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BASIC RESEARCH

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.

Key words: *Lactobacillus*; *Escherichia coli*; Gene expression; Probiotic; cDNA microarray

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INTRODUCTION

There has been an upsurge in clinical trials involving probiotics in gastrointestinal diseases. Although promising, these trials with specific probiotic bacteria have shown variable results, with limited elucidation of the underlying pathophysiology. In real life, these strains never act on the host cells in isolation and over 800 bacterial strains in the adult human colon are engaged in constant cross talk with intestinal epithelial cells. No detailed study so far has attempted to examine the effect of individual probiotic bacteria on host gastrointestinal cells, and the changes during co-infection with other enteric bacteria.

However, a lot of emphasis has recently been given to the normal bacterial flora in the intestine, including many *Lactobacillus* strains that are considered as probiotics with health-promoting effects on the host. A myriad of effects have been shown by these bacteria, spanning from bacterial killing *via* secretion of bacteriocins[1], to inhibition of attachment and invasion by pathogenic bacteria[2], and modulation of host inflammatory responses[3]. These commensal strains have been shown to modulate the expression of genes involved in important physiologic functions such as postnatal intestinal maturation, cell growth, proliferation, nutrient absorption, mucosal barrier function, and angiogenesis[4-6]. Multiple laboratory studies have shown beneficial effects of *Lactobacillus* strains against single pathogenic bacterial strains in *in vitro* and *in vivo* systems[7,8]. During the last few years, there has been an exponential increase of clinical trial reports and reviews in the literature pertaining to the utility of probiotics in gastrointestinal and allergic diseases[9-21]. Many small studies utilizing *Lactobacillus* and *Bifidobacteria* have shown beneficial effects such as better weight gain and improved

feeding tolerance[22] in neonates, and efficacy against neonatal necrotizing enterocolitis (NEC)[23-25] and sepsis[24]. Other reports have demonstrated no effect in NEC[26], 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[7,28-30].

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℃ 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, 1.0 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[34].

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[38].

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 cot-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℃ for 1 h with hybridization buffer. After overnight

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		
						L.p.	E.c.	Mix
1	GPR34	2857	G protein-coupled receptor 34	Integral to plasma membrane	G-protein coupled receptor activity	2.43	3.03	-0.53
2	GTPBP4	23560	GTP binding protein 4	Nucleus	Ribosome biogenesis - small GTPase mediated signal transduction	2.00	2.91	-0.30
3	TFPI2	7980	Tissue factor pathway inhibitor 2	Extracellular matrix	Serine-type endopeptidase inhibitor activity	2.10	2.93	-0.27
4	CYP26A1	1592	Cytochrome P450, family 26, subfamily A, polypeptide 1	Membrane	Metal ion binding	2.31	2.39	-0.88
5	ZNF35	7584	Zinc finger protein 35 (clone HF.10)	Nucleus	Transcription factor activity	2.18	2.19	-0.66
6	RTTN	25914	Rotatin		required for left-right specification in mouse embryos	2.21	2.13	-0.58
7	FXYD3	5349	FXYD domain containing ion transport regulator 3	Membrane	Chloride channel activity	2.44	2.03	-0.15
8	CYYR1	116159	Cysteine/tyrosine-rich 1	Integral to membrane	Molecular function unknown	-2.30	-2.20	1.05
9	BFAR	512836	Bifunctional apoptosis regulator		Apoptosis regulator	-2.40	-2.17	1.06
10	C19orf4	25789	Chromosome 19 open reading frame 4	Integral to membrane	Molecular function unknown	-2.51	-2.28	0.19
11	KIAA1305	57523	KIAA1305 protein		Hypothetical protein	-2.19	-2.29	0.55
12	PCDH9	5101	Protocadherin 9	Integral to membrane	Cell adhesion	-2.18	-2.30	0.50
13	IKIP	121457	IKK interacting protein			-2.23	-2.33	0.83
14	FLJ21963	79611	FLJ21963 protein		Hypothetical protein	-2.12	-2.37	1.88
15	SCRG1	11341	Scrapie responsive protein 1	Extracellular space	Nervous system development	-2.14	-2.54	0.74
16	ULK2	9706	Unc-51-like kinase 2 (C. elegans)		Similar to a serine/threonine kinase in C. elegans	-2.46	-2.72	0.26
17	LIFR	3977	Leukemia inhibitory factor receptor	Integral to plasma membrane	Receptor activity	-2.26	-2.96	0.29
18	BMF	90427	Bcl-2 modifying factor	Sequestrated by myosin	Apoptotic activator - protein binding	1.00	2.95	2.40
19	CD248	57124	CD248 antigen, endosialin		Marker of stromal fibroblasts	0.74	2.31	2.10
20	PPM1E	22843	Protein phosphatase 1E (PP2C domain containing)		Phosphatase	0.76	2.33	2.06
21	CARD8	22900	Caspase recruitment domain family, member 8		Involved in NFκB pathway	-0.59	-3.26	4.07

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

hybridization at 42℃, the slides were washed in 50 mL 2 × SSC and 0.1% SDS at 55℃ 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.

