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Administration of *Lactobacillus* evokes coordinated changes in the intestinal expression profile of genes regulating energy homeostasis and immune phenotype in mice

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Lactic acid bacteria are probiotics widely used in functional food products, with a variety of beneficial effects reported. Recently, intense research has been carried out to provide insight into the mechanism of the action of probiotic bacteria. We have used gene array technology to map the pattern of changes in the global gene expression profile of the host caused by *Lactobacillus* administration. Affymetrix microarrays were applied to comparatively characterize differences in gene transcription in the distal ileum of normal microflora (NMF) and germ-free (GF) mice evoked by oral administration of two *Lactobacillus* strains used in fermented dairy products today – *Lactobacillus paracasei* ssp. *paracasei* F19 (*L.* F19) or *Lactobacillus acidophilus* NCFB 1748. We show that feeding either of the two strains caused very similar effects on the transcriptional profile of the host. Both *L.* F19 and *L. acidophilus* NCFB 1748 evoked a complex response in the gut, reflected by differential regulation of a number of genes involved in essential physiological functions such as immune response, regulation of energy homeostasis and host defence. Notably, the changes in intestinal gene expression caused by *Lactobacillus* were different in the mice raised under GF *v.* NMF conditions, underlying the complex and dynamic nature of the host-commensal relationship. Differential expression of an array of genes described in this report evokes novel hypothesis of possible interactions between the probiotic bacteria and the host organism and warrants further studies to evaluate the functional significance of these transcriptional changes on the metabolic profile of the host.

***Lactobacillus*: Oligonucleotide microarray: Energy homeostasis: Immune regulation**

During the last decades the role of the diet in development, as well as in prevention and management, of many diseases has been subjected to intense research. The term ‘functional food’ has been adapted to denote foods that may provide a health benefit beyond basic nutrition (Saris *et al.* 1998). The oldest and probably best-known functional food products are health-promoting bacteria or probiotics, defined as live microbial dietary supplements that beneficially affect consumers through their effects in the intestinal tract (Roberfroid, 2000). At present, probiotics, most often belonging to the genera *Lactobacillus* and *Bifidobacteria*, are almost exclusively consumed as fermented dairy products, such as yoghurt or freeze-dried cultures. Several health-related effects associated with the intake of probiotics have been reported in different animal models as well as in human studies (Roberfroid, 2000). However, the scientific evidence is still scarce and the mechanisms by which probiotics influence the host organism are only beginning to be explored.

The initial step in the characterization of mechanism of action for functional food products is the identification of a specific interaction between the active component of this food and an effect in the host organism that is potentially beneficial for health. One approach to investigate these interactions is to map the changes in transcription profile of the host organism caused by nutrient intake. Recent development of expression profiling by the use of microarray technology has made it possible to monitor the expression of thousands of genes simultaneously, allowing systematic analysis of complex biological processes and offering an advantage of reducing bias in data collection, compared with the candidate gene-based approaches. In the present report, we have studied the interactions between the intake of two strains of probiotic bacteria and regulation of intestinal gene expression of the host organism. Oligonucleotide microarrays were applied to compare global transcriptional profiles in the distal ileum of mice receiving *Lactobacillus paracasei* ssp. *paracasei* F19 (*L.* F19) or *L. acidophilus* NCFB 1748 with the control group of mice receiving a placebo product. The effects of the two *Lactobacillus* strains were evaluated both in germ-free (GF) or normal microflora (NMF) mice. *L.* F19 as well as *L. acidophilus* NCFB 1748 has been used in products branded Cultura and Dofilus (Arla Foods, Stockholm,

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Abbreviations: GF, germ-free; *L.* F19, *Lactobacillus paracasei* ssp. *paracasei* F19; NMF, normal microflora.

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Sweden). Both strains have good survival in the gastrointestinal tract, which is considered an important characteristic for the health-promoting activity of probiotics (Mättö *et al.* 2006). Previously, *L.* F19 and *L. acidophilus* NCFB 1748, in combination with *Bifidobacterium lactis* Bb12, have been shown to efficiently restore the intestinal microflora during antibiotic treatment (Sullivan *et al.* 2003). The objective of this study was to provide a comparative insight into the molecular mechanisms by which *L.* F19 and *L. acidophilus* NCFB 1748 interact with the host organism in a gnotobiotic environment *v.* in the context of the complex gut microflora.

