, H., Calderwood, D., Grant, I. R., Grover, S., & Green, B. D. (2014). *Lactobacillus* strains isolated from infant faeces possess potent inhibitory activity against intestinal alpha- and beta-glucosidases suggesting anti-diabetic potential. European Journal of Nutrition, 53(7), 1465-1474.

Panwar, H., Calderwood, D., Grant, I. R., Grover, S., & Green, B. D. (2016). Lactobacilli possess inhibitory activity against dipeptidyl peptidase-4 (DPP-4). Annals of Microbiology 66(1), 505-509.

Prakash, S., Tomaro-Duchesneau, C., Saha, S., Rodes, L., Kahouli, I., Malhotra M. (2013). Probiotics for the prevention and treatment of allergies, with an emphasis on mode of delivery and mechanism of action. Current Pharmaceutical Design, 20(6), 1025-1037.

Rafferty, E. P., Wylie, A. R., Hand, K. H., Elliott, C. E., Grieve, D. J., Green, B. D. (2011). 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.

Ramshur, E. B., Rull, T. R., & Wice, B. M. (2002). Novel insulin/GIP co-producing cell lines provide unexpected insights into gut K-cell function in vivo. Journal of Cellular Physiology, 192(3), 339-350.

Rasouli, M., Ahmad, Z., Omar, A. R., & Allaudin, Z. N. (2011). 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.

Rogall, T. J., Wolters, T. F., & Bottger, E. C. (1990). Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. International Journal of Systematic Bacteriology, 40, 323–330.

Sanz, Y., Santacruz, A., & Gauffin, P. (2010). Gut microbiota in obesity and metabolic disorders. Proceedings of the Nutrition Society, 69(3), 434-441.

Simon, M. C., Strassburger, K., Nowotny, B., Kolb, H., Nowotny, P., Burkart, V., Zivehe, F., Hwang, J. H., Stehle, P., Pacini, G., Hartmann, B., Holst, J. J., MacKenzie, C., Bindels, L. B., Martinez, I., Walter, J., Henrich, B., Schloot, N. C., Roden, M. (2015). Intake of *Lactobacillus reuteri* Improves incretin and insulin secretion in glucose

514 tolerant humans: a proof of concept. Diabetes Care, pii: dc142690. [Epub ahead of
515 print]

516 Speck, M., Cho, Y. M., Asadi, A., Rubino, F., & Kieffer, T. J. (2011). Duodenal-jejunal
517 bypass protects GK rats from {beta}-cell loss and aggravation of hyperglycemia and
518 increases enteroendocrine cells coexpressing GIP and GLP-1. American Journal of
519 Physiology. Endocrinology and metabolism, 300(5), E923-932.

520 Tate, M., Chong, A., Robinson, E., Green, B. D., & Grieve, D. J. (2015). Selective targeting
521 of glucagon-like peptide-1 signalling as a novel therapeutic approach for
522 cardiovascular disease in diabetes. British Journal of Pharmacology, 172(3), 721-736.

523 Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating deep
524 phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA
525 sequence analysis. Journal of Eukaryotic Microbiology, 46(4), 327–338.

526 Umeki, U., Oue, K., Mochizuki, S., Shirai, Y., & Sakai, K. (2004). Effect of *Lactobacillus*
527 *rhamnosus* KY-3 and cellobiose as synbiotics on lipid metabolism in rats. Journal of
528 Nutritional Science and Vitaminology, 50(5), 330-334.

529 Walter, J., & Ley, R. (2011). The human gut microbiome: Ecology and recent evolutionary
530 changes. Annual Review of Microbiology, 65, 411-429.

531 Wehr, H. M., & Frank, J. F. (Eds.) (2004). *Standard Methods for the Examination of Dairy*
532 *Products*, American Public Health Association, Baltimore, United States of America
533 2004.

534 Yadav, H., Lee, J. H., Lloyd, J., Walter, P., & Rane, S. G. (2013). Beneficial metabolic
535 effects of a probiotic via butyrate-induced GLP-1 hormone secretion. The Journal of
536 Biological Chemistry, 288(35), 25088-25097.

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 ± SEM (n=6) and statistical significance is indicated (*P <0.05 and ***P<0.001 compared with control; One-way ANOVA).

