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Heat-killed probiotic bacteria differentially regulate colonic epithelial cell production of human β-defensin-2: dependence on inflammatory cytokines

N. Habil[1,2], W. Abate[1], J. Beal[1] and A.D. Foey[1*]

[1]School of Biomedical & Healthcare Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, United Kingdom; [2]Foundation of Technical Education (FTE), Alnakabat Street, 55555 Baghdad, Iraq; andrew.foey@plymouth.ac.uk

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RESEARCH ARTICLE

Abstract

The inducible antimicrobial peptide human β-defensin-2 (hBD-2) stimulated by pro-inflammatory cytokines and bacterial products is essential to antipathogen responses of gut epithelial cells. Commensal and probiotic bacteria can augment such mucosal defences. Probiotic use in the treatment of inflammatory bowel disease, however, may have adverse effects, boosting inflammatory responses. The aim of this investigation was to determine the effect of selected probiotic strains on hBD-2 production by epithelial cells induced by pathologically relevant pro-inflammatory cytokines and the role of cytokine modulators in controlling hBD-2. Caco-2 colonic intestinal epithelial cells were pre-incubated with heat-killed probiotics, i.e. *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* strain MS15 (LF), followed by stimulation of hBD-2 by interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α) in the absence or presence of exogenous IL-10 or anti-IL-10 neutralising antibody. Cytokines and hBD-2 mRNA and protein were analysed by real-time quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. LcS augmented IL-1β-induced hBD-2, whereas LF enhanced TNF-α- and suppressed IL-1β-induced hBD-2. LF enhanced TNF-α-induced TNF-α and suppressed IL-10, whereas augmented IL-1β-induced IL-10. LcS upregulated IL-1β-induced TNF-α mRNA and suppressed IL-10. Endogenous IL-10 differentially regulated hBD-2; neutralisation of IL-10 augmented TNF-α- and suppressed IL-1β-induced hBD-2. Exogenous IL-10, however, suppressed both TNF-α- and IL-1β-induced hBD-2; LcS partially rescued suppression in TNF-α- and IL-1β-stimulation, whereas LF further suppressed IL-1β-induced hBD-2. It can be concluded that probiotic strains differentially regulate hBD-2 mRNA expression and protein secretion, modulation being dictated by inflammatory stimulus and resulting cytokine environment.

Keywords: probiotics, β-defensin-2, epithelial cells, inflammation

1. Introduction

The gut mucosal barrier has a complicated relationship with both luminal contents and the underlying immune cells of the lamina propria and Peyer's patches. The fundamental function of this mucosal barrier is to protect host tissue from damage, mediated by luminal pathogens and toxic products obtained from either pathogenic bacteria or food, while at the same time allowing uptake of nutrients. The mucosal barrier thus determines gut immune cell fate as either immune activation in response to pathogens or inducing immune hypo-responsiveness, thereby tolerating non-harmful beneficial products, such as food and commensal microbes. Integral to this mucosal barrier function is the interrelationship with commensal non-pathogenic bacteria. These resident commensal bacteria or exogenous bacteria in the case of ingested probiotic bacteria are beneficial to the host by maintaining barrier integrity, competing with pathogens for nutrients and binding sites on epithelial cells and modulating immune function by either stimulating or tolerising immune responses (Walker, 2008). The recognition by and interaction of harmful or non-harmful beneficial bacteria with epithelial cells is thus of paramount importance to immune fate, i.e. tolerance or activation.

A protective mechanism elicited by epithelial cells in response to a broad range of Gram-positive and Gram-negative pathogens is the production and secretion of antimicrobial peptides (AMPs), such as defensins and cathelicidins (Kluver *et al*., 2006). Colonic epithelial cells have been described as potent producers of the inducible antimicrobial human β-defensin-2 (hBD-2). Defensins are small cationic peptides produced by epithelial cells, Paneth cells, neutrophils and macrophages contributing to broad spectrum innate immunity. hBD-2 is an inducible AMP with a molecular mass of 4-6 kD acting as an endogenous antibiotic in the defence against potential pathogenic microbes of the gut (for review, see Ganz, 2003). This AMP can be induced by endogenous stimuli, such as inflammatory cytokines (e.g. interleukin (IL)-1β, tumour necrosis factor alpha (TNF-α) and IL-22) as well as exogenous microbial products and their microbial associated molecular patterns (MAMPs) (Vora *et al.*, 2004). Due to their conserved nature, it is probable that MAMPs shared between pathogenic and probiotic bacteria display both beneficial and detrimental effects to the host, playing a pivotal role in homeostatic regulation in the gut mucosa as well as a pro-inflammatory pathological role.

Upon mucosal dysfunction and barrier breakdown, such as observed in inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), hBD-2 has been reported to be suppressed (Wehkamp *et al.*, 2005). Indeed, it has been observed that there is a differential expression of inducible and constitutive β-defensins in CD and UC (Wehkamp *et al.*, 2003). The overgrowth of hBD-2-sensitive pathogenic bacteria would represent a realistic mechanism of potentiation of such an inflammatory pathology of the gastro-intestinal tract (GIT). The immunopathology of these IBDs is driven by dysregulated T cell and macrophage function and the production of tissue-destructive proinflammatory cytokines (Foey, 2012). CD has been described to be driven by pathogenic mechanisms resulting from dysregulation in innate pattern recognition receptors, sensing and through the predominant differentiation and activation of T-helper Th_1 and Th_{17} subsets. This combination was found to result in dysregulated expression and functionality of IL-12, IL-23, IL-6, IL-1β, interferon gamma, IL-17 and TNF-α, which initiate and perpetuate inflammation. The suppression of hBD-2 observed in CD would seem to be integrally linked with this inflammatory cytokine profile, as hBD-2 enhances T cell IL-10 production whilst suppressing IL-17 (Kanda *et al.*, 2011). The lack of hBD-2 would thus have the reverse effect, suppressing the anti-inflammatory cytokine IL-10 and augmenting IL-17, potentiating inflammatory pathological mechanisms in IBD.

In addition to their antimicrobial role, defensins have been shown to display other functions that mediate immune responses and inflammation. For example, defensins have been described to augment IL-8 production (Kluver *et al.*, 2006; Van Wetering *et al.*, 1997a,b) and both α- and β-defensins to be chemotactic for T cells, DCs, macrophages and monocytes (Chertov *et al.*, 1996; Garcia *et al.*, 2001; Territo *et al.*, 1989; Yang *et al.*, 2000). Defensins have been demonstrated to augment TNF-α and IL-1β production at the site of microbial infection, thus amplifying inflammatory mechanisms (Chaly *et al.*, 2000), whereas hBD-2, as indicated above, also possesses an anti-inflammatory/regulatory role by enhancing IL-10 and suppressing the pro-inflammatory cytokine IL-17 (Kanda *et al.*, 2011). It would appear that hBD-2 induction can exhibit either pro- or anti-inflammatory effects, overall response being determined by environmental context.