Materials and methods

Animal experiments

GF and NMF male mice of Swiss Webster strain aged 6–8 weeks (Taconic, Lille Skensved, Denmark) were maintained under a standard 12-h light cycle regime, the relative humidity was between 45–55 %, the temperature was kept at 20°C. The animals had *ad libitum* access to purified ingredient diet D12450B (Research Diets Inc., New Brunswick, NJ, USA) and water. NMF mice were housed in individually ventilated standard cages. GF mice were housed in gnotobiotic isolators and handled according to established procedures; isolators were daily monitored for sterility by culturing animal faeces and isolator interiors for bacteria. After 1 week of acclimatization, the mice were divided into two test groups, which received *L.* F19 (*n* 6) or *L. acidophilus* NCFB 1748 (*n* 6) bacteria in acidified ultra high-temperature processed-milk (approximately 5×10^8 colony forming units/ml) and a control group (four in NMF and five in GF), which received acidified milk only. In NMF conditions, the products were administrated twice per d during a 10-d period: the first dosing was performed by oral gavage (1 ml per mouse) while the second dosing was done by sublingual injection (100 μl per mouse). To minimize the risk of compromising the sterility of the gnotobiotic isolators, the number of product administration events was reduced in GF conditions: the products were fed to GF mice once per d in 8 out of 10 d by oral gavage (1 ml per mouse). Following the period of product administration, the mice were killed by cervical dislocation. To avoid diurnal variations, all the mice were killed between 11.00 and 13.00 hours and approximately 4 h after the last dosing of *Lactobacillus*. The distal part of the ileum (1·5 cm) was excised for RNA extraction. Additional samples of ileum and colon were collected from each mouse for bacteriological analysis. To analyse the presence of *L.* F19 and *L. acidophilus* NCFB 1748 in the groups receiving an active product and the non-presence of these strains in the control groups, the samples were assayed on Rogosa agar (Merck, Darmstadt, Germany). Colonies were isolated and the presence of the two probiotic strains was confirmed by randomly amplified polymorphic DNA-PCR using the primers LBC-19 (5′-AGT AGC CAC-3′) and OPA-05 (5′-TGC CGA GCT G-3′) for screening and OPA-02 (5′-TGC CGA GCT G-3′) and OPA-13 (5′-CAG CAC CAC-3′) for confirmation of *L.* F19 and *L. acidophilus* NCFB 1748, respectively. Slanetz Bartley agar (Merck) was used for checking for the possible presence of enterococci. To confirm the absence of bacteria other than the administrated strains in ex-GF mice, these samples were spread on blood agar base (Oxoid, Basingstoke, Hants., UK) with defibrinated blood added to a final concentration of 7 %, and the plates were incubated both aerobically and anaerobically. The present study was performed after prior approval from the local ethical committee for animal experimentation.

Test products

Test products were produced from ultra high-temperature processed milk containing 1·5 % fat. Probiotic organisms, *L.* F19 or *L. acidophilus* NCFB 1748, were added to a final concentration of 1×10^9 colony forming units/ml and the milk was acidified to pH 4·5 by addition of glucono-δ-lactone to a final concentration of 1·6 %. The placebo product was identical to the active product despite no addition of probiotic organisms. The number of probiotic bacteria in the products was assayed by plating on MRS agar, pH 5·4 (de Man Rogosa Sharp; Oxoid). The absence of contaminating bacteria was confirmed by plating on count agar sugar free FIL-IDF (Fédération internationale de laiterie/International Dairy Federation) (Merck) and on plate count agar (Oxoid) with skimmed milk added to a final concentration of 0·1 %, for the active and placebo products, respectively.

RNA extraction

Tissues collected for RNA preparation were immediately submerged in RNA*later* RNA Stabilization Reagent (Qiagen, Hilden, Germany) to preserve the quality and quantity of RNA. Total RNA was isolated from the intestinal tissue samples using the RNeasy Mini Kit (Qiagen) followed by a DNase digestion step (RNase-Free DNase Set; Qiagen) according to the manufacturer's instructions. The RNA yield was quantified by spectrophotometric analysis and the RNA purity was determined based on the A_{260}:A_{280} ratio. All RNA samples were analysed by agarose gel electrophoresis to check for integrity of 18S and 28S rRNA. Further, the quality of the RNA was verified by Agilent 2100 Bioanalyzer analysis (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 Nano Assay Kit (Agilent Technologies).