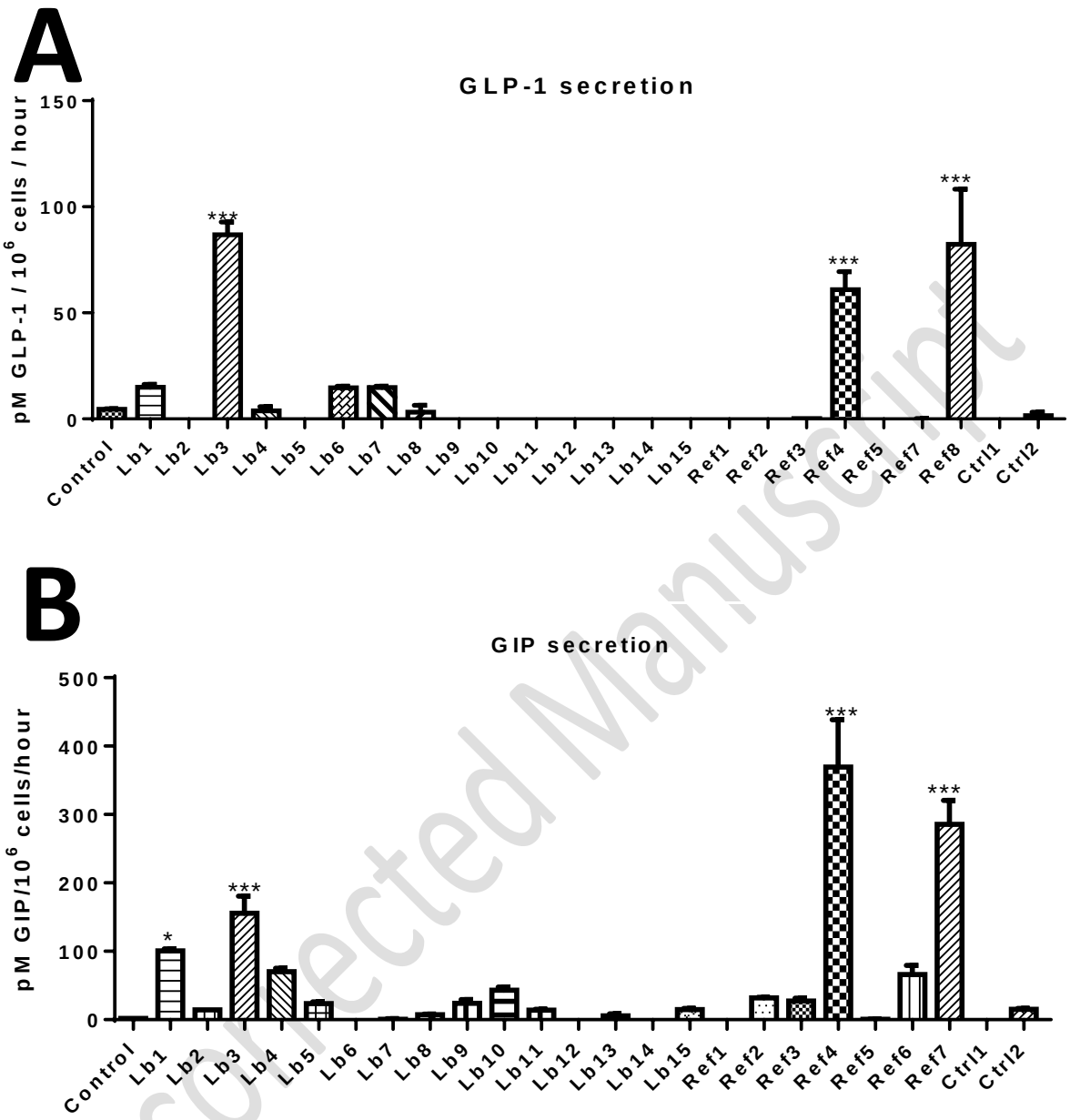
Figure 2 Co-culture of enteroendocrine cells with *Lactobacillus* strains upregulates incretin hormone gene expression. Graphs show effects of 15 *Lactobacillus* isolates (Lb1-15), 7 *Lactobacillus* reference cultures, a Gram positive control (*B. bifidum*) and a Gram negative control (*E. coli)* on 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 ± 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; $^{\Delta\Delta}$P<0.01; $^{\Delta\Delta\Delta}$P<0.001 compared with *L.rhamnosus*; One-way ANOVA).