Probiotic bacteria have been suggested to be beneficial to the GIT by reinforcing mucosal barrier defences via the induction of antimicrobial peptides, such as hBD-2. Indeed, earlier studies have shown that the probiotic strain *Escherichia coli* Nissle 1917 induced hBD-2 expression and production in Caco-2 epithelial cells and that the molecule predominating this induction is the Toll-like receptor 5 pathogen-associated molecular pattern (PAMP) flagellin (Schlee *et al.*, 2007; Wehkamp *et al.*, 2004). In addition, hBD-2 was found to be induced in Caco-2 cells by a range of specific lactic acid bacteria (LAB) strains and combinations, such as VSL#3 formulation; hBD-2 mRNA expression was induced by LAB, peaking at 6 h and persisting to 24 h, whereas secretion of hBD-2 peptide was very low in comparison to mRNA expression (Schlee *et al.*, 2008). Our previous investigations have demonstrated that probiotic bacteria exert differential effects on inflammation (immunoactivatory or immunoregulatory) and that the overall modulation of the resulting response was dependent on the inflammatory context of immune cell and probiotic strain being used (Habil *et al.*, 2011, 2012). When administered prophylactically, certain probiotic bacteria have been documented to afford health benefits to healthy subjects through the modulation of immune mechanisms via immunostimulation or -regulation. Indeed, this health benefit has been demonstrated in reduced incidence of allergic pathologies and cancer (Ishikawa *et al*., 2005; Kalliomaki *et al*., 2003; Rafter *et al*., 2007; reviewed in Hardy *et al.*, 2013). The specific health benefits of probiotics administered during chronic inflammatory pathology, however, are less well established. The aim of this study was to investigate the immunomodulatory effects of selected probiotic bacterial strains on the induction of hBD-2 expression and secretion by a cell line model of human intestinal epithelial cells in the presence of inflammatory cytokines that predominate and drive IBD pathogenesis.

2. Materials and methods

Bacterial culture and preparation of heat-killed extract

Lactobacillus fermentum strain MS15 (LF) was isolated from the crop of a chicken (Savvidou, 2009) and kindly provided by Dr. Jane Beal from internal microbiology stocks at the University of Plymouth (UK). *Lactobacillus casei* strain Shirota (LcS) was obtained from a commercial probiotic product (Yakult Honza Ltd., Tokio, Japan). All probiotic bacterial species were cultured in De Man Rogosa Sharp (MRS) broth at 37 °C for 18 h until the beginning of the stationary phase. Heat-killed (HK) bacterial samples was prepared according to the method described by Young *et al.* (2004). In brief, bacterial cells were centrifuged and washed twice in phosphate buffered saline; viable counts were adjusted to a density of 1×10^9 cfu/ml. Probiotic bacteria were heat-killed for 2 h at 90 °C. To confirm death of bacteria, all *Lactobacillus* spp. samples were plated on MRS agar and incubated for a minimum of 18 h. All HK bacterial extracts were Gram stained to check bacterial integrity after heating.

Cell culture

Caco-2 (human colon adenocarcinoma) epithelial cell line was kindly provided by Dr. Maria O'Connell (HNR, Cambridge, UK). Caco-2 cells were maintained in D10 medium, Dulbecocos' Modified Eagles' Medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Lonza, Wokingham, UK). Cells were plated at a density of 5×10^5 cells/ml and cultured in D10 medium in a humidified atmosphere at 37 °C and 5% CO_2 for 21 days to allow for full cell differentiation.

Modulatory effect of heat-killed probiotic bacteria

Differentiated Caco-2 cells were pre-incubated in D10 medium for 18 h without or with HK probiotic bacteria at a predefined optimal density equivalent of 1×10^8 cfu/ml. As HK probiotic bacteria gave similar hBD-2 mRNA induction time course profiles as live bacteria (shown in Results section), the former were adopted for subsequent experimentation to negate any effects of lactic acid on cell viability, hence indirectly modulating hBD-2 responses. Pre-incubation was followed by stimulation with pro-inflammatory cytokines prevalent in IBD, i.e. 10 ng/ml TNF-α or 10 ng/ml IL-1β, over a pre-determined time point of 18 h optimal for pro-inflammatory cytokine stimulation and HK bacteria to induce hBD-2 mRNA expression and protein secretion from Caco-2 cells (shown in Results section). TNF-α-induction of TNF-α protein is problematic, when measuring secretion of TNF-α protein. To circumvent it, Caco-2 cells were stimulated using a pulse-chase protocol. Cells were stimulated by 10 ng/ml exogenously added TNF-α for 6 h, whereafter TNF-α was washed off the cells, replaced by fresh D10 medium and incubated (chased) up to 18 h. Cell viability of differentiated Caco-2 cells was routinely monitored by the MTT assay and found to be unaffected by the reagent concentrations and the incubation times used.

Effect of endogenous IL-10 on hBD-2 induction

To find out whether the induction of hBD-2 and its modulation by probiotic bacteria was associated with endogenous IL-10 activity, a neutralising antibody was employed. Caco-2 cells were pre-incubated for 18 h without or with HK probiotic bacteria followed by stimulation with either TNF-α or IL-1β in the absence or presence of the neutralising antibody anti-human IL-10 (clone JES3-9D7; Biolegend, San Diego, CA, USA) at a concentration of 10 µg/ml. This concentration has previously been shown to neutralise 10 ng/ml IL-10 (Parry *et al.*, 1997). Epithelial cells were incubated for another 18 h, whereafter they were washed and collected for mRNA extraction and quantification of hBD-2 gene expression; culture supernatant was collected to detect secreted hBD-2 protein by enzyme-linked immunosorbent assay (ELISA).

Real-time qPCR analysis of gene expression

Expression of hBD-2, TNF-α, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was assessed by real-time quantitative polymerase chain reaction (RT-qPCR). Following each treatment, the cells were washed with ice-cold PBS and total RNA was extracted using GenElute RNA isolation kit (Sigma-Aldrich, Poole, UK) according to the manufacturer's instruction. The total RNA concentration was determined using NanoVue spectrophotometer (GE Healthcare, Freiberg, Germany). RNA purity was assessed by examining the absorbance ratio at 260 and 280 nm, while integrity was verified by electrophoresis on 1% denaturing agarose gel. One microgram of total RNA was reverse transcribed using MMLV Reverse Transcriptase reaction kit (Sigma-Aldrich) as suggested by the supplier.

Sequence specific primers for hBD-2, TNF-α, IL-10 and GAPDH (Table 1) were designed using Primer Express Software (Applied Biosystems, Paisley, UK) and synthesised by Eurofins MWG Operon (Ebersberg, Germany). RT- qPCR was performed using StepOnePlus thermal cycler and Power SYBR Green kit (Applied Biosystems, Foster City, CA, USA) using 10 pmol of the forward and reverse primers for each target. Target amplification was carried out under the following conditions: preheating at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. RT-qPCR data were analysed following the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) using GAPDH as an endogenous

Table 1. Sequence of real-time PCR primers and estimated product size.

Target name	Forward primer 5'-	Size (bp)	Reverse primer 3'-	Size (bp)	Product size (bp)
GAPDH	CTGCTCCTCCTGTTCGACAGT	21	CCGTTGACTCCGACCTTCAC	23	100
hBD-2	CACCTGTGGTCTCCCTGGAA	20	CTCTGATGAGGGAGCCCTTTC	17	100
TNF-α	ACATCCAACCTTCCCAAACG	20	GCCCCCAATTCTCTTTTTGAG	22	151
IL-10	AGGAGGTGATGCCCCAAGCTGA	22	TCGATGACAGCGCCGTAGCCT	21	110

Abbreviations used: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; hBD-2= human β-defensin-2; TNF- α = tumour necrosis factor alpha; IL-10 = interleukin-10.

control and resting cells as a reference sample. Thus, the relative quantity of the target transcript is described as fold increase (RQ, relative quantitation) relative to the reference sample and GAPDH.