Expression profiling by Affymetrix

Total RNA spiked with poly-A controls (pGIBS-TRP, –THR and –LYS; American Type Culture Collection, Manassas, VA, USA) was converted into cDNA utilizing a T7 promoter-polyT primer (Genset, Paris, France) and the RT Superscript II (Invitrogen Life Technologies, Paisley, UK) followed by a second strand cDNA synthesis (Invitrogen Life Technologies). ds cDNA was *in vitro* transcribed into biotinylated cRNA (Enzo Life Sciences, Farmingdale, NY, USA). Finally, fragmented cRNA (35–200 bases) was used as a target for hybridization. Hybridization spike controls (oligonucleotide B2 and a cRNA cocktail (BioB, BioC, BioD and Cre; GeneChip Eukaryotic Hybridization Control Kit; Affymetrix, Santa Clara, CA, USA)) were used as hybridization quality controls. Aliquots of each sample were hybridized (16 h at 45°C) to the Murine Genome Array U74Av2 (Affymetrix). The arrays were subsequently washed, stained and scanned according to the manufacturer's instructions (GeneChip Expression Analysis Technical Manual; Affymetrix). Data were analysed using robust multi-chip analysis in GeneTraffic UNO version 2.6–25 (Stratagene, La Jolla, CA, USA) and Spotfire

DecitionSite for Functional Genomics version 7.1 (Spotfire Inc., Göteborg, Sweden). The $\log_2$ fold change for each sample and probe set *v.* the control group was calculated using the formula: $\log_2$ fold change = $\log_2$ (probe set intensity/mean probe set intensity in the control group). The mean $\log_2$ fold change was calculated for each *Lactobacillus* strain *v.* the control group. Statistical significance of the difference in gene expression was determined using two-sided Student's *t* test. A transcript was considered differentially expressed if the mean absolute $\log_2$ fold change was >0·5 (corresponding to a mean absolute fold change >1·41) and the *P*-value was <0·05. In addition, the mean intensity in the group showing the highest expression should be >75. To view the microarray data in a biological context, Ingenuity Pathway Analysis Software tool (Ingenuity Systems, Redwood City, CA, USA; http:\\www.ingenuity.com) was used to generate a network connecting the differentially regulated targets and all other gene products, based on known mammalian gene product events (such as protein–protein, protein–nucleic acid interactions) defined in Ingenuity System's database.

Expression profiles of genes differentially regulated in GF mice in response to *Lactobacillus* administration were compared with the corresponding profiles in GF mice of the NMRI/KI strain mono-colonized by *Bacteroides thetaiotaomicron,* using the data published by Hooper *et al.* (2001). The .CEL files from this study (Mu11K and Mu19K Affymetrix array sets, duplicate microarray hybridizations performed on pooled ileal RNA samples corresponding to GF *v.* mono-colonized mice) kindly provided by Professor L.V. Hooper, were re-analysed using Microarray Suite Version 5.0 (MAS5.0) (Affymetrix) and Spotfire DecitionSite for Functional Genomics version 7.1 (Spotfire Inc.). The overall intensity across each array was scaled to a target intensity of 150. A transcript was considered differentially expressed if the mean absolute $\log_2$ fold change was >0·5 (corresponding to a mean absolute fold change >1·41). Additionally, the mRNA should be called Present by the MAS5.0 software in either GF or colonized mice, and the differential expression should be observed in all four comparisons performed on duplicate microarray hybridizations (see Hooper *et al.* (2001) for details concerning the design of the microarray experiment).

Quantitative real-time PCR

Expression profiles for selected targets were confirmed using quantitative real-time PCR with the ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Briefly, total RNA, isolated for the expression profiling, was converted into cDNA by utilizing the Superscript™ first-strand synthesis system (Invitrogen Life Technologies). PCR reactions (25 μl) contained each PCR primer (400 nm) designed by using PRIMER EXPRESS 2.0 software (Applied Biosystems) and 1x SYBR® Green PCR Master Mix (Applied Biosystems). The forward and reverse primer sequences are presented in Table 1. All reactions were performed in duplicate and a dissociation curve was completed for every PCR run to control the specificity of the amplification reaction. The relative quantities of different mRNA transcripts were calculated after normalization of the data against an endogenous control – acidic ribosomal phosphoprotein P0 (*Arbp*) – using the standard curve method (Applied Biosystems, 1997).