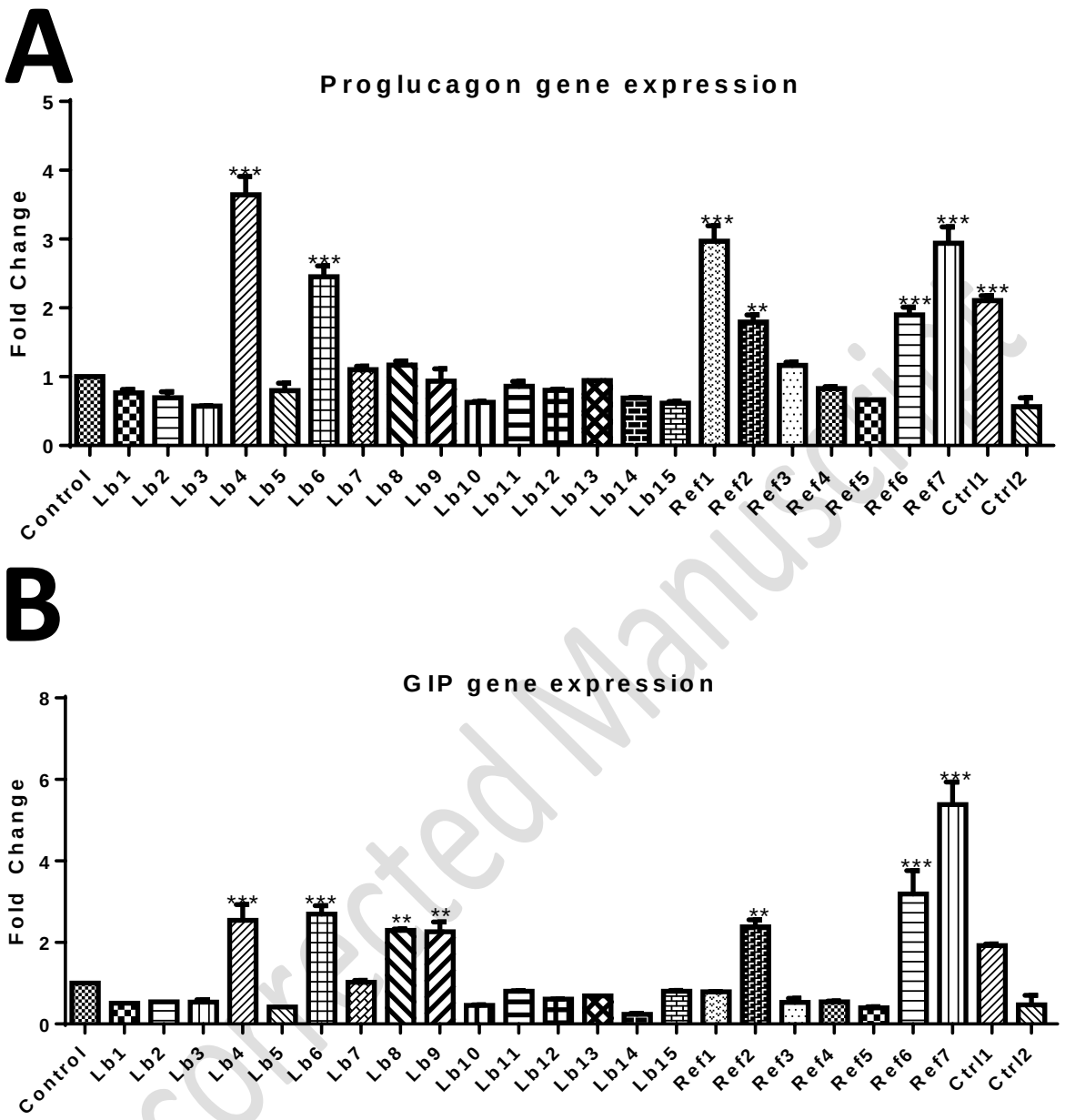
577 **Figure 1**



578