Quantification of hBD-2 and cytokine secretion

Secretion of hBD-2 and the cytokines IL-10 and TNF-α into cell culture supernatants by treated Caco-2 epithelial cells was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available paired capture and detection antibodies for TNF-α and IL-10 (BD-Pharmingen, Oxford, UK), and hBD-2 (PeproTech EC Ltd., London, UK). Protocols according to manufacturer's instructions were followed and compared to standard curves of recombinant human cytokines using recognised international cytokine standards available from NIBSC (Potter's Bar, UK) and recombinant hBD-2 (PeproTech EC Ltd.) over the range of 7 to 5,000 pg/ml. Colorimetric development was determined by an OPTIMax tuneable microplate reader at 450 nm and analysed by Softmax Pro software version 2.4.1 (Molecular Devices Corp., Sunnyvale, CA, USA).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Statgraphics Software version 5.1 (Informer Technologies, Inc.) was used to analyse the data. All data are expressed as the mean ± standard error of triplicate values for a representative experiment of at least three independent experiments. Statistical significance of treatment compared to stimulation control is indicated by a *P*-value <0.05.

3. Results

Induction of epithelial cell hBD-2 by probiotic bacteria and pro-inflammatory cytokines

The ability of probiotic bacteria and pro-inflammatory cytokines associated with inflammatory pathology (TNF-α and IL-1β) to modulate epithelial cell induction of hBD-2 expression was investigated. Live and HK probiotic bacteria were incubated over a time period of 24 h with Caco-2 epithelial cells. At $1x10^3$ and $1x10^6$ cfu/ml, no significant difference was observed between induction of hBD-2 mRNA expression by live and HK bacteria. However, a significant difference was observed at 1×10^9 cfu/ml. From 12 to 24 h, relative hBD-2 mRNA levels were increased by 1000% and 1,300% for live LcS and LF, respectively (Figure 1A), whereas HK LcS and LF resulted in 23,800% and 23,100% augmentation, respectively, over the same period (Figure 1B). hBD-2 was truly inducible; no detectable mRNA levels were observed in unstimulated epithelial cells over the same time period and no protein secretion was detected over the lower levels of sensitivity of the sandwich ELISA (7 pg/ml) (data not shown).

The pro-inflammatory cytokines TNF-α and IL-1β predominating the pathology of chronic IBD were found to induce both expression of hBD-2 mRNA (Figure 1C) and protein secretion (Figure 1D) over a time period of 24 h. No hBD-2 mRNA expression or secreted protein was detected in unstimulated cells. The kinetics of hBD-2 protein secretion were comparable for both TNF-α and IL-1β stimulation; protein secretion continued beyond 18 h, at which time point hBD-2 secretion was 81±1 and 91±4 pg/ml, respectively. Expression of hBD-2 mRNA peaked at 9 h for IL-1β and 18 h for TNF-α. Both TNFα- and IL-1β-induced hBD-2 mRNA persisted beyond 20 h incubation (Figure 1C).

Modulation of hBD-2 depending on inflammatory environment

The pro-inflammaotry cytokines TNF-α and IL-1β clearly induced hBD-2 in Caco-2 cells (Figure 2). Probiotic modulation of the epithelial cell defence is less clear with respect to inflammatory pathology of the gastrointestinal tract. Depending on disease classification, environmental factors and genetics either IL-1β or TNF-α can predominate in driving destructive inflammatory pathological mechanisms observed in IBD. It was hypothesised that TNF-α- and IL-1β-stimulated epithelial cells would exhibit differential probiotic modulation of hBD-2 induction.

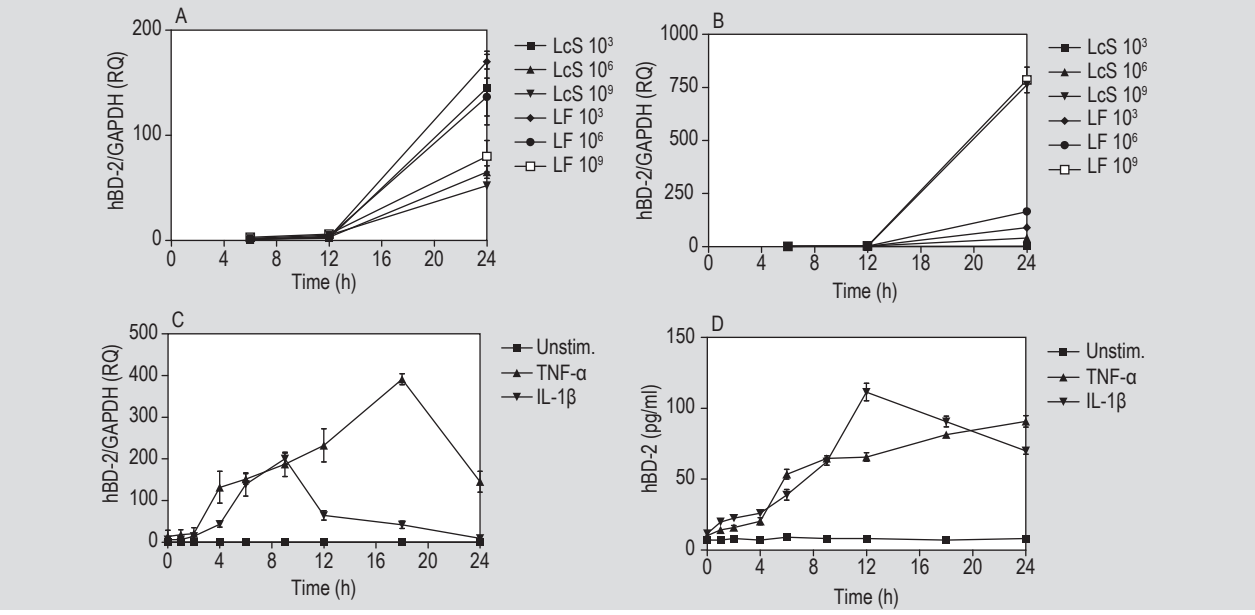

Figure 1. Induction of human β-defensin -2 (hBD-2) in intestinal epithelial cells by live and heat-killed probiotic bacteria, and pro-inflammatory cytokines. Caco-2 cells were incubated with (A) live and (B) heat-killed *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* (LF) at 1×103, 1×106 and 1×109 cfu/ml for an incubation time course up to 24 h. The effects of culture with the pro-inflammatory cytokines, TNFα (10 ng/ml) and IL-1β (10 ng/ml), on the induction of (C) hBD-2 mRNA expression and (D) protein secretion over 24 h is also shown. Induction of hBD-2 mRNA expression is presented as relative expression (RQ) compared to GAPDH housekeeping gene expression, and protein secretion as the mean hBD-2 protein (pg/ml) ± standard deviation. Data shown are of triplicate samples for a representative experiment of n=3 independent replicate experiments.

