



Research Paper

Heat-killed probiotic *lactobacilli* and cell wall components differentially induce production of human β -defensin-2 by intestinal Caco-2 cells *in vitro*

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ABSTRACT

Recent evidence suggests that probiotic bacteria may stabilize gut barrier function through induction of anti-microbial peptides such as defensins. This study aimed to elucidate the induction mechanism of the human beta defensin-2 (hBD-2) gene by different heat-killed (HK) probiotic lactobacillus strains and cell wall components. Caco-2 colonic intestinal epithelial cells were pre-incubated with heat-killed probiotics and cell wall components. hBD-2 mRNA and protein were analyzed using real-time quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. The results showed that HK and live lactobacillus strains differentially regulate hBD-2 mRNA expression and protein secretion. Isolated cell wall components (WCW, WPG, LTA) from probiotic lactobacilli induced hBD-2 mRNA significantly in contrast to the un-stimulated Caco-2 cells, and induction effects followed the trend WPG > HK > WCW > LTA > S in Caco-2. Significant increases of hBD-2 mRNA and protein were found following WPG incubation treatment at 8, 12, and 24 h post-incubation, respectively. Moreover, the expression of hBD-2 mRNA was synergistically up-regulated by cell wall components in combination with WPG under normal conditions, confirming that WPG is the major stimulatory factor of probiotic *lactobacilli*.

Lequan Qiu¹, Jialin Zhang¹, Huaxing Zhang², Qiaozhen Feng¹ and Shijin Wu^{1*}

¹College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310032, People's Republic of China.

²Ningbo Institute of Technology, Zhejiang University, Ningbo315100, People's Republic of China.

*Corresponding author. E-mail: wujian28@zjut.edu.cn. Tel: +86-571-88320243; Fax: +86-571-88320884.

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INTRODUCTION

Probiotics, defined as 'live microbial food supplements which improve the health of the host', have obtained increasing medical relevance (Isolauri et al., 2002). In the intestine they may prevent the overgrowth of pathogenic bacteria, increase the resistance of the gut to invasion by pathogens and ameliorate disease processes. The spectrum of probiotics, including *Escherichia coli*, lactobacilli, bifidobacteria and streptococci, as well as yeasts, is as complex as their clinical applications. For example, the duration of traveler's diarrhea and other self-limited gastrointestinal infections is shortened by probiotics especially in infants (Rembacken et al., 1999; Ganz, 2003).

Many mechanisms of action are proposed by which probiotics might enhance mucosal protection against gastrointestinal infections and reduce idiopathic

inflammation. The concept of a competition between probiotic and pathogenic bacteria for specific binding sites on intestinal epithelial cells is well established (Lee and Puong, 2002; Otte and Podolsky, 2004). It is postulated that probiotic effects on the barrier function, for example, enhanced phosphorylation of actinin and occludin in the tight junction region of epithelial cells which inhibited the invasion of enteroinvasive *E. coli* (Resta-Lenert and Barrett, 2003). Furthermore, probiotics also appear to directly affect mucosal immune function through modulation of immunoglobulin A (IgA) synthesis, mucus formation, or alterations of the pro-versus anti-inflammatory balance of local cytokines (Madsen et al., 2001; Cukrowska et al., 2002).

Intestinal mucosal epithelial cells play a crucial role in the

Table 1: Bacterial strains used in this study.

Species of subspecies (isolate)	Strain designation/serotype	Type of isolate	Source and/or reference
<i>Lactobacillus rhamnosus</i> HB3 (CCTCC53103)	LMG P-17806	Intestinal isolate	a
<i>Lactobacillus casei</i> HB9(CCTCC 334)	LMG P-17806	Intestinal isolate	a
<i>Lactobacillus plantarum</i> KB8(CCTCC 4356)	LMG P-17806	Intestinal isolate	a
<i>Esherichia coli</i> Nissle 1917 (EcN)	O6:K5:H1	Pharmaceutical	b, (Wehkamp et al., 2004)

a. China Center for Type Culture Collection (CCTCC, Wuhan, China). b. Ardeypharm Collection of Strains, Pharma-Zentrale GmbH, Herdecke, Germany.

local antimicrobial defense. These cells represent not only a physiologic barrier for pathogens, but also function as a part of the innate immune system as they for example, produce cytokines and antimicrobial peptides (Ganz and Lehrer, 1994). 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 and interaction of harmful or non-harmful beneficial bacteria with epithelial cells is thus of paramount importance to immune fate, that is, tolerance or activation.

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 (Ganz and Lehrer, 1994). The known human α -defensins include the human neutrophil peptides 1 to 4 as well as, the epithelial human defensin-5(HD-5) and HD-6.

Human β -defensin-1(hBD-1), -2, -3, and -4 are expressed in various epithelial cells. Defensins have a broad spectrum of antimicrobial activity against bacteria, fungi and some enveloped viruses and the mechanism is not fully understood (Yang et al., 1999; Ganz, 2003). Interestingly, Human beta-defensin 2 (HBD-2) is produced by epithelial cells primarily, but monocytes, macrophages and dendritic cells are also capable of HBD-2 production. The peptide is highly inducible due to various stimuli, such as inflammatory cytokines and bacterial, viral, fungal or protozoal infection and cell wall adhesive glycoprotein (Resta-Lenert and Barrett, 2003). Earlier studies have shown that the probiotic strain *E. 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 and cell wall peptidoglycan (Schlee et al., 2007). In addition, hBD-2 was found to be induced in Caco-2 cells by a range of specific lactic acid bacteria (LAB) strains and cell wall component combinations (Wehkamp

et al., 2002; Wehkamp et al., 2003). However, the mechanisms of cell wall components related stimuli hBD-2 induction are still largely unknown. Therefore, the topic of cell wall components related stimuli need for further study.