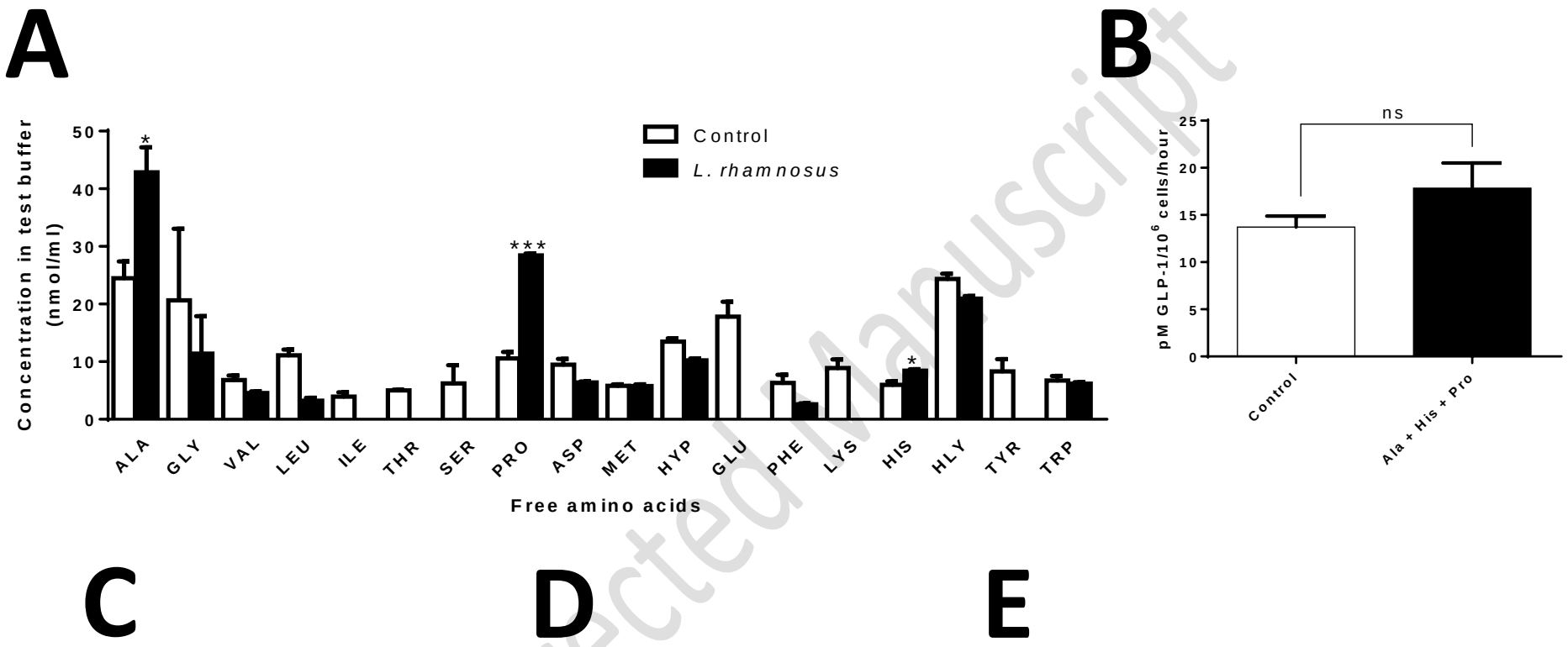
579

580 **Figure 2**



581