Results

Microarray analysis of the differential gene expression evoked by oral administration of Lactobacillus *strains*

We have used oligonucleotide microarray to analyse the host transcriptional responses caused by oral delivery of *Lactobacillus* bacteria. The test groups of mice received one strain of *Lactobacillus* – *L.* F19 or *L. acidophilus* NCFB 1748 – in acidified milk, while the control group of mice received acidified milk only. Experiments were performed using age-matched male mice raised under GF or NMF conditions. After the period of product administration of 10 d, the presence of the corresponding *Lactobacillus* strains in the intestinal contents of the test groups of mice, and the lack of these bacteria in the control group, were confirmed. Importantly, from the intestine of the test groups of mice raised under GF conditions, only the administrated strain could be re-isolated, while the corresponding control group remained GF during the whole study period. While previous studies have shown that the different strains of *Lactobacillus* bacteria are able to colonize the whole gastrointestinal tract of GF mice and are established in high numbers in both the small and large intestine (Wagner *et al.* 1997; Ibnou-Zekri *et al.* 2003), we found that *L.* F19 and *L. acidophilus* NCFB 1748 were present in the ileum of the GF mice in considerably larger numbers than compared with the colon (data not shown). The gene expression profiles in the distal ileum of the test groups of mice were compared with the corresponding control group using Affymetrix gene arrays.

Table 1. Primer sequences used for quantitative real-time-PCR analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession no.
Clu	CCACCGTGACCACCCATT	CAGCTTCACCACCACCTCAGT	NM_013492
Igh-6	ACTGCCTCCACCTTCATCGT	CTGAGAGTCATTTCACCTTGAACAG	BC098504
Cxcl13	AACGCAGGCTTCCAAAATAGTC	TGCTTTGCACCACCTCATGA	NM_018866
Ltb	ATCGGGTACGGGTCGTTATG	ATCACCGCCCCGAAGAAG	NM_008518
Serpina1c	CAAACTCTCAGCAAGGAGCTCAT	GGGAAGTGGATCTGGGCTAAC	NM_009245
Rbp2	ACATGAAGGCCCTAGATATTGATTTT	AGTGATGATCTTCGTCTGAGTCAGA	NM_009034
Apoa4	CAGCTGGGTCCCAATTCG	CAGGGTGCTCATAAAGGAGTTGA	NM_007468
Retnlb	CAATGCTCCTTTGAGTCTTTGGT	GCAGGAGATCGTCTTAGGCTCTT	NM_023881
Adipoq	TCAACGACTCTACATTTACTGGCTTT	GTTCCATGATTCTCCTGGTGTATG	NM_009605
Cfd	GCTATCCCAGAATGCCTCGTT	GGTTCCACTTCTTTGTCCTCGTA	NM_013459
Car3	CACACGTTAACATCATTGTAGATCTCA	CTTGGTAGTAGGCAAATTTTTAACGA	NM_007606
Arbp	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC	NM_007475

The global gene expression analysis demonstrated that genes altered by administration of *Lactobacillus* are involved in widely different functions. In the NMF mice, the expression of twenty-two probe sets was significantly ($P < 0{\cdot}05$) changed by a factor of more than 41 % (absolute $\log_2$ fold change $> 0{\cdot}5$), in at least one test group, relative to the control group (Table 2). Administration of *Lactobacillus* to GF mice led to the differential expression of thirty probe sets in one or both test groups, relative to control mice (absolute fold change $> 1{\cdot}41$, $P < 0{\cdot}05$; Table 3). From these transcripts, the identity of most of the genes is known and represents proteins of different functional classes, whereas three transcripts only show homology to sequences in the Expressed sequence tag (EST) or genomic databases. The vast majority of genes, which were significantly changed exclusively in mice receiving *L.* F19 or *L. acidophilus* NCFB 1748, tended to be regulated in the same manner in the other test group, even though this difference did not reach statistical significance and/or meet the fold change criteria. Importantly, transcriptional changes evoked by administration of *Lactobacillus* to NMF mice were different from the responses seen in GF mice. In fact, only one probe set, representing retinol binding protein 2, showed significant difference in response to the delivery of *Lactobacillus* bacteria both to NMF and GF mice (Tables 2 and 3).