The data discussed in this publication have been deposited in NCBIs 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 $\Delta\Delta C_T$ 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,

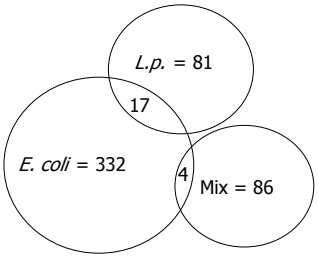

Figure 1 Schematic representation of the genes influenced by each treatment, and the overlapping (common) genes among treatments. The relative gene expression after treatment was > 2-fold compared with the control.

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://panigrahipeds.googlepages.com/suppl-tables.pdf; Raw data of all 19 200 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=nzyxdkkuwukuytk&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 mixed 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 chemoattractant 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 colonizing 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)[37,54] that expands to a limited but heterogenous 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:	HOXD10	3236	Homeobox D10	2.50
Regulation of transcription	PHF7	51533	PHD finger protein 7 (Zinc ion binding)	2.44
	EGR1	1958	Early growth response 1	-2.08
	TRIM24	8805	Tripartite motif-containing 24 (Zinc ion and DNA binding)	-2.15
	ENO1	2023	Enolase 1, (alpha) (DNA binding)	-2.34
Category 2: RNA processing	SSB	6741	Sjogren syndrome antigen B (autoantigen La)	-2.08
	FUSIP1	10772	FUS interacting protein (serine/arginine-rich) 1	-2.21
	NOLA5	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 3:	NEURL	9148	Neuralized homolog (Intracellular protein transport)	2.91
Protein biosynthesis, folding,	WDR36	134430	WD repeat domain 36	2.37
binding and transport	MTFMT	123263	Mitochondrial methionyl-tRNA formyltransferase	2.04
	ARF4	378	ADP-ribosylation factor 4	-2.03
	CEP57	9702	Centrosomal protein 57 kDa	-2.11
	ETF1	2107	Eukaryotic translation termination factor 1	-2.27
	HSPA1A	3303	Heat shock 70 kDa protein 1A	-2.28
	LGALS3	3958	Lectin, galactoside-binding, soluble, 3 (galectin 3)	-2.75
	HSPH1	10808	Heat shock 105 kDa/110 kDa protein 1	-2.93
	HSPA8	3312	Heat shock 70 kDa protein 8	-2.97
Category 4: Structural protein	AMPH	273	Amphiphysin (Actin cytoskeleton)	3.04
	MAP1B	4131	Microtubule-associated protein 1B	2.13
	ABCC10	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
	TWF1	5756	Twinfilin, actin-binding protein, homolog 1 (Tyrosin kinase)	-2.01
	AKR1C1	1645	Aldo-keto reductase 1, member C1 (Bile acid binding)	-2.09
	HMGCR	3156	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-2.10
	DC2	58505	DC2 protein (Glycotransferase activity)	-2.12
	GSTA1	2938	Glutathione S-transferase A1	-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	3320	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	-2.46
	AHCY	191	S-adenosylhomocysteine hydrolase	-2.48
	MAT2A	4144	Methionine adenosyltransferase Ⅱ, alpha	-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, mitochondrial F0 complex, subunit C3	-2.26
	HSPA1L	3305	Heat shock 70 kDa protein 1-like	-2.26
Category 7: Cell proliferation	FOSL1	8061	FOS-like antigen 1 (transcription factor activity)	-2.09
	FGG	2266	Fibrinogen gamma chain	-2.36
	FGG	2244	Fibrinogen beta chain	-2.45
Category 8: Cell adhesion	NELL2	4753	NEL-like 2 (Calcium ion binding)	2.25
	ITGB3	3690	Integrin, beta 3 (platelet glycoprotein Ⅲa, antigen CD61)	2.11
	RHOB	388	Ras homolog gene family, member B	-2.19
	ADRM1	11047	Adhesion regulating molecule 1	-2.25
Category 9: Ubiquitination	UBE2N	7334	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	-2.02
	UBE2S	27338	Ubiquitin-conjugating enzyme E2S	-2.05
	ANAPC7	51434	Anaphase promoting complex subunit 7	-2.15
	CACYBP	27101	Calcyclin 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
	RPS3A	6189	Ribosomal protein S3A	-2.08
	TWF1	5756	Twinfilin, 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.

period, on gut cells. We took advantage of a microarray chip that allowed us to examine 19 200 human genes in this simulated microbial gut environment. In this *in vitro* model, single and combined bacteria were allowed to interact with cultured cells, and our results were analyzed under high-stringency conditions to identify specific genes expressed during defined bacteria-gut cell interactions.