Figure 3



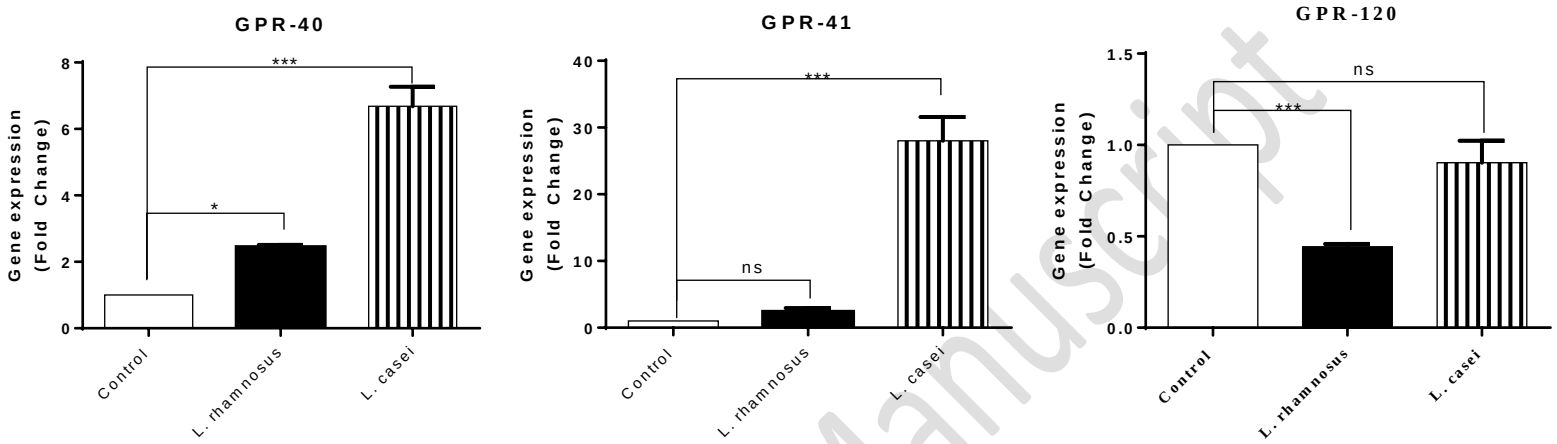