Quantitative real-time PCR validation of differentially expressed genes

To minimize erroneous conclusions due to technical variability and multiple testing effects inherent to the microarray technology, quantitative real-time PCR analysis was applied to validate expression profiles of eleven genes selected on the basis of biological interest (Fig. 1). For all the genes examined, quantitative real-time PCR data are in good agreement with the gene array results with regard to the direction of observed changes. Furthermore, individual animal-to-animal comparison of the expression profiles for these genes showed good correlation comparing the two techniques (data not shown).

Administration of Lactobacillus *to normal microflora mice modulates the immune phenotype expression*

Several genes with a potential role in the intestinal immune system – *Clu, C3, Bcl6, Ptprc, Serpina1, Laptm5* and *Vcam1* – were significantly up-regulated in the NMF mice receiving *L. acidophilus* NCFB 1748 and showed identical transcription profiles. Searching for similar expression patterns using Profile Search (Spotfire DecitionSite for Functional Genomics version 7.1; Spotfire Inc.) with correlation as similarity measure (0·92 as cut off) identified thirty-five additional probe sets up-regulated by a factor of more than 41 % in at least one test group, compared with the control mice (Table 2). Ingenuity Pathway Analysis (Ingenuity Systems) reveals that these targets form an integrated functional network controlling different aspects of immune system development and function and are regulated by common upstream factors (Fig. 2(A)). Most of the transcripts in this cluster are expressed predominantly or exclusively in B cells, e.g. the components of the B cell receptor for antigen including Ig molecule (encoded by *Ighs*), *Cd79a* and *Cd79b*, as well as factors involved in downstream signalling from the B cell receptor, such as *Ptprc* (alias *Cd45*), *Cd19, Blk, Lck* and *Prkcb1*. A key mediator responsible for the organization of B cells within lymphoid structures, *Ltb*, together with three additional markers potentially involved in this function, Cxcl13*, Vcam1* and *Bcl6*, were up-regulated in response to *Lactobacillus* administration. Additionally, genes with potential function in phagocytosis (*Mfge8* and *Coro1a*) and complement function (*C3* and *Clu*) were increased in the test groups. Several probe sets supported an up-regulation in *Serpina1* transcript, encoding for α1-antitrypsin, in response to *Lactobacillus* administration.

Administration of Lactobacillus *strains in germ-free mice evokes coordinated changes in expression profiles of genes regulating energy homeostasis and host defence*

Three transcripts encoding for key regulators of fat and sugar metabolism and insulin sensitivity at the whole body level – adipsin (*Cfd*), adiponection (*Adipoq*) and resistin like β (*Retnlb*) – were differentially expressed in the test groups of mice compared with the control group (see Table 3 for statistical significance notes). Adipsin and adiponectin, an insulin-sensitizing hormone (Pajvani & Scherer, 2003), were up-regulated, while resistin like β, known to induce insulin resistance (Rajala *et al.* 2003), was down-regulated, in response to *Lactobacillus* administration. Transcripts for three cytosolic proteins, Scd1, Car3 and Thrsp, with possible functions in lipid metabolism, were significantly up-regulated in the groups of mice receiving *Lactobacillus* (Table 3). Stearoyl-Coenzyme A desaturase 1 is an Fe-containing enzyme that catalyses a rate-limiting step in the synthesis of unsaturated fatty acids. Although very little is known about the specific function of *Car3* and *Thrsp*, both genes are implicated in fatty acid metabolism (Stanton *et al.* 1991; Kinlaw *et al.* 1995). Additionally, several genes with a potential role in intestinal defence against bacterial infections – *Mmp7, Lyzs, Pla2g2a, Sprr1a, Igh6* – were up-regulated in response to *Lactobacillus* administration in GF mice. Ingenuity Pathway Analysis (Ingenuity Systems) indicated that the differentially expressed targets involved in regulation of energy homeostasis as well as host defence form a complex network regulated by common upstream mediators, with TNF having a central regulatory role (Fig. 2(B)).

