Probiotic bacteria change Escherichia coli-induced gene expression in cultured colonocytes: Implications in intestinal pathophysiology

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ABSTRACT

AIM: To investigate the change in eukaryotic gene expression profile in Caco-2 cells after infection with strains of Escherichia coli and commensal probiotic bacteria.

METHODS: A 19,200 gene/expressed sequence tag gene chip was used to examine expression of genes after infection of Caco-2 cells with strains of normal flora E. coli, Lactobacillus plantarum, and a combination of the two.

RESULTS: The cDNA microarray revealed up-regulation of 155 and down-regulation of 177 genes by E. coli, L. plantarum up-regulated 45 and down-regulated 36 genes. During mixed infection, 27 genes were up-regulated and 59 were down-regulated, with nullification of stimulatory/inhibitory effects on most of the genes. Expression of several new genes was noted in this group.

CONCLUSION: The commensal bacterial strains used in this study induced the expression of a large number of genes in colonocyte-like cultured cells and changed the expression of several genes involved in important cellular processes such as regulation of transcription, protein biosynthesis, metabolism, cell adhesion, ubiquitination, and apoptosis. Such changes induced by the presence of probiotic bacteria may shape the physiologic and pathologic responses they trigger in the host.
feeding tolerance\(^{22}\) in neonates, and efficacy against neonatal necrotizing enterocolitis (NEC)\(^{23-25}\) and sepsis\(^{26}\). Other reports have demonstrated no effect in NEC\(^{20}\), and in some cases, deterioration of specific conditions with probiotic therapy\(^{25}\). Results of clinical trials done by our group have shown a wide range (0%-60%) of colonization rates in newborn infants when three different probiotic strains were used\(^{27}\). These mixed and non-reproducible results have raised more questions than providing answers, and have strongly suggested complex interactions among bacterial strains and epithelial cells in the human intestine\(^{28-30}\).

At this time, our understanding of the response of eukaryotic cells (e.g., intestinal cells) is limited to nutrients and local factors\(^{31}\), and virulence mechanisms involving individual microorganisms. Although contrasting signal transduction mechanisms in bacterial and eukaryotic gene transcription have been described\(^{32}\), reports on cross talk between bacteria and epithelial cells have focused on single bacterial strains\(^{33}\). As a result, the physiologic and pathologic changes in the host cells as a response to multiple bacteria have not been addressed. Since the mammalian gut is colonized with multiple bacterial strains very quickly after birth, it is conceivable that the ultimate effect of probiotic treatment will depend greatly on the presence of other bacteria in the host intestine at that time.

In the current study, we examined the difference between gene expression in intestinal cells in response to infection with a single bacterial strain, compared to that during mixed infection. Caco-2 cells were utilized to discern the effect of Lactobacillus plantarum (the most common Lactobacillus species in humans)\(^{34}\), Escherichia coli (a common Gram-negative enteric strain) and the combination of the two strains. A high-density cDNA glass microarray and standard techniques were employed to identify bacteria-induced gene expression in this eukaryotic system.

MATERIALS AND METHODS

Caco-2 cell culture model

Caco-2 cells, obtained from American Type Culture Collection (ATCC HTB-37), were used at passage 10-12. This human colon-adenocarcinoma-derived cell line has been used extensively for physiologic and enteric bacterial pathogenesis studies\(^{35}\). The cells were cultured in a humidified atmosphere containing 5% CO\(_2\) at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal calf serum (Sigma, St. Louis, MO, USA), 2 mmol/L glutamine, 0.1 mmol/L sodium pyruvate, 0.1% non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. All experiments were performed without serum or antibiotics in 8-10-d-old cells after they reached confluence.

Bacterial strains

E. coli strain 6-1 was isolated from a healthy infant, and has been used previously in in vitro and in vivo studies in our laboratory\(^{36}\). This strain does not possess any known virulence genes\(^{37}\). We used a human strain of L. plantarum (ATCC 202195), the species most commonly isolated from humans\(^{38}\).