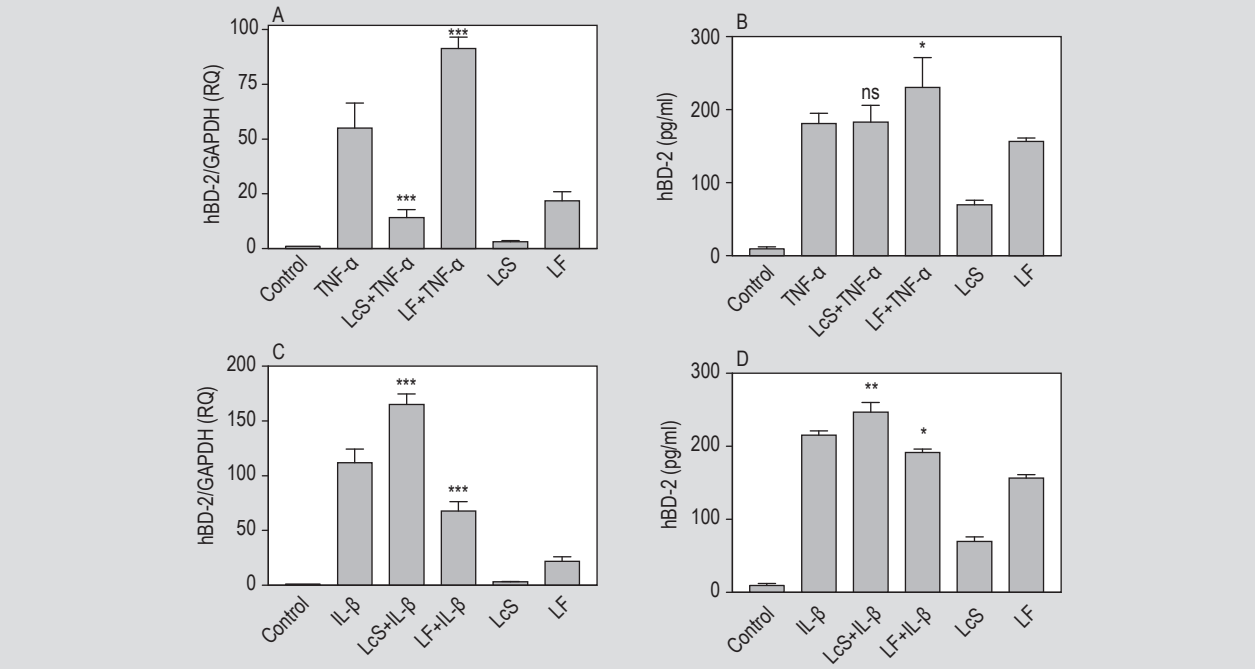

Figure 2. Heat-killed probiotic bacteria differentially modulate epithelial cell hBD-2 induced by the pro-inflammatory cytokines, IL-1β and TNFα. Caco-2 cells were pre-treated without or with heat-killed *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* (LF) for 18 h followed by stimulation with 10 ng/ml TNFα (A and B) or 10 ng/ml IL-1β (C and D) for a further 18 h. Induction of hBD-2 mRNA expression (A and C) is presented as relative expression (RQ) compared to GAPDH housekeeping gene expression, and protein secretion (B and D) as the mean hBD-2 protein (pg/ml) ± standard deviation. Data shown are of triplicate samples for a representative experiment of n=3 independent replicate experiments. Significant effects of the probiotic strains are compared to the cytokine-stimulated control and are indicated as * P<0.05, ** P<0.01, *** P<0.001 and ns (not significant).

Indeed, the probiotics studied modulated hBD-2 in a strain-dependent manner. TNF-α induction of hBD-2 mRNA was suppressed by LcS (75%; $P<0.001$) and augmented by LF (65%: $P<0.001$) (Figure 2A). TNF-α-induced hBD-2 protein secretion was augmented by LF (28%; $P=0.045$), whereas LcS did not modulate hBD-2 protein secretion ($P=0.126$) (Figure 2B). Compared to TNF-α, IL-1β induction of hBD-2 mRNA showed a different pattern. hBD-2 mRNA (Figure 2C) and protein secretion (Figure 2D) were suppressed by pretreatment with LF and augmented by LcS. LF suppressed induction of hBD-2 mRNA by 39% ($P<0.001$) and protein secretion by 12% ($P=0.018$; LcS augmented mRNA expression by 47% ($P<0.001$) and protein secretion by 15% ($P=0.005$).

Modulation of TNF-α- and IL-1β-induced cytokines by heat-killed probiotic bacteria

In the homeostatic regulatory mucosal environment, mucosal epithelial cells and tissue as a whole exhibit a tolerogenic or regulatory/anti-inflammatory environment characterised by anti-inflammatory cytokines, such as IL-10. On the other hand, expression of TNF-α is prevalent in inflammatory mucosal environments. It is likely that both IL-10 and TNF-α exert modulatory effects on the production of hBD-2 and that they are differentially modulated in intestinal epithelial cells by HK probiotic bacteria. The probiotic bacteria LcS and LF differentially modulated epithelial cell TNF-α and IL-10 in a manner dependent on inflammatory stimuli, TNF-α or IL-1β, and probiotic bacterial strain. LcS suppressed TNF-α-induced TNF-α mRNA (48%; $P=0.125$), whereas LF augmented it (270%; $P<0.001$); protein levels, however, were unchanged in the presence of LcS ($P=0.583$) but increased by LF (37%; $P=0.031$) (Figures 3A and B). TNF-α-induced IL-10 mRNA was augmented by 10% by LcS ($P=0.629$) and suppressed by LF by 61.5% ($P=0.021$) (Figure 3C). This trend was also seen for IL-10 protein secretion, which was not modulated by LcS ($P=0.707$) but suppressed by LF (73%; $P=0.003$) (Figure 3D).

In contrast, probiotic modulation of IL-1β-induced Caco-2 cell cytokines exhibited a different pattern compared to TNF-α-induced inflammatory cytokines. IL-1β induced TNF-α mRNA; LcS and LF enhanced this induction by 4,300% ($P<0.001$) and 200% ($P=0.125$), respectively (Figure 4A). This result was not reflected in TNF-α protein secretion, which was not modulated by LcS and LF (Figure 4B). IL-1β induction of IL-10 expression displayed a similar trend regarding mRNA and protein, but also showed a probiotic strain-dependent modulation. LcS suppressed IL-10 (83% for mRNA, $P<0.001$; 65% for protein secretion, $P<0.001$) and LF augmented this anti-inflammatory/regulatory cytokine (316% for mRNA, $P<0.001$; 75% for protein secretion, $P<0.001$) (Figures 4C and D).

Endogenous IL-10 activity differentially regulates cytokine-induced hBD-2

Due to the rather modest levels of protein secreted in the supernatant, and the need to make a direct link between probiotic modulation of the cytokines and the subsequent induction of hBD-2, the role of endogenous cell-associated anti-inflammatory IL-10 was investigated using a neutralising antibody. Data clearly show that endogenous IL-10 differentially modulates TNF-α- and IL-1β-induced hBD-2 in intestinal epithelial cells. The profile obtained for hBD-2 protein produced (Figure 5B) and secreted into the supernatant follows that of hBD-2 mRNA expression (Figure 5A). In the absence of inflammatory stimuli, epithelial cells do not express or secrete hBD-2; neutralisation of endogenous IL-10 activity, however, resulted in augmentation of both hBD-2 mRNA (2,500%; $P<0.001$) and protein (1,600%; $P<0.001$). TNF-α-induced hBD-2 mRNA and protein were augmented upon neutralisation of endogenous IL-10 by 250% ($P<0.001$) and 180% ($P<0.001$), respectively. In contrast to these observations, endogenous IL-10 exhibited a differential effect on IL-1β-stimulated hBD-2. Neutralisation of endogenous IL-10 suppressed mRNA expression by 83% ($P<0.001$) and hBD-2 protein secretion by 50% ($P<0.001$).