To test our hypothesis that cell wall components of probiotic lactobacilli may stimulate the colonic epithelial chemical defense system differentially, we decided to study the effect of various heat-killed probiotic lactobacilli and cell wall components of probiotics on the production of hBD-2 in human colonic epithelial cell lines *in vitro*. In order to reveal the molecular mechanism of the regulation of intestinal function by probiotics and to provide new ideas and basis for the selection, genetic modification and application of probiotics were projected in the future.

MATERIALS AND METHODS

Bacterial culture and preparation of heat-killed extract

Table 1 shows bacterial strains used in this study. 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 were 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 wall components were prepared according to the method described by Lomolino and Andrea (2007).

Cell culture

Caco-2 (human colon adenocarcinoma) epithelial cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 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% CO₂ for 21 days to allow for full cell differentiation.

RNA isolation and cDNA synthesis

At the end of the stimulation experiment, cells were washed with PBS and harvested with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the supplier's protocol. Subsequently, 1 µg of total RNA was reverse-transcribed into cDNA with oligo (dT) primers and 15 U/µg avianmyeloblastosis virus (AMV) reverse transcriptase (Promega), according to standard procedures.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. cDNA corresponding to 10 ng of RNA served as a template in a 10 µl reaction mixture containing 3 mM MgCl₂, 0.5 µM of each primer and 1×light Cycler- Fast Start DNA Master SYBR Green I mix (Roche Diagnostics GmbH). Initial denaturation at 95°C for 10 min was followed by 45 cycles, each cycle consisting of 95°C for 15 s, the primer-specific annealing temperature for 5 s and elongation at 72°C for 15 s. For hBD-2 (sense 5'-ATCAGCCATGAGGGTCTTGT-3'; anti-sense 5'-GAGACCACAGGTGCCAATTT-3') the annealing temperature was set at 62°C.

Amplification using these primers resulted in a 172 base pair (bp) fragment. As an internal control gene we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most commonly used housekeeping genes. For GAPDH (sense 5'-CCAGCCGAGCCACATCGCTC-3'; anti-sense 5'-ATGAGCCCCAGCCTTCTCCAT-3') we used a touchdown protocol with a primary temperature of 66°C and a target temperature of 60°C. At the end of each run melting curve profiles were achieved by cooling the sample to 65°C for 15 s and then heating slowly at 0 to 20°C/s up to 95°C with continuous measurement of fluorescence to confirm amplification of specific transcripts.

Cycle-to-cycle fluorescence emission readings were monitored and analyzed using Light Cycler software (Roche Diagnostics GmbH). Melting curves were generated after each run to confirm amplification of specific transcripts. The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2% agarose gel and visualization by ethidium bromide staining. The quantitative evaluation plasmids served as an external homologous DNA standard of known number of

copies. To create standard curves, the plasmids were serially diluted (1:10) covering the appropriate concentration range (Wehkamp et al., 2004). The mRNA expression is given as a ratio between the target gene and GAPDH gene expression.

Western blot assays

Cells (1×10^6) were homogenized in ice-cold lysis buffer containing RIPA buffer and protease inhibitor cocktail (Sigma) and the mixture was then centrifuged at 10,000 g for 10 min to remove cell debris. Protein concentrations of cell lysates were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Supernatants were mixed with Laemmli's sample buffer and boiled for 3 min. To detect beta defensin, aliquots of the supernatants containing 25 mg of total protein were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). Preblocked blots were reacted for 4 h with goat IgG anti-human beta-defensin 2 antibody (R&D Systems, Minneapolis, MN, USA) in PBS containing 0.05% (v/v) Tween-20, 1% (w/v) dried non-fat milk (Difco Laboratories, Detroit, MI, USA) and 1% (w/v) BSA (fraction V; Sigma). Blots were then incubated for 2 h with a peroxidase-conjugated anti-goat IgG antibody (Sigma-Aldrich). Filters were washed five times in PBSTween for 5 min after each step and developed using a chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Immunofluorescence and semi-quantitative assessment of fluorescence intensities

Expressions of HBD-2 peptide in Caco-2 cells were investigated from Cytospin preparations by immunofluorescence analysis. Cells were fixed in 1% acetone for 15 min at room temperature. Cells were stained with goat anti-HBD-2 antibody (R and D Systems) for 1 h. The secondary antibody was anti-goat IgG NorthernLights 493 (NL493) fluorochrome-labeled antibody (R and D Systems), which was applied for 45 min. After each incubation step, the cells were washed thrice with PBS containing 0.2% BSA. Fluorescence signals were analyzed through confocal microscopy. Eight serial images of each immunostained sample were captured by Olympus FV1000 confocal laser scanning microscope with standard parameter settings. The immunofluorescence of control cells was quantitatively analyzed by ImageQuant software (Molecular Dynamics) as follows: 6-6 equally sized circular areas covering the cells were randomly selected on each image. The backgrounds of the selected areas were eliminated by threshold set up and the fluorescence intensities/pixel values of the randomly selected cells quantified.