Defined bacterial treatment of epithelial cells

Cells were washed in PBS and re-fed with experimental DMEM without serum or antibiotics before the experiments. Following previously described methods in which a maximal effect of Lactobacillus was seen, Caco-2 cells were infected with E. coli and/or L. plantarum at 1:10 multiplicity of infection, and incubated for 2 h\(^{39}\).

cDNA microarray

For examination of Caco-2 cell gene expression under our experimental conditions, we used a high-density glass microarray H19K (University Health Network Microarray Centre, Toronto, www.microarrays.ca/home.html) that had 19 200 genes/expressed sequence tags (ESTs). These included fully characterized, partially characterized and some uncharacterized human gene elements. Each gene/EST was printed in duplicate in this array. The genes in the array represented constitutively expressed genes/ESTs and the manufacturer did not include genes that are transiently expressed, such as cytokines and chemokines. In our experiments, we used dye swapping procedures and bioinformatics tools considered as standard techniques that have been reported in similar studies in the past\(^{39}\).

Sample preparation

Total RNA was extracted from Caco-2 cells grown in 75-cm\(^2\) tissue culture flasks using the TRIZOL method (Invitrogen, Carlsbad, CA, USA), following manufacturer’s instructions. RNA samples were treated with RNase-free DNase to remove contaminating genomic DNA, examined by 260/280 nm UV absorption ratio (> 1.8) followed by assessment of integrity by running in a 1.2% agarose gel and ethidium bromide staining.

Preparation of fluorescent-labeled cDNA\(^{40}\), hybridization\(^{41}\) and signal detection

Total mRNA (10 μg) was reversely transcribed using 20 mmol/L dNTP mix including amino-allyl dUTP (AA-dUTP; Sigma) and 400 U SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting aa-cDNA, cleaned with a QIAquick column (Qiagen, Valencia, CA, USA), was coupled with Cy3 or Cy5 dye (Amersham Biosciences, Piscataway, NJ, USA) in the presence of sodium bicarbonate for 1 h in the dark. After adding 10 μL 4 mol/L hydroxylamine and 125 μL buffer PB (Qiagen supplied) to each, the control and treatment samples were combined and cleaned using another QIAquick column. The elute was transferred to a Microcon YM 30 centrifugal filter device (Amicon Millipore, Bedford, MA, USA), and after adding 20 μL of 1 human DNA (Gibco-BRL), the whole volume was concentrated to 5 μL. Ten microliters of 1 μg/μL poly (A) RNA (Sigma), 1 μL 10 μg/μL tRNA (Gibco-BRL), 4 μL water and 5 μL hybridization buffer (50% formamide, 5 × SSC (3 mol/L sodium chloride, 0.3 mol/L sodium citrate and 0.1% SDS) were added. The array was pretreated at 42°C for 1 h with hybridization buffer. After overnight

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hybridization at 42°C, the slides were washed in 50 mL 2 × SSC and 0.1% SDS at 55°C for 5 min, once in 0.1 × SSC and 0.1% SDS for 5 min at room temperature (RT), and for 5 min with 0.1 × SSC at RT, air-dried and scanned with 555 nm and 647 nm lasers in a Scan Array 5000 (GSI Lumonics, Novi, MI, USA). Images of the fluorescence intensity for each dye were analyzed using Imagene 4.2 software (Biodiscovery, CA, USA).

RNA from each experimental condition and control Caco-2 cells were hybridized on the same microarray. To eliminate the color bias, duplicate reactions were carried out in which the dyes (Cy3, Cy5) for the control and experimental samples were swapped.

Data interpretation

Individual gene intensity data files for each experimental condition were compared with the control values using the GeneSight 2.1 program (Biodiscovery). After correction for the local background, normalization using all the spots, removal of the outliers, averaging of the replicates and transforming to base 2, each gene was assigned a relative expression value when compared with the control. A twofold or larger difference in the relative gene expression was considered significant.

Table 1  Common genes induced by bacterial treatment (Seventeen genes were influenced by both E. coli and L. plantarum and four genes by both E. coli and combination treatment)