Exogenous IL-10 differentially regulates cytokine-induced hBD-2

As suggested by the neutralisation of endogenous IL-10 activity, IL-10 could both negatively and positively regulate hBD-2 under certain inflammatory environments. This was further investigated by the addition of exogenous IL-10 in the presence of the pro-inflammatory stimulators of epithelial cell hBD-2. The addition of exogenous IL-10 to TNF-α-stimulated epithelial cells resulted in a clear suppression of hBD-2 mRNA by 96% and protein by 83%. Exogenous IL-10 also suppressed IL-1β-stimulated hBD-2, but to a lesser extent; mRNA was suppressed by 57% and protein by 27% (Figures 6). In addition, the effect of the probiotic strains LcS and LF on this modulation of hBD-2 by exogenous IL-10 was also investigated. The modulation observed was dependent on the inflammatory cytokine inducing hBD-2 and the probiotic strain. When stimulated by TNFα, both probiotic strains partially rescued IL-10-induced suppression of hBD-2 mRNA and secreted protein. The suppression of hBD-2 mRNA to 4% of the TNF-α stimulus control was increased to 13% ($P=0.121$) and 48% ($P<0.001$) following the addition of LcS and LF, respectively, whereas hBD-2 protein suppressed to 17% of the TNF-α stimulus control was increased to 32% ($P=0.036$) and 49% ($P<0.001$) by the probiotic strains (Figures 6A and B). Regarding IL-1β-induced hBD-2, LcS completely rescued mRNA expression and even augmented expression by 41% of the IL-1β stimulus control ($P<0.001$); LF rescued IL-10 suppression from 43% to 88% ($P<0.001$) (Figure 6A).

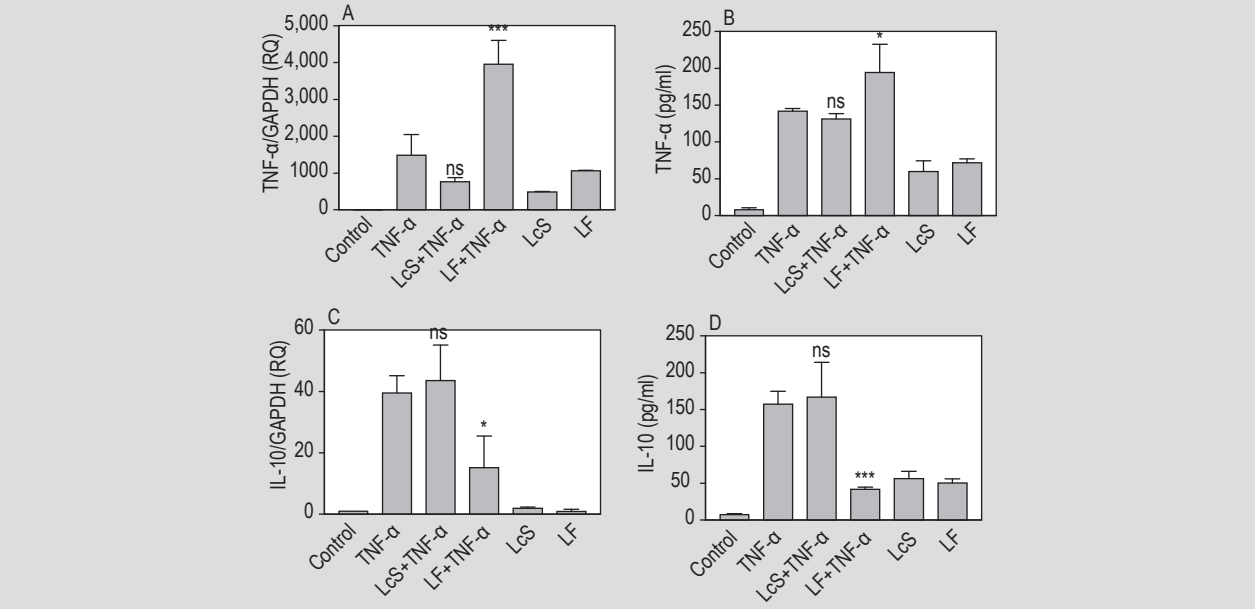

Figure 3. Modulation of TNFα-induced epithelial cell cytokines by heat-killed probiotic bacteria. Caco-2 cells were pre-treated without or with heat-killed *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* (LF) for 18 h followed by stimulation with 10 ng/ml TNFα for a further 18 h. Induction of mRNA expression for (A) TNFα and (C) IL-10 is presented as relative expression (RQ) compared to GAPDH housekeeping gene expression and protein secretion as the mean (B) TNFα and (D) IL-10 protein (pg/ml) ± standard deviation. Data shown are of triplicate samples for a representative experiment of n=4 independent replicate experiments. Significant effects of probiotic strains are compared to the TNFα-stimulated control and are indicated as * P<0.05, *** P<0.001 and ns (not significant).