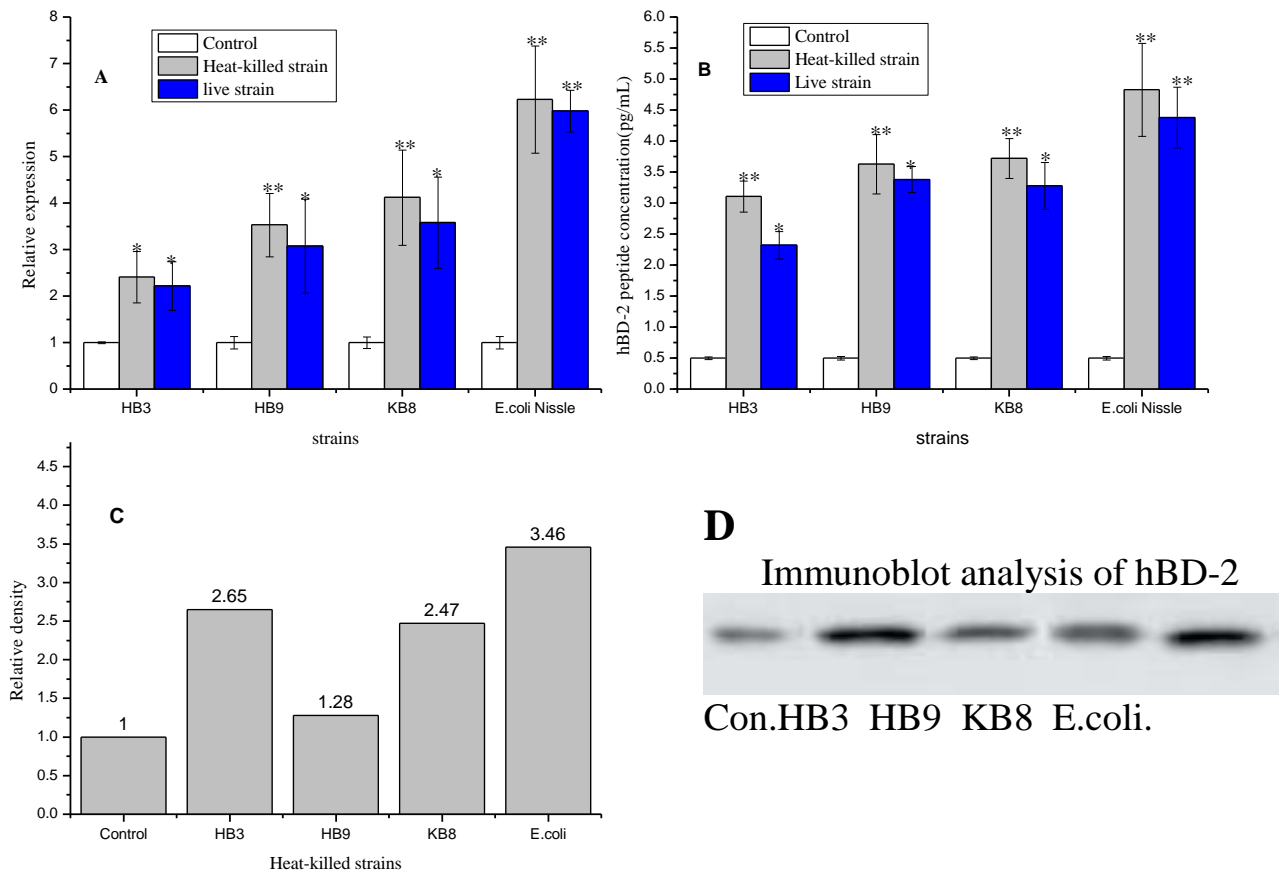


Figure 1: Panel A: hBD-2 mRNA relative expression in Caco-2 cells after stimulation with heat-killed and live probiotic lactobacilli strains. Caco-2 cells were infected with *Lactobacillus rhamnosus* HB3, *Lactobacillus casei* HB9 and *Lactobacillus plantarum* KB8, at 1×10^7 for 24 h. Uninfected Caco-2 cells or cells challenged with *E. coli* Nissle 1917 served as negative and positive controls, respectively. The hBD-2 mRNA levels were normalized to GAPDH, using quantitative real-time PCR. Panel B: hBD-2 peptide concentration in culture supernatants from Caco-2 cells infected with lactobacilli strain. Caco-2 cells were infected with *Lactobacillus rhamnosus* HB3, *Lactobacillus casei* HB9 and *Lactobacillus plantarum* KB8 at 1×10^7 for 24 h. Uninfected Caco-2 cells or cells challenged with *E. coli* Nissle 1917 served as negative and positive controls, respectively. The secreted hBD-2 peptide levels in the supernatants were measured by ELISA. Panel C and Panel D: Immunoblot analysis of hBD-2 peptide expression in heat-killed lactobacilli strain-stimulated intestinal epithelial cells. Caco-2 cell cultures were infected with heat-killed lactobacilli strain at 1×10^7 for 24 h. Total cellular protein (25 mg) from each culture was loaded on a 15% SDS-PAGE gel for immune-blotting. After transfer, the membranes were incubated with antibody to hBD-2, and the labeling was detected with ECL. Lane 1 represents non-stimulated lactobacilli strain cells whereas the other lanes are lactobacilli strain cells incubated with different heat-killed lactobacilli strain for 24 h. These results are representative of three experiments performed independently. Relative density to negative control cells were set at 1. Results are mean \pm S.E.M. ($n = 8$). The asterisks are indicator of statistical differences obtained separately at different time points compared to their controls shown in figure as "0". * $P < 0.01$, ** $P < 0.001$.