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

Table 3 Primers used for RT-PCR

Gene symbol	Gene ID (NCBI)	Gene name	Gene role	Primer	Primer sequence 5′-3′
BMF	90427	Bcl2 modifying factor, transcript variant 1	Has a single Bcl2 homology domain 3 (BH3), binds Bclk2 proteins and functions as an apoptotic activator	F	GCTTCAGTTGCATTGCAGACCAGTT
				R	AGAGCCCTTGGGAATTCTCACCAT
CD248	57124	CD248 antigen = endosialin	A gene regulated by the cell density *in vitro*. Has a calcium binding domain	F	TCAACTACGTTGGTGGCTTCGAGT
				R	AGTTGGGATAATGGGAAGCTGGGT
PPM1E	22843	Protein phosphatase 1E	Member of the PP2C family of Ser/Thr phosphatases known to be negative regulators of stress response pathways	F	ATGCCTCCATTCACCTCCACGTTA
				R	TGTCATAGAAGCCATCACAGGCCA
FXYD3	5349	FXY domain containing ion transport reg. 3	The protein encoded by this gene may function as a chloride channel or as a chloride channel regulator	F	AATGCAAGTTTGGCCAGAAGTCCG
				R	TTGCATATGAGGTCCCATGGCTGA
OAS2	4939	2′-5′-oligoadenylate synthetase 2	This enzyme family plays a significant role in the inhibition of cellular protein synthesis	F	AGAAGCCAACGTGACATCCTCGAT
				R	TGCTGGAGTTCAGTGAAGCAGACT
FY	2532	Duffy blood group antigen	Helps in leukocyte recruitment to sites of inflammation by facilitating movement of chemokines across the endothelium	F	TGACTCTGCACTGCCCTTCTTCAT
				R	TTGACAACAGCAACAGCTTGGACC
CERK	64781	Ceramide kinase	Integral to membranes, has roles in arachidonic acid release and production of eicosanoids	F	TGAGAAGAAACGGTGGTTGGGTCT
				R	AGCATTTCCGGATGAGGATGAGGT
HPSE	10855	Heparanase	Cell surface expression and secretion markedly promote tumor angiogenesis and metastasis	F	ACCTTTGCAGCTGGCTTTATGTGG
				R	CTTGCACGCTTGCCATTAACACCT
GAPDH	2597	Glyceraldehyde-3-phosphate dehydrogenase	Used as reference	F	GACCACAGTCCATGCCATCAC
				R	GAGCTTCAGAAAGTGGTCGTTGA

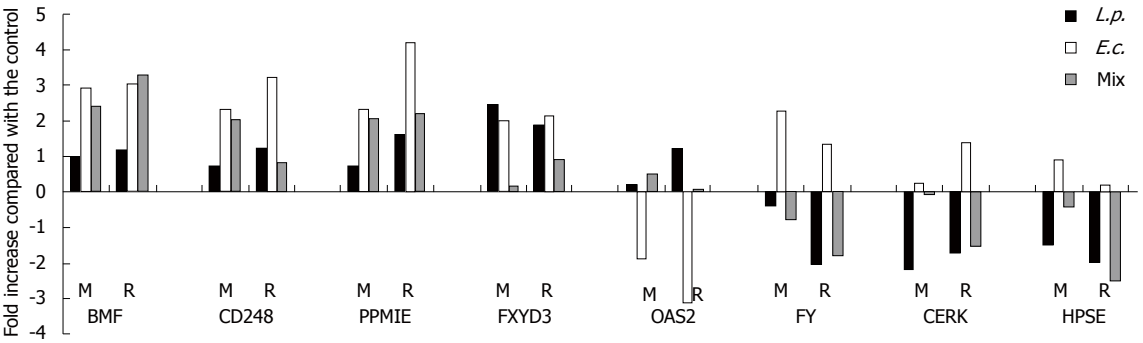

Figure 2 Effect of different bacterial treatments on expression of eight genes assessed by microarray (M) and RT-PCR (R).

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]. The numbers of unique genes presented in this study are in the range reported in previous studies in which Gram-negative enteric pathogens modified the expression of 0.5%-13% of the genes in epithelial cells[50,56-58], and commensal bacteria induced differential expression of 0.35%-6.2% of examined genes in mouse colonocytes[59]. 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 19200 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[49], 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[60,61]. 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

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, Ⅶc and Ⅷ 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 I-κB stabilization *via* 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[36]. 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 increasing clear that specific *Lactobacillus* species posses 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 vivo* 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 *Bifidobacteria* 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 19 200 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[50].

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[69,70] 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[71,72]. 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

endeavors, 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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