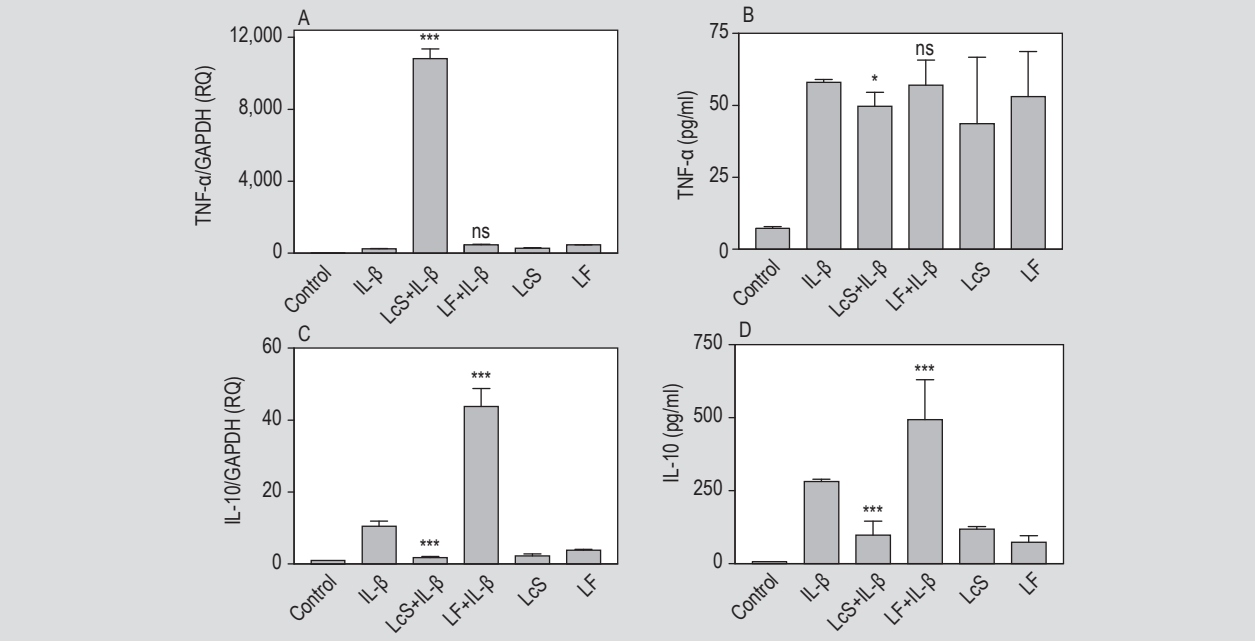

Figure 4. Modulation of IL-1β-induced epithelial cell cytokines by heat-killed probiotic bacteria. Caco-2 cells were pre-treated without or with heat-killed *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* (LF) for 18 h followed by stimulation with 10 ng/ml IL-1β for a further 18 h. Induction of mRNA expression for (A) TNFα and (C) IL-10 is presented as relative expression (RQ) compared to GAPDH housekeeping gene expression, and protein secretion as the mean (B) TNFα and (D) IL-10 protein (pg/ml) ± standard deviation. Data shown are of triplicate samples for a representative experiment of n=3 independent replicate experiments. Significant effects of probiotic strains are compared to the IL-1β-stimulated control and are indicated as * P<0.05, *** P<0.001 and ns (not significant).

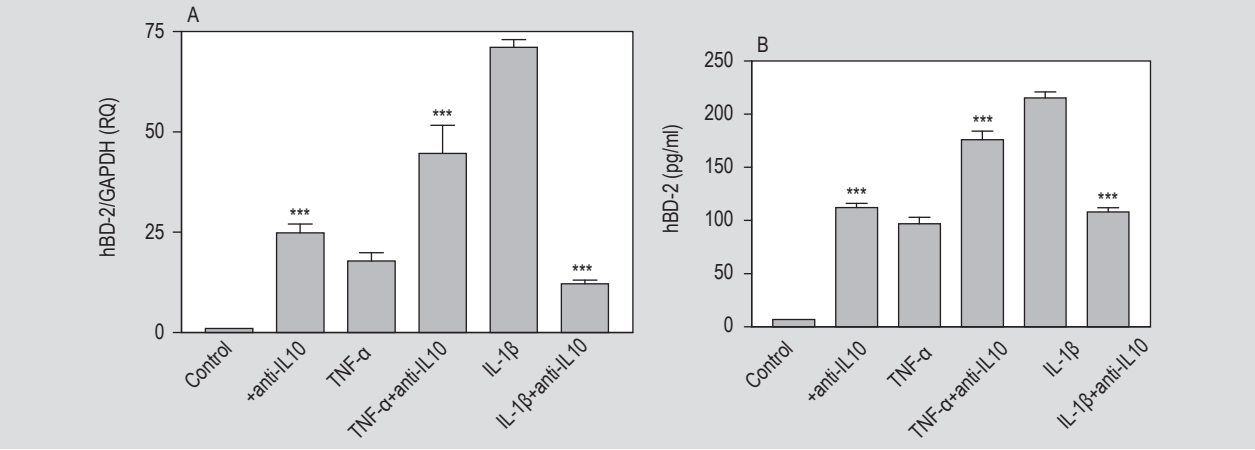

Figure 5. Endogenous IL-10 activity differentially regulates cytokine-induced hBD-2. Caco-2 cells were pre-treated without or with 10 mg/ml anti-IL-10 neutralising antibody (+anti-IL10) for 4 h followed by stimulation with 10 ng/ml IL-1β or 10 ng/ml TNFα for a further 18 h. Induction of (A) hBD-2 mRNA expression is presented as relative expression (RQ) compared to GAPDH housekeeping gene expression, and (B) protein secretion as the mean protein (pg/ml) ± standard deviation. Data shown are of triplicate samples for a representative experiment of n=3 independent replicate experiments. Significant effects of endogenous IL-10 are compared to the respective unstimulated or cytokine-stimulated control and significance indicated as *** P<0.001.

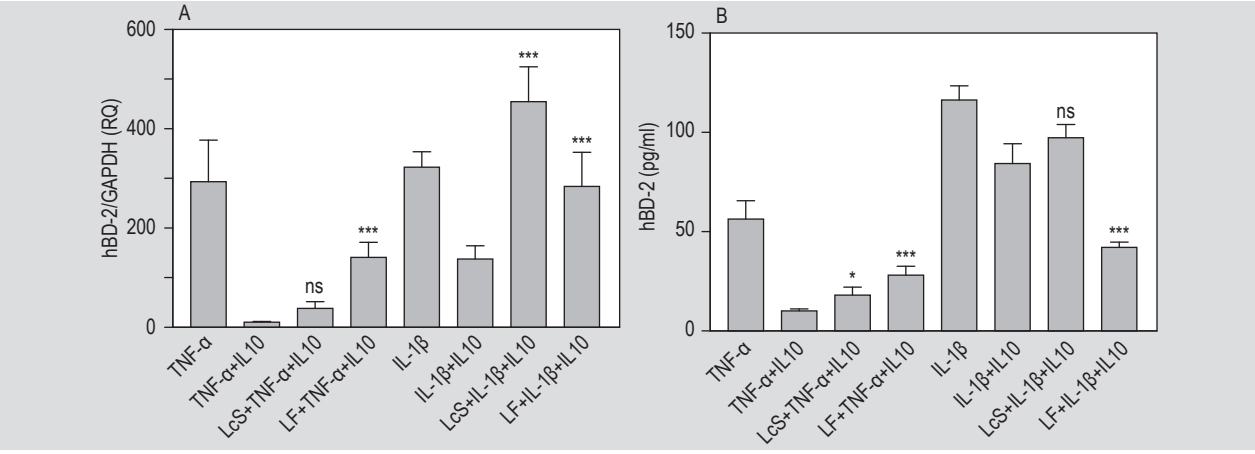

Figure 6. Exogenous IL-10 suppresses cytokine-induced hBD-2 production: modulation by heat-killed probiotic bacteria. Caco-2 cells were pre-treated without or with heat-killed *Lactobacillus casei* strain Shirota (LcS) or *Lactobacillus fermentum* (LF) for 18 h followed by stimulation with 10 ng/ml TNFα or 10 ng/ml IL-1β for a further 18 h in the absence or presence of 10 ng/ml IL-10. Modulation of (A) hBD-2 mRNA expression and (B) protein secretion by exogenous IL-10 and the rescue effect of heat-killed probiotic bacteria on IL-10-mediated suppression are presented as relative expression (RQ) compared to GAPDH housekeeping gene expression and mean protein (pg/ml) ± standard deviation, respectively. Data shown are of triplicate samples for a representative experiment of n=3 independent replicate experiments. Significant effects of modulation of exogenous IL-10 response by heat-killed probiotic strains are compared to the cytokine stimulus + exogenous IL-10; significance is indicated as * P<0.05, *** P<0.001 and ns (not significant).

This profile of probiotic modulation of IL-10 suppression of IL-1β-induced hBD-2 mRNA was not repeated when observing the effects on secreted protein induction. LcS partially rescued the IL-10-mediated suppression from 73% to 84% of the IL-1β stimulus control (P=0.065), whereas LF showed a further suppression from 73% to 36% (P<0.001) (Figure 6B).