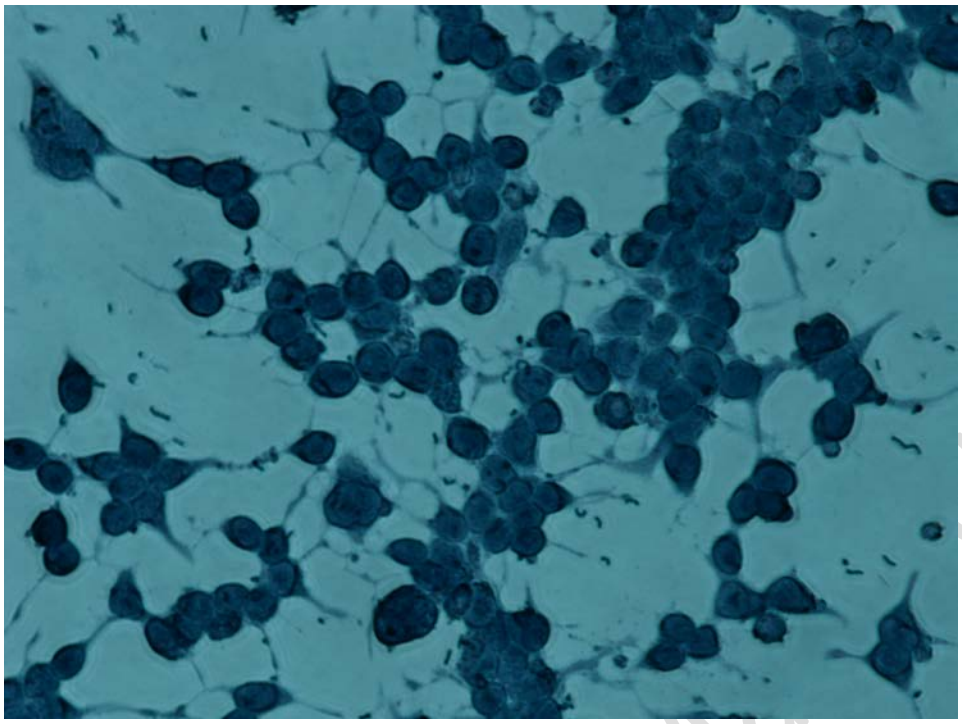
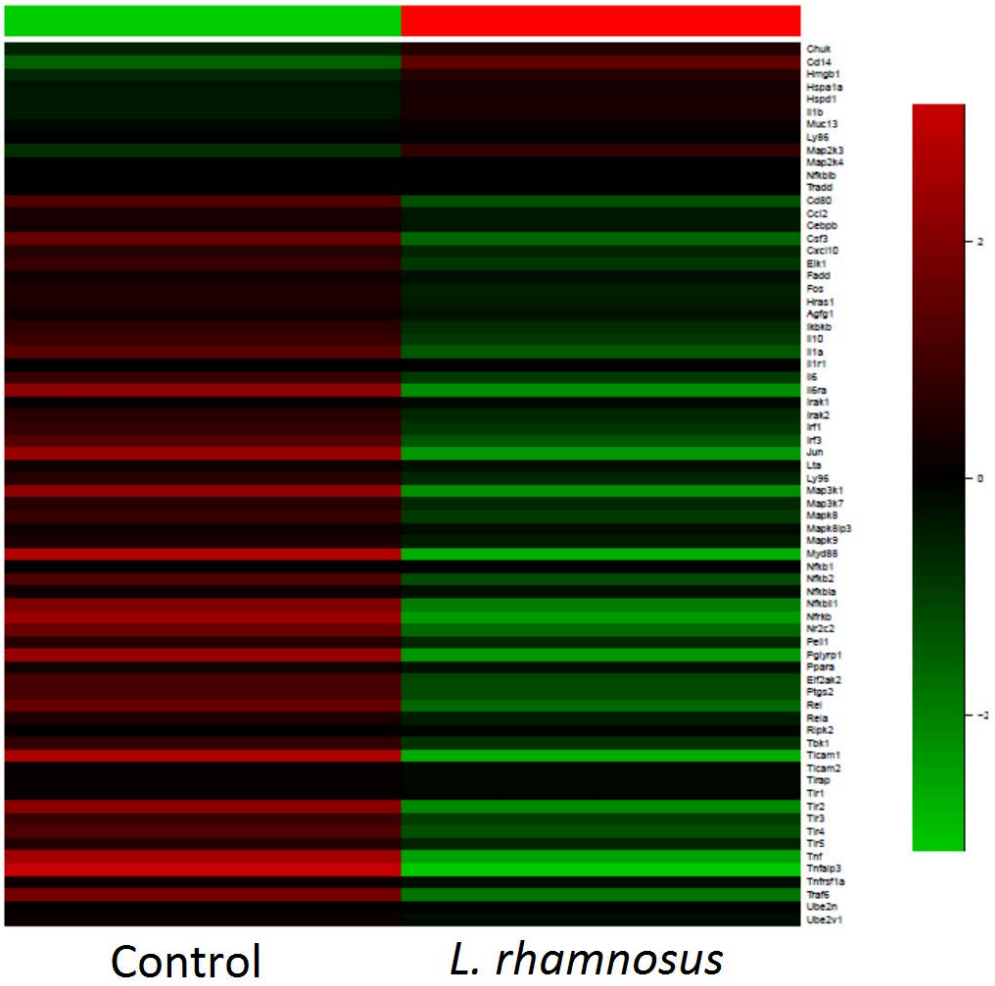