Statistical analysis

All values are expressed as mean \pm standard deviation (SD). For the quantification of HBD-2 expression using RT-PCR and ELISA, results from infected samples were compared with non-infected controls. The data were subjected to one way ANOVA test with Dunnett and Bonferroni post test according to the data set. For immunofluorescence intensity analysis, intensity data from infected cells were compared with those of the non-infected control.

For all statistical evaluations, $p < 0.05$ was considered statistically significant. Data analyses were performed by Graph PadPrism 5 (GraphPad Software Inc., San Diego, CA, USA) statistical program.

RESULTS

Expression of hBD-2 mRNA in Caco-2 cells induced with probiotic lactobacilli

Live and HK probiotic bacteria were incubated over a time period of 24 h with Caco-2 epithelial cells. RT-PCR was performed to test whether hBD-2 gene expression is induced in Caco-2 cells upon stimulation with live and HK probiotic bacteria. The relative expression of inducible hBD-2 mRNA was significantly increased in the cells infected with live and HK probiotic bacteria as compared to uninfected samples after 24 h of the stimulation and HK probiotic bacteria more strongly induced hBD-2 than live bacteria in Caco-2 cells (Figure 1A).

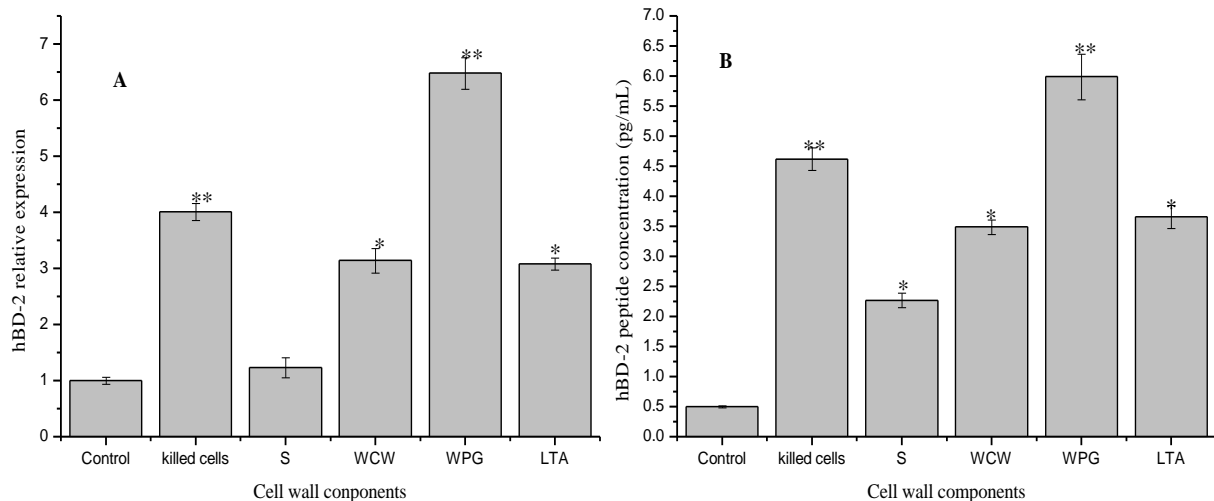


Figure 2: Panel A: hBD-2 mRNA relative expression in Caco-2 cells after stimulation for 24 h with different cell wall components. Caco-2 cells were stimulated with heat-killed *Lactobacillus plantarum* KB8 (1×10^7), cell supernatant (S), whole cell wall components (WCW), whole peptidoglycan (WPG) and lipoteichoic acid (LTA) at a concentration of 100 $\mu\text{g/ml}$ for 24 h. Un-stimulated Caco-2 cells served as negative controls, respectively. The hBD-2 mRNA levels were normalized to GAPDH, using quantitative real-time PCR. Panel B: hBD-2 peptide concentration in culture supernatants from Caco-2 cells stimulated with different cell wall components. Caco-2 cells were stimulated with heat-killed *Lactobacillus plantarum* KB8, cell supernatant (S), whole cell wall components (WCW), whole peptidoglycan (WPG) and lipoteichoic acid (LTA) for 8 h. Unstimulated Caco-2 cells served as negative controls, respectively. The secreted hBD-2 peptide levels in the supernatants were measured by ELISA.

Among the lactobacilli, KB8 was the most potent hBD-2 mRNA inducers (4.01 ± 0.5 fold increase), although none of them reached the level induced by the positive control, *E. coli* Nissle 1917 strain (6.46 ± 0.3 fold increase). Induction effects followed the trend *E. coli* Nissle 1917 > KB8 > HB9 > HB3 in Caco-2. Lactobacilli HB3 induced the least hBD-2 mRNA expression in this system (2.46 ± 0.3 fold increase). ANOVA test was used to evaluate whether the increase in hBD-2 expression was statistically significant. There was a significant difference between the values: $p = 0.0085$; but according to the Dunnett post test the difference was statistically significant ($p < 0.001$) only between control vs. HB9 and between control vs. KB8. The differences between species were statistically significant according to the Bonferroni post test (Figure 1A).