| Nr. | Gene symbol | Gene ID NCBI | Gene name | Location | Function | Relative fold modification |
|-----|-------------|--------------|-----------|----------|----------|---------------------------|
| 1   | GPIR34      | 2857         | G protein-coupled receptor 34 | Integral to plasma membrane | G-protein coupled receptor activity | E.c. 2.43; L.p. 3.03; Mix -0.53 |
| 2   | GTPBP4      | 23560        | GTP binding protein 4 | Nucleus | Ribosome biogenesis - small GTPase mediated signal transduction | E.c. 2.00; L.p. 2.91; Mix -0.30 |
| 3   | TFPI2       | 7980         | Tissue factor pathway inhibitor 2 | Extracellular matrix | Serine-type endopeptidase inhibitor activity | E.c. 2.10; L.p. 2.93; Mix -0.27 |
| 4   | CYP26A1     | 1592         | Cytochrome P450, family 26, subfamily A, polypeptide 1 | Membrane | Metal ion binding | E.c. 2.31; L.p. 2.39; Mix -0.88 |
| 5   | ZNF35       | 7584         | Zinc finger protein 35 (clone HF.10) | Nucleus | Transcription factor activity required for left-right specification in mouse embryos | E.c. 2.18; L.p. 2.19; Mix -0.66 |
| 6   | CD248       | 25914        | CD248 antigen, endosialin | GTP binding protein 4 | Apoptosis regulator | E.c. 2.21; L.p. 2.13; Mix -0.58 |
| 7   | FLJ21963    | 5349         | FLJ21963 protein | Membrane | Chloride channel activity | E.c. 2.44; L.p. 2.03; Mix -0.15 |
| 8   | CYYR1       | 116159       | Cysteine/tyrosine-rich 1 | Integral to plasma membrane | Molecular function unknown | E.c. -2.30; L.p. -2.20; Mix 1.05 |
| 9   | BFAR        | 512836       | Bifunctional apoptosis regulator | Membrane | Apoptosis regulator | E.c. -2.40; L.p. -2.17; Mix 1.06 |
| 10  | C19orf14    | 25789        | Chromosome 19 open reading frame 4 | Membrane | Molecular function unknown | E.c. -2.51; L.p. -2.28; Mix 0.19 |
| 11  | KIAA1305    | 57523        | KIAA1305 protein | Integral to plasma membrane | Hypothetical protein | E.c. -2.19; L.p. -2.29; Mix 0.55 |
| 12  | PCDH9       | 5101         | Protocadherin 9 | Integral to plasma membrane | Cell adhesion | E.c. -2.18; L.p. -2.30; Mix 0.50 |
| 13  | IKIP        | 121457       | IKK interacting protein | Membrane | Hypothetical protein | E.c. -2.23; L.p. -2.33; Mix 0.83 |
| 14  | FLJ21963    | 79611        | FLJ21963 protein | Membrane | Hypothetical protein | E.c. -2.12; L.p. -2.37; Mix 1.88 |
| 15  | SCRG1       | 11341        | Scrapie responsive protein 1 | Membrane | Nervous system development | E.c. -2.14; L.p. -2.54; Mix 0.74 |
| 16  | ULK2        | 9706         | Unc-51-like kinase 2 (C. elegans) | Membrane | Similar to a serine/threonine kinase in C. elegans | E.c. -2.46; L.p. -2.72; Mix 0.26 |
| 17  | LIFR        | 3977         | Leukemia inhibitory factor receptor | Integral to plasma membrane | Receptor activity | E.c. -2.26; L.p. -2.96; Mix 0.29 |
| 18  | BMF         | 90427        | Bel-2 modifying factor | Sequestered by myosin | Apoptotic activator - protein binding | E.c. 1.00; L.p. 2.95; Mix 2.40 |
| 19  | CD248       | 57124        | CD248 antigen, endosialin | Integral to plasma membrane | Marker of stromal fibroblasts | E.c. 0.74; L.p. 2.31; Mix 2.10 |
| 20  | PPM1E       | 22843        | Protein phosphatase 1E (PP2C domain containing) | Integral to plasma membrane | Phosphatase | E.c. 0.76; L.p. 2.33; Mix 2.06 |
| 21  | CARD8       | 22900        | Caspase recruitment domain family, member 8 | Membrane | Involved in NFkB pathway | E.c. -0.59; L.p. -3.26; Mix 4.07 |

E.c., E. coli; L.p., L. plantarum.

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE5874.

Real-time quantitative PCR

We randomly selected eight genes (BMF, CD248, PPM1E, FXYD3, OAS2, FY, CERK and HPSE) from our pool of expressed genes/ESTs that are well characterized in the literature and appear to have some biologic significance. ESTs were not included. Real-time quantitative PCR (Bio-Rad iQ SYBR Green Supermix and iCicler) was done using GAPDH for normalization. The levels of expression detected by microarray were compared with PCR results. The primers used to amplify specific gene segments are presented in Table 1. The relative gene expression was calculated using the comparative ΔΔCt method. Each sample was tested twice in triplicate.

RESULTS

Gene expression after bacterial infection

After 2 h treatment, E. coli, L. plantarum and their combination changed the expression (by twofold) of 332,
was reversed in the mixed

Schematic representation of the genes influenced by each treatment, and the overlapping (common) genes among treatments.