Figure 4

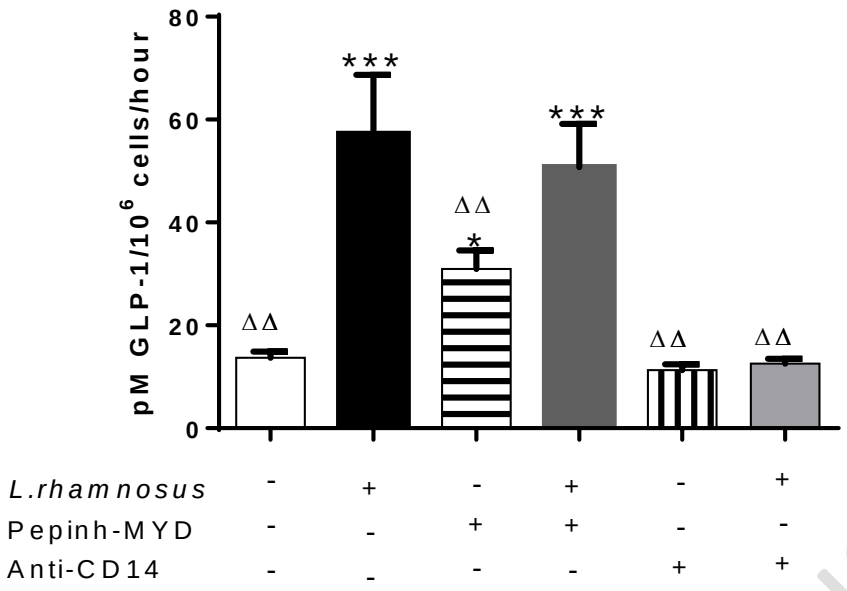
A



B



C



	Strain code	Type	Identification	% Sequence Similarity	Accession or culture collection no.
1	Lb1	Faecal isolate	Strain of *Lactobacillus plantarum*	98 %	Unknown
2	Lb2	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	Unknown
3	Lb3	Faecal isolate	*Lactobacillus plantarum subsp. argentorotensis*	99 %	KC491380
4	Lb4	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	KF678450
5	Lb5	Faecal isolate	Strain of *Lactobacillus plantarum*	95 %	Unknown
6	Lb6	Faecal isolate	Strain of *Lactobacillus plantarum*	96 %	Unknown
7	Lb7	Faecal isolate	Strain of *Lactobacillus fermentum*	98 %	Unknown
8	Lb8	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	KF678451
9	Lb9	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	KF678452
10	Lb10	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	KF678453
11	Lb11	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	Unknown
12	Lb12	Faecal isolate	*Lactobacillus* sp.	99 %	Unknown
13	Lb13	Faecal isolate	Strain of *Lactobacillus fermentum*	97 %	KC866340
14	Lb14	Faecal isolate	Strain of *Lactobacillus plantarum*	99 %	Unknown

15	Lb15	Faecal isolate	Strain of *Lactobacillus acidophilus*	99 %	Unknown
16	Ref1	Reference culture	*Lactobacillus acidophilus*	n/a	NCIMB701748
17	Ref2	Reference culture	*Lactobacillus casei*	n/a	NCIMB4114
18	Ref3	Reference culture	*Lactobacillus fermentum*	n/a	NCIMB2797
19	Ref4	Reference culture	*Lactobacillus johnsonii*	n/a	NCIMB8795
20	Ref5	Reference culture	*Lactobacillus paracasei*	n/a	NCIMB1407
21	Ref6	Reference culture	*Lactobacillus plantarum*	n/a	NCIMB1406
22	Ref7	Reference culture	*Lactobacillus rhamnosus*	n/a	NCIMB6375
23	Ctrl1	Gram positive control	*Bifidobacterium bifidum*	n/a	NCIMB702715
24	Ctrl2	Gram negative control	*Escherichia coli*	n/a	NCTC 10538

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.

Table 2 Primer sequences used in this study

Target	Forward	Reverse	Reference
Proglucagon (GLP-1)	Proglucagon-F 5’- ggcacattcaccagcgactac -3’,	Proglucagon-R 5’- caatggcgacttcttctggg -3’	Rasouli et al.,2011
GIP	GIP-F 5’- gaagacctgctctctgttgctggt -3’	GIP-R 5’- cagagctctgcttggtccaccatc -3’	Jepeal et al., 2008
β-actin	β-actin-F 5’- gtgtgatggtgggaatgggtc -3’	β-actin-R 5’- aggaagaggatgcggcagtg -3’	Rasouli et al.,2011
GPR40	GPR40-F 5’- agtcctcgtcacacatattg -3’	GPR40-R 5'- aatgcctccaatgtggatag -3'	Katsuma et al., 2005
GPR41	GPR 41-F 5’- ttcttgcagccacactgctc -3’	GPR 41-R 5'- gcccaccacatgggacatat -3'	Brown et al. 2003
GPR120	GPR 120-F 5'- gcataggagaaatctcatgg -3,	GPR 120-R 5'- gagttggcaaacgtgaaggc -3'	Katsuma et al., 2005
LbLMA1/R-161	LbLMA1/R-161-F 5'- ctcaaaactaaacaaagtttc -3'	LbLMA1/R-161-R 5'-ctcgtacttgtacacaccgcccgtca -3'	Dubernet et al., 2002
16SrRNA	16SrRNA-F 5'- ccagagtttgatcmtggctcag -3'	16SrRNA-R 5'- cggttaccttgttacgacttcacc -3'	Turner et al., 1999; Rogall et al., 1990
Phe	Phe-F 5'- tatttcaaaattgcraaacgr -3';	Phe-R 5'- cccwgcwcgtgatatgca -3’	Naser et al., 2007