Probiotic lactobacilli induce hBD-2 secretion by Caco-2 cells

Experiments were performed to determine whether hBD-2 is secreted by the intestinal epithelial cells following live and HK probiotic lactobacilli bacteria stimulation. hBD-2 ELISAs showed that the concentration of hBD-2 protein was significantly elevated in the supernatant 24 h after stimulation (Figure 1B). KB8 induced the highest release of hBD-2 relative to the other lactobacilli species with HB3 and HB9 producing similar results. hBD-2 levels were low in the unstimulated control supernatant. These results imply that human epithelial cells release hBD-2 upon stimulation with lactobacilli spp., especially KB8. Again, ANOVA test was used to assess whether the increase in hBD-2 protein levels were statistically significant. There was a significant difference between the

values: $p = 0.0025$; but according to the Dunnett post test the difference was statistically significant only between control vs. live HB3 and HB9 ($p < 0.05$), and between control vs. HK KB8 ($p < 0.01$). The differences between species were statistically significant according to the Bonferroni post test.

hBD-2 peptide expression in Caco-2 cells induced with Probiotic lactobacilli

The expression of hBD-2 peptide in probiotic bacteria-stimulated epithelial cells was determined by Western blot analysis. Using the hBD-2 binding antibody, we identified a single band of about 4 kDa from epithelial cell lysates (Figure 1C and D). The signal intensity of the band increased in lysates of cells challenged with probiotic bacteria cells, with HB3 inducing the largest increase. The level of hBD-2 was only weakly detectable after the processing of the control cell lysate without probiotic bacteria infection.

Expression of hBD-2 mRNA in Caco-2 cells induced with cell wall components

Caco-2 cells were stimulated with heat-killed *Lactobacillus plantarum* KB8 (1×10^7), cell supernatant (S), whole cell wall components (WCW), whole peptidoglycan (WPG) and lipoteichoic acid (LTA) at a concentration of 100 $\mu\text{g/ml}$ for 8 h. The relative expression of inducible hBD-2 mRNA was significantly increased in the cells infected with cell wall components as compared to uninfected samples after 24 h of the stimulation (Figure 2A). WPG was the most potent hBD-2

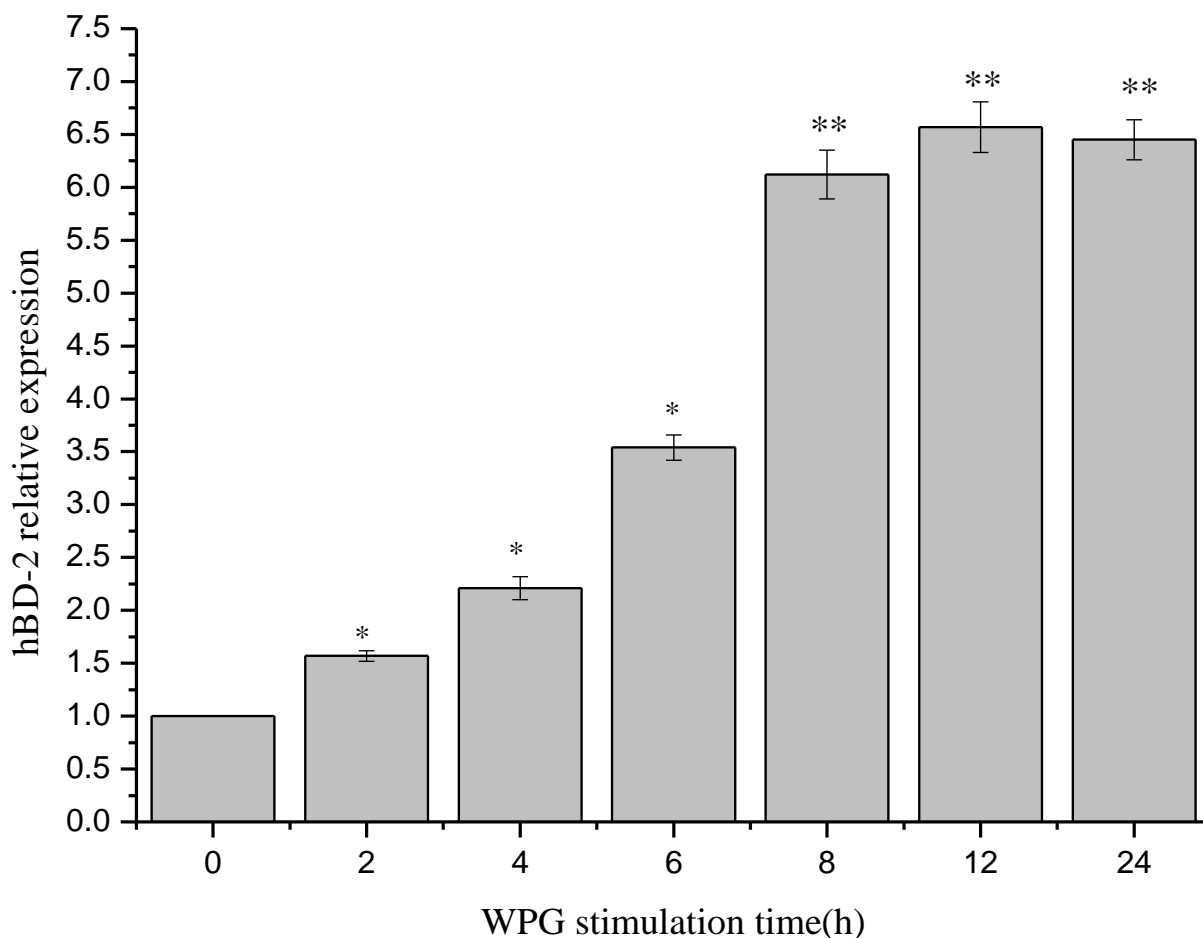


Figure 3: hBD-2 mRNA relative expression in Caco-2 cells after stimulation for 2, 4, 6, 8, 12, 24 h with WPG components. Caco-2 cells were stimulated with whole peptidoglycan(WPG) at a concentration of 100 $\mu\text{g}/\text{mL}$ for different time. Unstimulated Caco-2 cells served as negative controls, respectively. The hBD-2 mRNA levels were normalized to GAPDH, using quantitative real-time PCR. These results are representative of three experiments performed independently. Relative density to negative control cells were set at 1. Results are mean \pm S.E.M.($n = 8$). The asterisks are indicator of statistical differences obtained separately at different time points compared to their controls shown in figure as "0". * $P < 0.01$, ** $P < 0.001$.