The relative gene expression after treatment was > 2-fold compared with the control.

81 and 86 genes, respectively, compared to uninfected control Caco-2 cells (Figure 1). After infection with \( E. \ coli \), 155 genes were up-regulated and 177 were down-regulated (Table 1 and Supplementary Table 1). \( L. \ plantarum \) induced up-regulation of 45 genes and 36 genes were down-regulated (Table 1 and Supplementary Table 2). The combination treatment up-regulated 27 genes and down-regulated 59 (Table 1 and Supplementary Table 3) [Note: The supplementary tables above can be accessed at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nnzuydkkuwukuyt&acc=GSE5874]. Raw data of all 19200 genes during each treatment can be accessed from the NCBI/GEO data base (GSE5874) at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nnzuydkkuwukuyt&acc=GSE5874]. Mixed infection nullified the previously demonstrated stimulatory and inhibitory effects of \( E. \ coli \) on 152 and 177 genes and of \( L. \ plantarum \) on 38 and 26 genes, respectively. Stimulation of 23 and inhibition of 59 genes were noted after mixed infection that was not influenced by either bacterium alone.

There were 21 genes influenced by two different treatment conditions (Table 1). Seventeen genes were affected by \( E. \ coli \) and \( L. \ plantarum \), and four by \( E. \ coli \) and the combination of bacteria. Genes nos. 1-7 were up-regulated by both \( E. \ coli \) and \( L. \ plantarum \); and genes nos. 8-17 were down-regulated by both bacteria. For each of the 17 genes in this group, the effects of the individual bacteria were brought to baseline by the combination treatment. In contrast, for three genes BMF, CD248 and PPM1E (nos. 18, 19 and 20 in Table 1), the stimulatory effect of \( E. \ coli \) was maintained after mixed infection with \( L. \ plantarum \). For one gene (no. 21, CARD8), the 3.26-fold down-regulation by \( E. \ coli \) was reversed in the mixed infection, with demonstration of a four fold increase.

Apart from the specific up- and down-regulation of genes by either \( E. \ coli \) or \( L. \ plantarum \), and reversal of \( E. \ coli \)-induced effects when \( L. \ plantarum \) was used as a co-infectant, several genes of physiologic importance were noted in our system. Table 2 describes 58 genes under 10 specific categories that were expressed during infection. While the function of a small number of genes was not very well defined, most of the genes could be grouped into important cellular functions. These include genes involved in transcription regulation, RNA processing, protein biosynthesis, and other important processes such as ubiquitination, cell adhesion, proliferation and apoptosis.

Confirmation of selected gene expression by real-time quantitative PCR

Eight genes were randomly tested by quantitative real-time PCR to verify the expression detected by microarray (Table 3). For each of these genes, RT-PCR confirmed their expression after the three bacterial treatments in the same direction (stimulation or inhibition) as in the microarray experiments (Figure 2).

DISCUSSION

The infant gut is essentially sterile at birth and is first colonized with Enterobacteriaceae, which change the redox potential in the intestine and allow more microaerophilic and anaerobic species to colonize[42,43]. The latter group, which is comprised primarily of \( Bifidobacteria \) and \( Lactobacillus \) organisms[44], are considered as normal flora that coexist in the human colon, as new species are introduced to ultimately provide a stable flora in the human gut[45], in which over 800 bacterial species coexist in harmony[46]. In such a healthy state, the intestinal mucosa serves as the first line of defense against infections by providing an important mechanical and immunologic barrier between the host's internal milieu and the gut environment. These intestinal epithelial cells generate and transmit signals between bacteria and deeper layers in the intestine[47]. In the event of specific infections, epithelial cells express and secrete proinflammatory and chemotactic cytokines[48] that further transmit signals to the underlying cells in the reticuloendothelial system[47]. The virulence factors and the host responses to these factors in various diseases have been studied in a fair amount of detail (\( E. \ coli \), \( Vibrio \) cholerae, \( Salmonella \) and \( Pseudomonas \)) using tissue culture and in vivo models, and specific genes and gene functions have been described[49-52]. These experiments have utilized single bacterial strains.