4. Discussion

The HK probiotic strains LcS and LF were shown to exhibit a differential modulatory activity on hBD-2 induced during an inflammatory signal, such as provided by TNF-α or IL-1β. These cytokines are representative of inflammatory pathology and associated with IBD pathogenesis (Strober *et al.*, 2010). However, the design of the present experimental procedure was representative of acute inflammatory

episodes and the modulation of these episodes using a regimen of probiotic transient pretreatment. This study should be extrapolated to consider chronic inflammation, in which TNF-α or IL-1β are present for much longer time periods. Chronic stimulation by pro-inflammatory cytokines has been indicated to induce immune hyporesponsiveness (Cope, 1998; Cope *et al.*, 1994, 1997); whether such an effect is observed with epithelial cells awaits investigation. In addition, the modulatory effects observed with the HK probiotic bacteria might be completely different on chronically stimulated epithelial cells. These studies are vital to our understanding of the efficacy of probiotic use for early or late-stage IBD.

Probiotics are generally viable beneficial microbes. The use of HK bacteria in this study was not ideal but needed for the interpretation of responses observed, in the context of studying the direct modulatory activity of probiotic bacteria on induction of hBD-2 in intestinal epithelial cells. Probiotics, such as LABs, produce acid endproducts, which have grave effects on epithelial cell functionality and viability. In the absence of a complete gut microbiome, digesta, mucus glycocalyx, and family of interacting immune cells and lamina propria cells, epithelial cells are extremely sensitive to these acid endproducts. Heat-killing probiotic bacteria was adopted to minimise acid effects. This represented a compromise at a defined metabolic snap-shot of bacterial growth. More eloquent approaches considered included acid neutralisation, reduction of metabolic activity by low glucose concentrations and the use of antibiotics; alternative methods that are not without problems when considering effects of metabolic activity.

The HK probiotic strains LcS and LF, and the pro-inflammatory cytokines TNFα and IL-1β induced hBD-2 mRNA expression. Optimal expression occurred from 12 h onwards and persisted to 24 h for each stimulus, secreted protein levels being optimal at 18 h of stimulation. This would suggest that (1) hBD-2 mRNA was very stable, as it persisted up to and beyond 18 h, and p38 mitogen-activated protein kinase (MAPK) might play a predominant role in hBD-2 expression, as this signal component is well established to result in stabilisation of mRNA transcripts (Tudor *et al.*, 2009) through the binding of factors such as tristetraprolin (TTP) to the 3'UTR of TNF-α; and (2) levels of hBD-2 protein being secreted did not conform to such high induction levels of mRNA. This is suggestive of significant posttranscriptional and posttranslational regulation, where relatively low levels of protein secreted may be explained by intracellular protein or endogenous cell membrane-associated hBD-2 activity being induced by cytokines (Hugo *et al.*, 2010). This observation appears to conform to the data of Schlee *et al.* (2008). These authors observed very low hBD-2 secretion upon heat-inactivated probiotic stimulation; LF strain PZ1162 induced secretion up to 0.4 pg hBD-2 per 10^6 Caco-2 cells. In contrast to the present study, Schlee *et al.* (2008) described that hBD-2 mRNA was rapidly induced, peaking at 6 h. This might be the consequence of using undifferentiated and 21-day-differentiated Caco-2 cells. The present study demonstrated early mRNA and protein secretion when Caco-2 cells were stimulated by TNF-α and IL-1β. With respect to modulation of pro-inflammatory cytokine-induced hBD-2, LF suppressed IL-1β-induced hBD-2 mRNA and secreted protein, whereas LcS augmented hBD-2. This was in contrast with TNF-α-induced hBD-2; LcS suppressed TNF-α-induced hBD-2 mRNA expression, while LF augmented TNFα-induced mRNA and protein secretion. This suggests that TNFα-induced hBD-2 protein is strain-dependent. There is need to fully investigate probiotic modulation of posttranscriptional and posttranslational mechanisms involved in hBD-2 production.

In these longer-term cultures (pretreatment with HK bacteria for 18 h followed by a stimulation period of 18 h), it is not clear whether HK probiotic bacteria modulate TNF-α- and IL-1β-induced production of hBD-2 or, conversely, pro-inflammatory cytokines modulate probiotic-driven hBD-2 response. This study attempted to investigate how probiotics modulate hBD-2 induced in the presence of an inflammatory stimulus predominating in gut mucosal pathologies, such as CD. The longer time duration of probiotic pretreatment adopted was representative of a time where direct probiotic effects on hBD-2 are on the decrease (Schlee *et al.*, 2008) and mimicked the real-life delivery of probiotics over a protracted time period. Whether this approach is relevant to short-term early-stage or chronic IBD and probiotic treatment is beneficial or detrimental awaits clarification. In addition to the direct induction of this antimicrobial peptide, hBD-2 may also exert both a positive feed-back auto-induction loop and directly regulate inflammatory cytokines. Thus, modulation of hBD-2 will exhibit immunomodulatory properites (Biragyn *et al.*, 2002; Chaly *et al.*, 2000; Kanda *et al.*, 2011; Presicce *et al.*, 2009; and reviewed in Kluver *et al.*, 2006).

Cytokines are pivotal to the immune-responsiveness of gut mucosal tissue. Here, the anti-inflammatory/regulatory cytokine, IL-10 is integral to mucosal tolerance and tissue homeostasis, whereas TNF-α and IL-1β predominate in inflammatory pathology arising from a breakdown in mucosal tolerance (reviewed in Foey, 2012). The balance between such pro-inflammatory and regulatory cytokines plays a vital role in the induction of expression and secretion of hBD-2, which acts as both an AMP and a modulator of immune responses. TNF-α-stimulation induced both expression and secretion of TNF-α and IL-10 by colonic epithelial cells. These cytokines were regulated by HK bacteria in a strain-dependent manner, where LF augmented both TNF-α mRNA expression and protein secretion, and LcS failed to significantly modulate TNF-α. Of particular interest, however, was the observation that TNF-α-induced

TNF-α mRNA and secreted protein levels correlated with each other, although induction of TNF-α mRNA appeared to be disproportionately large compared to the small levels of protein secreted. With respect to induction of IL-10, LcS failed to modulate both TNF-α-induced IL-10 mRNA expression and protein secretion. This lack of effect on protein secretion, despite a relatively high TNF-α-induction of IL-10 mRNA, may reflect the fact that IL-10 can also be produced as a membrane-bound activity (Fleming *et al.*, 1996), which may or may not be cleaved for secretion. On the other hand, HK LF suppressed TNF-α-induced IL-10 mRNA and secreted protein levels. HK probiotic bacteria also differentially modulated IL-1β-induction of epithelial cell cytokines. LcS greatly augmented IL-1β-induced TNF-α mRNA, which failed to be reflected by secreted protein. LF only partially upregulated TNF-α mRNA but had no effect on secreted protein. This again could have been reflected by mRNA stability and posttranscriptional processes resulting in membrane-bound, endogenous protein rather than secreted protein. In addition, this may also be indicative of TNF-α scavenging systems via TNF-Rp75, perpetuating an inflammatory response whilst concurrently reducing secreted TNF-α protein levels or shed TNF-R binding to and interfering with the TNF-α ELISA detection system. A different pattern of modulation was displayed for IL-10, where probiotic strain-specific responses were observed; LcS suppressed IL-1β-induced IL-10 and LF augmented IL-10. Taken together, it would appear that LcS modulation exhibits a pro-inflammatory profile and LF an anti-inflammatory profile when being induced by IL-1β, whereas TNF-α-stimulation of Caco-2 cells resulted in LF exhibiting a pro-inflammatory profile and LcS a balanced pro- and anti-inflammatory profile. This effect on modulatory behaviour is also demonstrated in macrophages, where differential regulation of pro-inflammatory cytokines is dependent on both probiotic bacterial strain and differentiation status of the responding cell (Habil *et al.*, 2011, 2012).