mRNA inducers (6.52 ± 0.3 fold increase), and WCW (3.12 ± 0.2 fold increase) and LTA (3.11 ± 0.1 fold increase). Induction effects followed the trend WPG > HK > WCW > LTA > S in Caco-2. WPG induced the highest release of hBD-2 relative to the other cell wall components, with WCW and LTA producing similar results. hBD-2 levels were low in the supernatant and unstimulated control samples. These results imply that human epithelial cells release hBD-2 upon stimulation with different cell wall components, especially WPG (Figure 2B).

Caco-2 cells were incubated with whole peptidoglycan (WPG) at a concentration of 100 $\mu\text{g}/\text{ml}$ for an incubation time course of 24 h. The result showed that in the first incubation time from 0 to 6 h, no significant difference was observed in the control group for the relative expression of hBD-2 genes among three time points. After 8 h, the relative expression of hBD-2 were significantly higher than in controls at time points of 8 and 12 h, but began to decrease

after 24 h. Thus, we deduced the best incubation time should be controlled to less than 12 to 24 h, and the optimum inducible yield of 6.52 ± 0.3 fold increase was observed (Figure 3).

Expression of hBD-2 mRNA in Caco-2 cells induced with different inducer concentration of cell wall components.

The different cell wall components inducer concentration was added into medium before cultivation, and their effects on the relative expression of hBD-2 genes investigated in this experiment. The results showed that whole peptidoglycan (WPG) was the best inducer for the expression of hBD-2 mRNA, resulting in the highest relative expression of 6.47 ± 0.3 fold increase with the addition of 50 $\mu\text{g}/\text{ml}$ for 12 h (Figure 4). However, it was also found

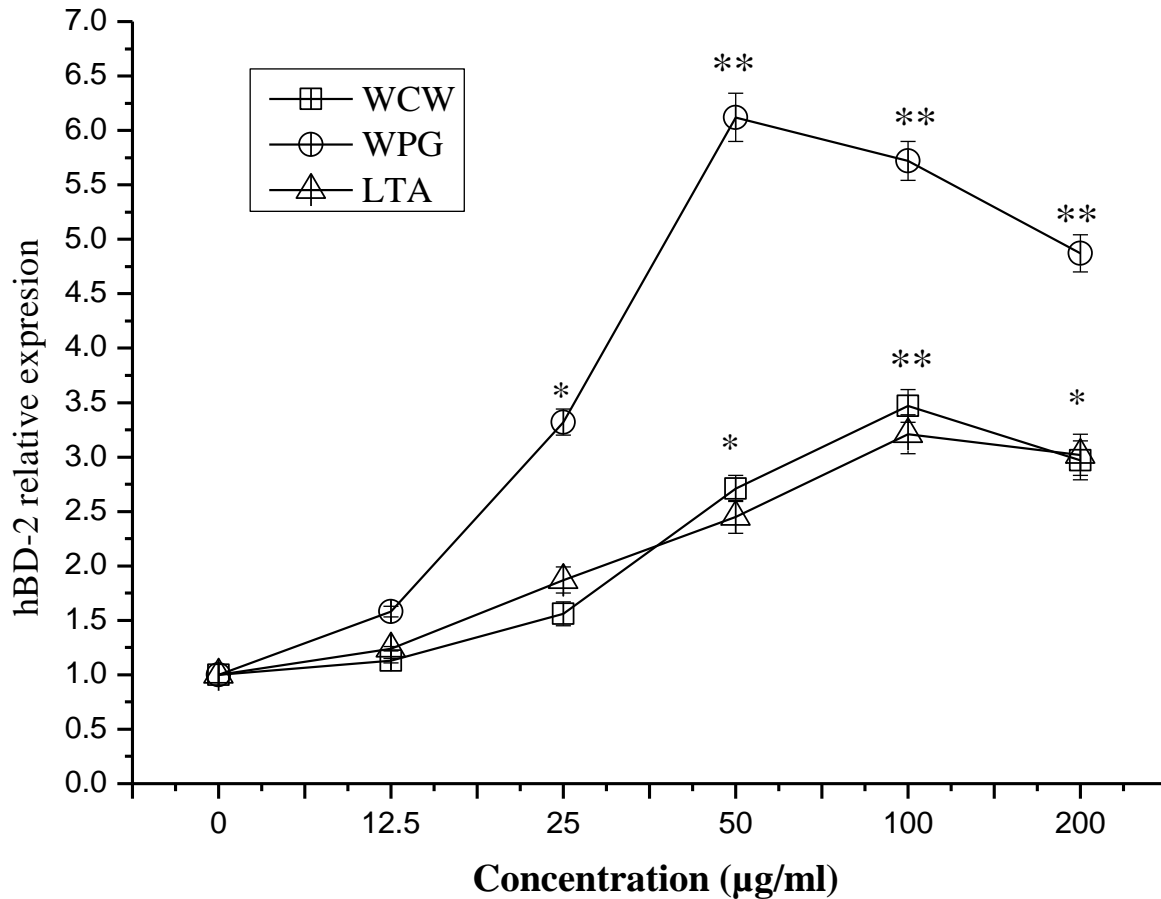


Figure 4: hBD-2 mRNA relative expression in Caco-2 cells after stimulation for 24 h with different cell wall components and different concentration. Caco-2 cells were stimulated with whole cell wall components (WCW), whole peptidoglycan (WPG) and lipoteichoic acid (LTA) at a concentration of 12.5, 25, 50, 100, 200 µg/mL for 24 h. The hBD-2 mRNA levels were normalized to GAPDH, using quantitative real-time PCR. These results are representative of three experiments performed independently. Relative density to negative control cells were set at 1. Results are mean ± S.E.M. (n = 8). The asterisks are indicator of statistical differences obtained separately at different time points compared to their controls shown in figure as "0". *P < 0.01, **P < 0.001.