In an attempt to mimic the natural gut environment, communication systems among bacteria have also been studied relatively well. Chemical signals produced and detected by bacteria can be directed at other bacteria and self. This phenomenon, called as quorum sensing, is important for the microorganism's adaptation to the local environment[53]. This fundamental prokaryotic behavior (among bacteria) is known to affect the symbiotic or antagonistic environment created within the gut milieu. However, the effect of single versus multiple bacterial species on eukaryotic cells has not been addressed in the literature.

The stimulus for us to conduct the current study came from our observation that a large number of probiotic trials have been conducted and reported in the recent past, with almost no basis for selection of the strain, and more importantly, with no data on changes in physiologic or pathologic parameters in the host, other than analysis of the primary and secondary clinical endpoints. Although a live bacterial supplement was used in all of these reported studies, there was also a serious lack of data on the colonization ability of the probiotic strain and changes in the colonization by other bacteria in the host gut. Since the newborn gut is colonized with a paucity of bacteria (an average 2.5 species in preterm infants)[53,54] that expands to a limited but heterogeneous flora by 10 d of age[55], we designed the current simple system to examine the effects of \( L. \ plantarum \), a common human probiotic strain, and \( E. \ coli \), the most common colonizing strain in the neonatal
Table 2  Modulation of gene expression during mixed (E. coli and L. plantarum) infection

| Biological process                  | Gene symbol | Gene ID NCBI | Gene name                                                                 | Fold change |
|-------------------------------------|-------------|--------------|---------------------------------------------------------------------------|-------------|
| Category 1: Regulation of transcription | HOXD10      | 3236         | Homeobox D10                                                              | 2.50        |
| Category 2: RNA processing           | FGF7        | 51333        | PHD finger protein 7 (Zinc ion binding)                                    | 2.44        |
|                                     | EGRI        | 1958         | Early growth response 1                                                  | -2.08       |
|                                     | TRIM24      | 8605         | Tripartite motif-containing 24 (Zinc ion and DNA binding)                  | -2.15       |
|                                     | ENO1        | 2023         | Enolase 1, (alpha) (DNA binding)                                          | -2.34       |
| Category 3: Protein biosynthesis, folding, binding and transport | SSB         | 6741         | Sjogren syndrome antigen B (autoantigen)                                  | 2.08        |
|                                     | FUSIP1      | 10772        | FUS interacting protein (serine/arginine-rich) 1                          | -2.22       |
|                                     | NOLC1A5     | 10528        | Nucleolar protein 5A (56 kDa with KKE/D repeat)                           | 2.27        |
|                                     | DDX5        | 1655         | DEAD (Asp-Glu-Ala-Asp) box polypeptide 5                                  | -2.71       |
| Category 4: Structural protein       | AMPH        | 273          | Amphiphysin (Actin cytoskeleton)                                           | 3.04        |
|                                     | MAP1B       | 4131         | Microtubule-associated protein 1                                          | 2.13        |
|                                     | ARCC10      | 89845        | ATP-binding cassette, sub-family C (CFTR/MRP), member 10                   | -2.05       |
|                                     | SLC26A2     | 1836         | Solute carrier family 26 (sulfate transporter), member 2                   | 2.06        |
|                                     | TUBB2A      | 7280         | Tubulin, beta 2A                                                          | -2.15       |
| Category 5: Metabolism              | C5orf14     | 79770        | Chromosome 5 open reading frame 14                                        | 2.91        |
|                                     | NAV2        | 89797        | Neuron navigator 2                                                        | 2.83        |
|                                     | SLC24A4     | 123041       | Solute carrier family 24 (sodium/potassium/calcium), member 4             | 2.35        |