Genes	Refseq	Fold Changes		Genes	Refseq	Fold Changes	
Toll-like receptors				**NF kappa B pathway**			
Tlr1	NM_030682	1.84		Ccl2	NM_011333	1.16	
Tlr2	NM_011905	-9.71	*	Chuk	NM_007700	4.38	*
Tlr3	NM_126166	-1.75		Csf2	NM_009969	2.02	*
Tlr4	NM_021297	-2.79	*	Csf3	NM_009971	-4.62	*
Tlr5	NM_016928	-1.06		Agfg1	NM_010472	1.31	
Tlr6	NM_011604	2.02	*	Ikbkb	NM_010546	-1.27	
Tlr7	NM_133211	2.02	*	Il1a	NM_010554	-3.20	*
Tlr8	NM_133212	2.02	*	Il1b	NM_008361	3.41	*
Tlr9	NM_031178	2.02	*	Il1r1	NM_008362	1.88	
Muc13	NM_010739	2.50	*	Il2	NM_008366	2.02	*
Adaptors & interacting proteins				Il6	NM_031168	-1.72	
Btk	NM_013482	-2.48	*	Il10	NM_010548	-1.73	
Cd14	NM_009841	17.52	*	Il12a	NM_008351	2.02	*
Hmgb1	NM_010439	4.93	*	Map3k1	NM_011945	-	*
Hras1	NM_008284	1.14		Nfkb1	NM_008689	1.88	
Hspa1a	NM_010479	3.18	*	Nfkb2	NM_019408	-2.64	*
Hspd1	NM_010477	3.53	*	Nfkbia	NM_010907	1.43	
Lta	NM_010735	1.45		Nfkbib	NM_010908	2.17	*
Ly86	NM_010745	2.03	*	Nfkbil1	NM_010909	-7.89	*
Ly96	NM_016923	-1.15		Nfrkb	NM_172766	-	*
Mapk8ip3	NM_013931	1.57		Rel	NM_009044	-4.72	*
Myd88	NM_010851	-23.41	*	Rela	NM_009045	1.06	
Peli1	NM_023324	-1.09		Tnf	NM_013693	-	*
Pglyrp1	NM_009402	-12.99	*	Tnfaip3	NM_009397	-	*
Ripk2	NM_138952	1.83		Tnfrsf1a	NM_011609	1.50	
Ticam1	NM_174989	-21.69	*	Tradd	NM_001033161	2.07	*
Ticam2	NM_173394	1.63		**JNK/p38 pathway**			
Tirap	NM_054096	1.75		Elk1	NM_007922	-1.62	
Tollip	NM_023764	2.02	*	Fos	NM_010234	-1.01	
Effectors				Jun	NM_010591	-	*
Casp8	NM_009812	3.73	*	Map2k3	NM_008928	5.82	*
Fadd	NM_010175	1.41		Map2k4	NM_009157	2.04	*
Irak1	NM_008363	1.67		Mapk8	NM_016700	-1.73	
Irak2	NM_172161	-1.23		Mapk9	NM_016961	1.07	
Map3k7	NM_172688	-1.18		**NF/IL6 pathway**			
Nr2c2	NM_011630	-4.89	*	Cebpb	NM_009883	1.28	
Ppara	NM_011144	1.35		Clec4e	NM_019948	2.02	*
Eif2ak2	NM_011163	-2.31	*	Il6ra	NM_010559	-	*
Ube2n	NM_080560	1.92		Ptgs2	NM_011198	-2.41	*
Ube2v1	NM_023230	1.50		**Adaptive Immunity**			
IRF pathway				Cd80	NM_009855	-2.71	*
Cxcl10	NM_021274	-1.12		Cd86	NM_019388	2.02	*
Ifnb1	NM_010510	2.02	*	Traf6	NM_009424	-6.14	*
Ifng	NM_008337	2.02	*				
Irf1	NM_008390	-1.59					
Irf3	NM_016849	-3.14	*				
Tbk1	NM_019786	-1.44					

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.