that the relative expression of hBD-2 was influenced by addition of the inducer. Addition of inducer showed a negative effect on relative expression of hBD-2 at a higher concentration of 100 µg/ml. In order to reduce the inhibition influence, the concentration of WPG should be adjusted to less than 100 µg/ml (Figure 4).

Immunofluorescent staining of Caco-2 cells for HBD-2

Immunofluorescent staining images of cell wall components infected Caco-2 cells revealing that hBD-2 was diffusely distributed within the cytoplasm of the epithelial cells, as expected for a peptide within granules. Fluorescence was minimal in the uninfected controls. The calculated fluorescence intensity data (Figure 5) indicate that the hBD-2 peptide was highly inducible upon infection with cell wall components. Considerable fluorescence intensity was detected following infection with heat-killed

Lactobacillus plantarum KB8 (1×10^7), cell supernatant (S) and lipoteichoic acid (LTA) ($p < 0.01$ vs. control according to the ANOVA test with Dunnett post test, respectively and with whole cell wall components (WCW) and whole peptidoglycan (WPG) ($p < 0.001$).

DISCUSSION

The present and a previous report suggest that several apathogenic probiotic bacteria including lactobacilli and others induce innate immunity through defensin induction (Wehkamp et al., 2004). Notably, lactobacilli and other probiotics appear to induce the intestinal barrier defence system without provoking inflammatory events in patients. However, some lactobacilli can also cause severe as infections in immunocompromised individuals or when natural barriers are damaged (Gow and Hube, 2012). This induction may be mediated by classical pro-inflammatory

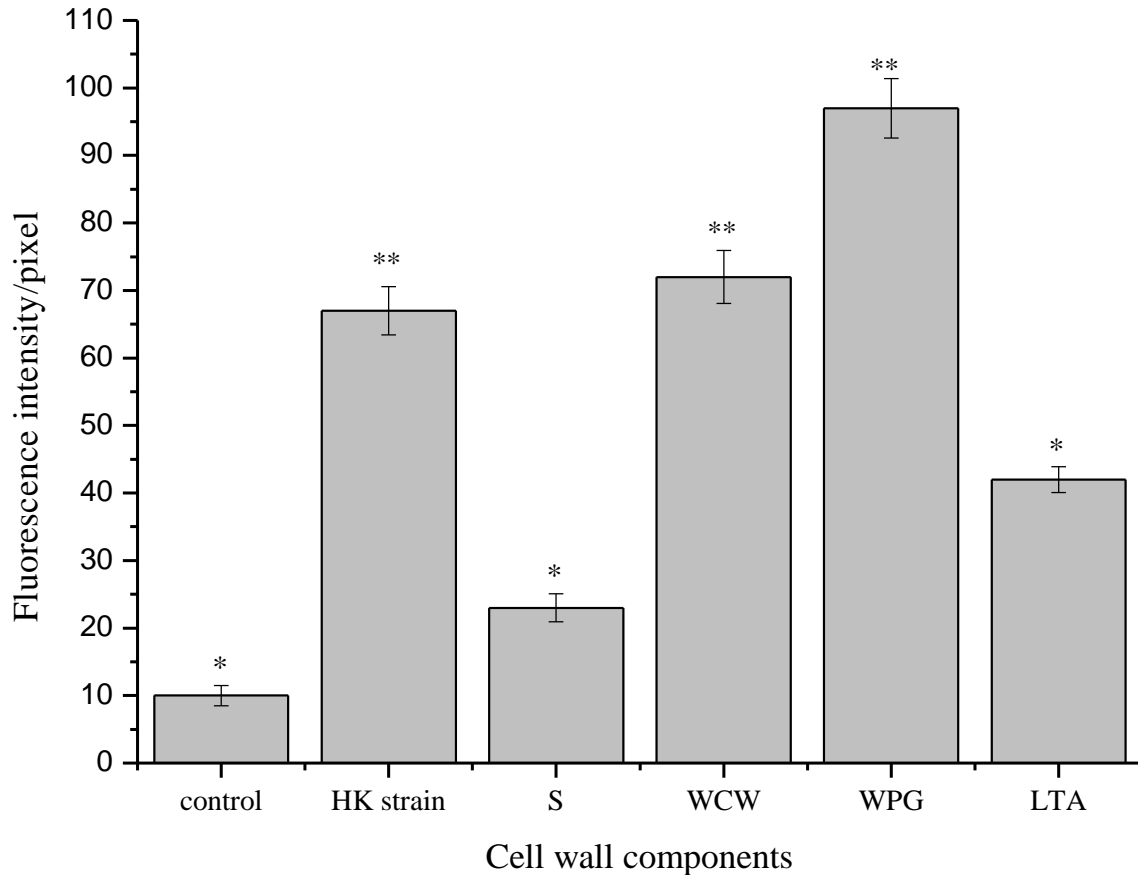


Figure 5: Fluorescence intensity of hBD-2 peptide in Caco-2 cells infected heat-killed *Lactobacillus plantarum* KB8 (1×10^7), cell supernatant (S), whole cell wall components (WCW), whole peptidoglycan (WPG) and lipoteichoic acid (LTA) at a concentration of $100 \mu\text{g/mL}$ for 24 h. Expression of hBD-2 was investigated by immunofluorescence analysis. The immunofluorescence of control and infected cells was quantitatively analyzed by ImageQuant software. Control: non-infected Caco-2 cells. Results are expressed as mean \pm SD of the data from three individual experiments. Statistical analysis. ANOVA one way test, $p = 0.0008$, and Dunnett post test. * $p < 0.01$ vs. control, ** $p < 0.001$ vs. control.