|                                     | PLEKHM2     | 23207        | Pleckstrin homology domain containing, family M, member 2                  | 2.10        |
|                                     | TWIF1       | 5756         | Tufinfilin, actin-binding protein 1 (Tyrosin kinase)                      | -2.01       |
|                                     | AKR1C1      | 1645         | Aldo-keto reductase 1, member C1 (Bile acid binding)                       | -2.09       |
|                                     | HMGC2R      | 3156         | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase                            | 2.10        |
|                                     | DC2         | 58505        | DC2 protein (Glycogen synthase activity)                                   | -2.12       |
|                                     | GSTA1       | 2938         | Glutathione S-transferase A                                                | -2.15       |
|                                     | GAPD        | 2597         | Glyceraldehyde-3-phosphate dehydrogenase                                  | -2.16       |
|                                     | GCLC        | 2729         | Glutamate-cysteine ligase, catalytic subunit                               | 2.21        |
|                                     | SRM         | 6723         | Spermidine synthase                                                        | -2.39       |
|                                     | HSP90AA1    | 3520         | Heat shock protein 90 kDa alpha (cytosolic), class A member 1              | -2.46       |
|                                     | AHCY        | 191          | S-adenosylhomocysteine hydrolase                                           | -2.74       |
| Category 6: Cell physiology         | NCF4        | 4689         | Neutrophil cytosolic factor 4, 40 kDa                                     | 2.39        |
|                                     | CYCS        | 54205        | Cytochrome c, somatic                                                     | -2.02       |
|                                     | DBI         | 1622         | GABA receptor modulator, acyl-Coenzyme A binding protein                   | -2.25       |
|                                     | ATP5G3      | 518          | ATP synthase, H+ transporting, mitochondrion F0 complex, subunit C         | -2.26       |
| Category 7: Cell proliferation      | FOSL1       | 8061         | FOS-like antigen 1 (transcription factor activity)                        | 2.06        |
|                                     | FGG         | 2266         | Fibrinogen gamma chain                                                    | -2.36       |
|                                     | FGG         | 2244         | Fibrinogen beta chain                                                     | -2.45       |
| Category 8: Cell adhesion           | NEL2        | 4753         | NEL-like 2 (Calcium ion binding)                                          | 2.25        |
|                                     | ITGB3       | 3690         | Integrin, beta 3 (platelet glycoprotein IIb, antigen CD61)                 | 2.11        |
|                                     | RHOB        | 388          | Ras homolog gene family, member B                                         | 2.19        |
| Category 9: Ubiquitination          | UBE2N       | 7334         | Ubiquitin-conjugating enzyme E2N (UBE1C1 homolog, yeast)                  | 2.02        |
|                                     | UBE2S       | 72738        | Ubiquitin-conjugating enzyme E2S                                           | 2.05        |
|                                     | ANAPC7      | 51434        | Anaphase promoting complex subunit 7                                      | -2.15       |
|                                     | CACYBP      | 27101        | Cyclin binding protein                                                    | -2.18       |
|                                     | UBA52       | 7311         | Ubiquitin A-52 residue ribosomal protein fusion product 1                 | -2.29       |
|                                     | COL6A3      | 1293         | Collagen, type VI, alpha 3                                               | 2.33        |
|                                     | RPS5A       | 6189         | Ribosomal protein S5A                                                    | -2.08       |
|                                     | TWIF1       | 5756         | Tufinfilin, actin-binding protein, homolog 1 (Tyrosin kinase)              | -2.01       |
|                                     | AKR1C1      | 1645         | Aldo-keto reductase 1, member C1 (Bile acid binding)                       | -2.09       |