IBD is a chronic inflammatory pathology that exhibits a wide range of inflammatory mechanisms. These mechanisms involve a variety of cells and molecules, which depend on trigger, genetics and immune-inflammatory stimuli. There is a cytokine expression hierarchy in which TNF-α and IL-1β predominate this pathology. These cytokines represented an appropriate focus for investigation, as they are generally co-expressed in inflammatory pathologies. The study of single cytokine effects is valid as most inflammatory pathologies exhibit differential cytokine dominance, however, the present investigation represents a starting point from which to interpret complex multi-cytokine responses. Future studies will focus on probiotic modulation of expression and functionality of more appropriate cytokine combinations, including TNF-α and IL-1β with other pro-inflammatory cytokines expressed in IBD, such as IL-12, IL-6, IL-15, IL-18, and IL-17, reflective of the 3-dimensional nature of the disease characterised by multifactoral and multicellular interactions.

This study demonstrated that the induction and modulation of both hBD-2 and cytokines TNF-α and IL-10 were probiotic bacterial strain- and inflammatory stimulus-dependent. The role of such cytokines in inducing/regulating epithelial cell hBD-2 became an important focus. Prior to inflammatory stimulation, Caco-2 cells expressed an endogenous IL-10 activity negatively regulating hBD-2, as evidenced by the augmentation of hBD-2 mRNA and secreted protein by anti-IL-10 neutralising antibody in the absence of stimulation. This observation alone conforms to the homeostatic, regulatory nature of healthy tolerant, un-stimulated intestinal epithelial cells. This negative regulatory aspect of endogenous IL-10 was reproduced when hBD-2 was stimulated by TNF-α. Surprising, however, was the observation that in the case of IL-1β-induced hBD-2 endogenous IL-10 exhibited a positive regulatory nature, as neutralisation suppressed hBD-2. In the context of IBD, where CD and UC exhibit differential modulation of hBD-2 (Aldhous, 2009; Wehkamp, 2005), hence anti-microbial activity, this variation in hBD-2 in response to endogenous IL-10 may be reflective of which pro-inflammatory cytokine predominates specific IBD pathologies.

To confirm the regulatory effect of endogenous IL-10, this anti-inflammatory cytokine was added exogenously to IL-1β- and TNF-α-stimulated epithelial cells in the presence or absence of LF and LcS. Exogenous IL-10 suppressed TNF-α-induced hBD-2 mRNA and secreted protein to basal levels. hBD-2 mRNA and secreted protein were only partially suppressed by IL-10 in the case of IL-1β-activated epithelial cells. This contrasts with the endogenous IL-10 result, where neutralisation suppressed hBD-2. It is possible that this unexpected sensitivity between exogenous and endogenous IL-10 reflects that endogenous, membrane-associated IL-10 requires direct cell-to-cell contact. The fact this observation is in contrast to the TNF-α-induced response may reflect differential IL-10 induction responses and IL-10R subunit patterns between IL-1β- and TNFα-stimulated Caco-2 cells. This suggests that IL-1β and TNF-α induce a differential sensitivity to IL-10 activity, possibly through differential IL-10R expression or utilisation of IL-10-dependent signalling components, such as JAK2 and STAT-3 (see review by Moore *et al.*, 2001). This observation alone may explain the rather poor anti-inflammatory effects of IL-10 when used to treat IBD (Tilg *et al.*, 2002); prognosis being dependent on prevalence of inflammatory cytokine. Regarding the effect of HK probiotic strains, both LcS and LF partially rescued the IL-10-induced suppression of hBD-2 (mRNA and secreted protein) observed in TNF-α-stimulated epithelial cells. In the case of IL-1β-induced hBD-2, LcS and LF totally rescued IL-10-mediated suppression of mRNA. In contrast, LF further suppressed IL-10-mediated suppression of hBD-2 secreted

protein. This LF-dependent response may be indicative of the need for careful investigation and future therapeutic intervention, where LF use, in combination with IL-10, maybe detrimental to IBD resulting in the decreased hBD-2 secretion associated with IBD pathology.

This study demonstrated that hBD-2 was differentially regulated by IL-10 and the HK probiotic strains LcS and LF in a manner dependent on inflammatory stimulus encountered by the intestinal epithelial cell. It is however, difficult to rationalise these results in the context of inflammatory pathology, such as IBD. hBD-2 was reported to be reduced in CD (Wehkamp, 2005), hence potentially resulting in overgrowth of pathogenic hBD-2-sensitive bacteria and perpetuation of inflammatory pathology. It is counterintuitive that the anti-inflammatory cytokine IL-10 suppresses hBD-2 and probiotic bacteria positively regulating IL-10 display a rescue effect. In addition, defensins have been shown to modulate cytokine production and DC functionality (Biragyn *et al.*, 2002; Presicce *et al.*, 2009). It is possible that reduced hBD-2 expression observed in CD not only results in a weakened anti-pathogen response, but also a weakened protective immune response mediated by DCs and other innate immune cells. Indeed, the complexity of hBD-2 function is being unravelled as hBD-2, and defensins in general, have been shown to be immune-modulatory, whereby regulating cytokine production, such as IL-1β, and inducing adhesion/migration of inflammatory Th_{17} subsets to endothelial cells (Ghannam *et al.*, 2011; Shi *et al.*, 2007). Exactly what modulatory function hBD-2 may feedback to epithelial cells and underlying mucosal immune cells awaits investigation.

Immune responses are generally weakened or dysfunctional in chronic inflammatory environments. This is induced by chronic inflammatory responses mediated by cytokines, such as TNF-α, resulting in suppressed receptor expression or dysfunctional receptor transduction events (reviewed by Cope, 1998). Long-term exposure to the inflammatory cytokines IL-1β or TNF-α, and pathogenic microbes/PAMPs may dysregulate immune inflammatory responses resulting in reduced protection to microbes and uncontrolled perpetuation of inflammation. The role of HK probiotic bacterial strains is quite different with respect to hBD-2 modulation in the context of IL-1β or TNF-α inflammatory stimuli, displaying both positive and negative responses. It is probable that host epithelial responses to beneficial microbes will differ according to probiotic strain, inflammatory stimuli and duration of stimuli (acute vs. chronic). With respect to chronic inflammatory stimuli, such as is the case in IBD, probiotics may exert different responses to those observed in acute situations, possibly inducing or perpetuating destructive, harmful inflammatory mechanisms. Future research efforts will help clarify these issues of probiotic effects in acute or chronic inflammatory pathology. Until then, a cautionary approach to probiotic usage by IBD patients is recommended.

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Conflict of interest disclosure

There are no conflicts of interest with this study.

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