pathways. Whether different cell wall components from lactobacilli species have different induction effects to induce hBD-2 expression in epithelial cells, the mechanisms are still largely unknown. Therefore, there is the need further study as regards the topic of cell wall component related stimuli.

In this study, we examined the hBD-2 production by Caco-2 intestinal epithelial-like cells upon stimulation with several different heat-killed probiotic lactobacillus strains and cell wall components. Our results show that all three heat-killed probiotic lactobacillus strains and cell wall components (WCW, WPG and LTA) used in our study are able to induce hBD-2 mRNA and protein production in human intestinal epithelial cells, suggesting that locally released hBD-2 may have a role in regulating the amount of commensal yeasts in the gut.

Moreover, the present study also suggests that WPG is the major factor expressed by the probiotic bacterium *L. plantarum* KB8 responsible for the induction of hBD-2. In order to characterize the responsible factor for hBD-2

induction, we tested known genes, which code for surface expressed or secreted proteins. The genes of known fitness factors, differing from other strains were therefore deleted to evaluate their relevance for hBD-2 expression. Interestingly, this species-dependent induction of hBD-2 could only be seen at the protein and not at the mRNA level. This is most probably the consequence of differences in post-translational regulation of defensin synthesis, as these molecules are known to be synthesized as inactive precursors in human cells (Wilson et al., 2009).

However, the high induction of hBD-2 by WPG may be associated with the fact that *L. plantarum* KB8 is one of the most prominent lactobacilli species in the human intestinal tract. Although there are several studies investigating the antifungal activity of defensins (Joly et al., 2004), little is known about the induction of these antimicrobial peptides by different cell wall components in the intestines. Notably, it may be interesting to examine the defensin-inducing capacity of different components of these species in the future, as strong strain-to-strain differences have been

noted among lactobacilli species regarding susceptibility to defensins. A deeper understanding of the mechanisms by which components stimulate immune responses in the gut may even lead to better therapies for inflammatory bowel disease (IBD) in the future (Iliev et al., 2012).

The time-dependence experiments of WPG showed a similar pattern as already described for *E. coli* Nissle 1917 (Wehkamp et al., 2004), with a maximum of hBD-2 induction after 12 h of incubation. At the concentration of 50 µg/ml the effect of WPG reached a maximum of hBD-2 induction. Our results revealed that the potential to induce hBD-2 depends on the lactobacilli tested and merely no class effect. This observation is in accordance with the varying magnitude of dendritic cell activation exerted by different lactobacilli strains (Christensen et al., 2006). When taking the induction of hBD-2 promoter activation and protein secretion into consideration, *L. plantarum* KB8 was the most potent of the lactobacilli tested and second only to *E. coli* Nissle 1917, while WPG induced the highest amount of hBD-2 mRNA as compared to the other cell wall components used, although it exerted a weaker capacity of hBD-2 peptide induction than *E. coli* Nissle 1917. The differences between promoter activation, mRNA expression and peptide secretion with respect to the order of cell wall components are minor and constitute no evidence for post-transcriptional regulation.

In previous investigations a 13-fold higher hBD-2-inducing capacity was found by the bacterial culture supernatant of *E. coli* Nissle 1917 than by the pelleted bacteria indicating a soluble factor as the main responsible inducer (Yan et al., 2007). We also therefore compared whether supernatants of *L. plantarum* KB8 exerted a stronger activity than the bacterial pellet. The lactobacillus pellet actually displayed no significantly higher hBD-2-inducing capacity than the culture supernatant showing that these strains might rather induce hBD-2 by a bacterial cell wall component.

Despite the heat treatment of the bacteria, this component seems not to be largely secreted or shed into the supernatant, as reported for the flagellin-mediated hBD-2 induction by *E. coli* Nissle 1917 (Wehkamp et al., 2004). However, secreted bacterial glycoproteins of probiotics seem to act as important mediators to promote epithelial cell survival, differentiation and growth (Yan et al., 2007). The culture supernatant of probiotics has also been shown to exert bacterial killing activity and therefore possesses a potent immunostimulatory capacity (Coconnier-Polter et al., 2005).

In the present study, we provide the first evidence that cell wall component from a probiotic bacterial strain specifically stimulates the expression of an antimicrobial peptide by epithelial intestinal cells. Whole peptidoglycan (WPG) and lipoteichoic acid (LTA) are the identified probiotic bacterial cell wall components regulating intestinal epithelial homeostasis through specific cellular signal transduction pathways. Although the precise

mechanisms involved in preferentially triggering the protective (defensin) rather than the inflammatory (cytokine) pathways are still unclear.

In conclusion, we believe that the induction of defensins by probiotics lactobacilli including cell wall components might be an interesting new therapeutic strategy to strengthen innate defence mechanisms.

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