Negative value indicates reduction in gene expression.

In our system, we observed a change (up- or down-regulation) in the expression of 333, 81 and 86 genes upon infection with E. coli, L. plantarum and the combined...
treatment, respectively. Our real-time PCR experiments confirmed the modifications demonstrated in the microarray experiments, albeit at a lower level, a phenomenon also reported in other studies[50,56-58]. The numbers of unique genes presented in this study are in the range reported in previous studies in which Gram-negative enteric pathogens modified 0.5%-13% of the genes in epithelial cells[50,56-58], and commensal bacteria induced differential expression of 0.5%-6.2% of examined genes in mouse colonocytes[50]. Our strain of E. coli modified 1.73%, and L. plantarum modified 0.43% of genes. The slightly lower number of genes identified in our 19,200 array may have been due to the use of a non-pathogenic strain of E. coli, a commensal Lactobacillus, and an array that included only constitutively expressed genes. Genes expected to be expressed after a bacterial insult such as pro-inflammatory cytokines were not spotted on this array. Additionally, a slightly low number might have resulted from our conservative choice of a twofold increase in expression as being significant in our analysis.

There are several comparisons that can be made between our results and those of others using a similar approach but with single bacterial infection. For example, from the six genes up-regulated by enteropathogenic E. coli in HeLa cells[59], we found only one (zyxin, a cytoskeletal protein) to be in common with our microarray results. There was a similar increase (1.72-fold) in expression of this gene when our E. coli strain 6-1 was used to infect Caco-2 cells. Two previous studies with commensal flora have reported that bacterial reconstitution of germ-free mice increased the expression of the colon-specific serum amyloid A1 gene[50,51]. In our model, serum amyloid A2 gene expression was increased by 2.22-fold. From the 12 genes down-regulated by non-pathogenic bacterial reconstitution of germ-free mice, reported by Fukushima et al[59] in colonic epithelial cells, three were in common with our microarray; selenoprotein P, 3-hydroxy-3 methylglutaryl-coenzyme A synthase and metallothionein. All three were also down-regulated in our combination treatment model. The authors also showed a down-regulation of solute carrier family 20 - member 1. Our results were very similar to this observation in that we also noted a decrease in the expression of other members of the solute carrier families, i.e., family 2, 9, 12, 20, 24, 25 and 35. Fukushima et al[59] have shown overexpression of heat shock protein (60 kDa) in germ-free mice compared to specific pathogen-free rodents that had received treatment with normal mouse flora. We observed a similar phenomenon in our system in which down-regulation of heat shock proteins 75, 105 and

Table 3 Primers used for RT-PCR

| Gene symbol | Gene ID (NCBI) | Gene name | Gene role | Primer | Primer sequence 5’-3’ |
|-------------|---------------|-----------|-----------|--------|-------------------|
| BMP         | 90427         | Bcl2 modifying factor, transcript | Has a single Bcl2 homology domain 3 (BH3), binds variant 1 | F  | GCCTCACATTGCAATGCCAGCACAGT |
| CD248       | 57124         | CD248 antigen α endosialin | A gene regulated by the cell density in vitro. Has a calcium binding domain | R  | AGAGCCCTTGGAAATTCCTACCAT |
| PPM1E       | 22843         | Protein phosphatase 1E | Member of the PP2C family of Ser/Thr phosphatases known to be negative regulators of stress response pathways | F  | TCAACTACGTTGGTGCTTCGAGAT |
| FXDY3       | 5349          | FXD domain containing ion transport reg. 3 | The protein encoded by this gene may function as a chloride channel or as a chloride channel regulator | R  | AGATGGGGATTATCACTGGAGGT |
| OAS2        | 4939          | 2’-5’-oligoadenylate synthetase 2 | This enzyme family plays a significant role in the inhibition of cellular protein synthesis | F  | ATGCCTTCCATCACCTTCCAGTTA |
| FY          | 2532          | Duffy blood group antigen | Helps in leukocyte recruitment to sites of inflammation by facilitating movement of chemokines across the endothelium | R  | TGTCAAGAAGAAGACAGCGGCA |
| CERK        | 64781         | Ceramide kinase | Integral to membranes, has roles in arachidonic acid release and production of eicosanoids | F  | AATGGCAACTTTGCGAGGAAAGTTGCC |
| HPSE        | 10855         | Heparanase | Cell surface expression and secretion markedly promote tumor angiogenesis and metastasis | R  | TTGCAGGTTCAGTGAACAGCAGAT |
| GAPDH       | 2597          | Glyceraldehyde-3-phosphate dehydrogenase | Used as reference | F  | TGACTTGCTGCTGCCCTTCTCAT |

Figure 2 Effect of different bacterial treatments on expression of eight genes assessed by microarray (M) and RT-PCR (R).
an ortholog of mouse heat shock protein 70 kDa were noted after combined bacterial treatment. We observed cytochrome c oxidase subunits IV isoform 1, Va, VIb, VIc, VIIa, VIIb, VIc, and VII to be up-regulated after *L. plantarum* treatment, similar to that described by Hooper et al., who demonstrated up-regulation of cytochrome c oxidase subunit 1 by *Bacteroides*, another species also considered as commensal flora. Hooper and colleagues have also shown up-regulation of calmodulin after treatment with *Bacteroides*.[61] Similar increases in expression were noted for calmodulin 1, 2, and 3, calmodulin-dependent protein kinase and phosphodiesterase in our system.

We observed modulation of multiple genes known to have an impact on cellular and physiologic processes in the eukaryotic system (Table 2). These genes ranged from basic transcriptional regulators to those involved in protein synthesis, cellular metabolism, cell proliferation and apoptosis. During mixed infection, we observed down-regulation of three genes involved in ubiquitination. Ubiquitin-conjugating enzyme E2N, ubiquitin-carrier protein E2-EPF and ubiquitin A-52 residue ribosomal protein fusion product 1 were reduced 2.02, 2.05 and 2.29-fold, respectively. In a recent study that investigated anti-inflammatory properties of *Lactobacillus casei*, expression of several genes involved in ubiquitination was reduced, including E2N, a gene (common to our system) that was reported to be decreased 2.88-fold.[62] The authors concluded IκB stabilization *in vivo* reduced ubiquitination and downstream modulation of inflammatory response driven by NF-κB in *Shigella*-infected Caco-2 cells. We used a non-pathogenic commensal strain of *E. coli* in our experiments, and while the aim of the current study was not to assess or examine the effects of *L. plantarum* during bacterial infection or inflammation, our results strongly suggest that *Lactobacillus* strains do indeed affect common physiologic pathways in gut cells, which may ultimately shape the host response in health and disease.

In our study, it was important and intriguing to note that the three experimental infections induced quite unique gene-expression profiles. Even the mixed infection with *E. coli* and *L. plantarum* had a very small overlap with the expression profiles of the strains when they were used alone. This illustrates how colonization can change the gene expression of host cells as they are exposed to more than one species of bacteria. In real life, the gut cells are exposed to a multitude of bacterial strains, and hence, it may be of limited value to study the effect of infection or colonization by single bacterial species in a clean tissue culture environment, and use the results as the basis for designing treatment or preventive strategies. Using neonatal models of gut colonization, we have previously shown that bacterial ecology (combination of Gram-negative and Gram-positive organisms), rather than individual virulent bacterial strains, plays a more important role in diseases such as NEC.[63] The results of our current study are in line with previous observations, and now provide an additional line of support and offer a possible explanation for the varied results of recent probiotic trials. On a broader scale, this report provides an insight into the complex host response that can be expected at mucosal sites such as the gastrointestinal tract. Based on the results obtained from tissue culture with only two bacteria in the system, it can be speculated that our findings are only the tip of the iceberg, and the real *in vivo* picture in mammals will be even more complex. While it is becoming increasingly clear that specific *Lactobacillus* species possess unique health-promoting characteristics,[29] knowledge gained from the current study further indicates that a "one strain fits all" approach may not always succeed in the treatment or prevention of specific diseases. A more global approach needs to be taken with proper emphasis on the microbial ecology, while addressing the pathogenesis of unique bacterial diseases in the mammalian intestine at different ages and stages of development.

In the context of *in vitro* or clinical trial environment, it should be noted that our current model and results do not represent a universal phenomenon, nor provide a comprehensive picture of the human intestine. For example, genes expressed will probably be different if other probiotic strains such as *Bifidobacterium* and *L. casei* were used in our system. Similarly, combinations of other aerobic and anaerobic Gram-negative and Gram-positive strains may induce different sets of genes. We can utilize other microarray systems with cytokine and signaling-molecule genes (not spotted in the current 19200 gene array), when our aim would be to identify modifications in inflammatory mediators. The relative concentrations of each bacterium in the system may also change the gene-expression profile. In the current study, we selected a 1:10 ratio of *E. coli* to *Lactobacillus* infecting dose to simulate the human intestinal microflora, in which anaerobic and microaerophilic organisms form the dominant flora.[63] Since enteric bacteria such as *E. coli* are sometimes present at <0.1% of the total bacterial population, with a predominance by obligate anaerobes,[64] it is not unexpected to observe a different gene-expression profile when a 10-100-fold higher proportion of *Lactobacilli* are used in the system. Nevertheless, such manipulations and experiments can be done, and despite some limitations, assessment of mRNA-expression profiles by cDNA array analysis can be utilized as a useful technique for expanding our understanding of the colonocyte-bacteria interaction.[65]

While it may appear difficult to analyze complex microflora (400-800 species) and their interactions with gut cells in the mature intestine, this is now made feasible with the availability of new techniques. Fluorescent *in situ* hybridization utilizing bacterial rRNA can identify and quantify major genera of bacteria, even if they are non-culturable in stools.[65,66] Bacterial microarray chips developed during the last year can identify thousands of bacterial species in stools in one experiment.[67,68] Denaturing gradient gel electrophoresis can be utilized to monitor changes in microflora pattern over time and after administration of probiotic supplements. Live colonocytes can be isolated from stool samples and used to examine the expression of genes and proteins during different experimental and/or disease states.[69,70] At this juncture, there is a need for the scientific community to engage in careful evaluation of probiotic strains in *in vitro* and *in vivo* systems prior to initiation of clinical trials. With the new non-invasive tools at hand, such preclinical
endavors, coupled with concurrent examination of changes in the gut flora and host responses during clinical trials, hold great promise in discerning the difference between "snake oil" and "magic bullets" when it comes to the role of probiotic therapy in human medicine.

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