To evaluate if the changes in gene expression profile we describe are specific to *Lactobacillus* or can be elicited by inoculation of the gnotobiotic intestine with non-probiotic bacteria as well, we turned to the previously published study describing the effects of colonization with *Bacteroides thetaiotaomicron*, a prominent component of the normal intestinal microflora (Hooper *et al.* 2001). In this report, the global gene expression profile in the distal ileum of mice inoculated with *Bacteroides* was compared with the age-matched mice remaining GF by Affymetrix microarrays, thus providing a good comparison to our dataset. We compared the transcription profiles of genes differentially regulated in response to *Lactobacillus* (Table 3) with the corresponding profiles in mice colonized by *Bacteroides,* using the raw data files kindly provided by Professor L.V. Hooper (see Materials and Methods for details on data analysis). The probe sets for

Table 2. Differentially expressed genes in normal microflora mice receiving *Lactobacillus paracasei* ssp. *paracasei* F19 (F19) or *Lactobacillus acidophilus* NCFB 1748 (NCFB) compared with the control group of mice receiving placebo product‡§

		Fold change		
Probe set ID	Gene symbol	F19	NCFB	Potential function
Immune regulation				
92 740_at†	*Igh-5*	2·13	2·61	Ig heavy chain of IgD
93 584_at†	*Igh-6, MGC60843*	1·67	2·41	Ig heavy chain of IgM
99 446_at†	*Ms4a1*	1·71	2·27	a B-lymphocyte surface marker involved in regulation of B-cell proliferation and differentiation
95 286_at	*Clu*	1·75	2·24*	a multifunctional glycoprotein involved in complement regulation
102025_at†	*Cxcl13*	1·85	2·23	a chemokine required for the architectural organization of B-cells within lymphoid follicles
102940_at†	*Ltb*	1·50	1·95	a membrane protein involved in organization of secondary lymphoid structures in the intestine
93 583_s_at†	*Igh-6, MGC60843*	1·57	1·90	Ig heavy chain of IgM
100468_g_at†	*Lyl1*	1·44	1·83	a cytosolic protein expressed in most B-lineage cells
101048_at	*Ptprc*	1·49	1·81*	a transmembrane tyrosine phosphatase, positive regulator of BCR signalling
92 880_at†	*Mfge8*	1·59	1·74	a secreted glycoprotein involved in phagocytosis
161294_f_at†	*Clu*	1·56	1·70	a multifunctional glycoprotein involved in complement regulation
102778_at†	*Cd79a*	1·68	1·67	a B-lineage-specific member of the Ig superfamily, together with CD79b forms the signal transducing part of the BCR
95 893_at†	*Blk*	1·45	1·65	a Src-family protein tyrosine kinase activated by BCR
94 278_at†	*Lcp1*	1·48	1·63	a major lymphocyte cytosolic protein
93 497_at	*C3*	1·51	1·62*	complement factor, complement-coated antigens cross-link CD21 with the BCR increasing the signal through the receptor
96 648_at†	*Coro1a*	1·43	1·59	an actin-binding protein required for phagosome formation
100329_at	*Serpina1c*	1·58	1·51*	a protease inhibitor, implicated in protection against mucosal damage in inflammatory bowel disease
101576_f_at†	*Serpina1b*	1·53	1·55	
93 109_f_at†	*Serpina1a,b,c,d*	1·47	1·53	
161012_at†	*Cd79b*	1·40	1·83	a B-lineage-specific member of the Ig superfamily, together with CD79a forms the signal transducing part of the BCR
93 915_at†	*Pou2af1*	1·29	1·77	B-cell-specific transcriptional co-activator regulating expression of Ig genes
104606_at†	*Cd52*	1·35	1·74	a cell surface protein expressed in lymphocytes, macrophages and monocytes
102823_at†	*Ighg*	1·23	1·58	Ig heavy chain of IgG
103015_at	*Bcl6*	1·39	1·56*	a transcriptional repressor expressed in B-cells, controls germinal centre formation
94 939_at†	*Cd53*	1·37	1·56	a glycoprotein expressed in leukocytes
100012_at	*Laptm5*	1·41	1·55*	a lysosomal protein expressed mostly in haematopoietic cells
102824_g_at†	*Ighg*	1·23	1·54	Ig heavy chain of IgG
102809_s_at†	*Lck*	1·37	1·52	a Src-family protein tyrosine kinase activated by BCR signalling
92 558_at	*Vcam1*	1·33	1·52*	a cell surface glycoprotein expressed in endothelium, where it mediates the adhesion of monocytes and lymphocytes
103040_at†	*Cd83*	1·35	1·51	a cell surface molecule expressed at haematopoietic cells
93 957_at†	*Vpreb3*	1·35	1·50	expressed exclusively in B-cells, associates with membrane Ig heavy chains early in the course of BCR biosynthesis
98 980_at†	*Cd37*	1·34	1·47	a membrane protein expressed predominantly on the surface of B cells
101876_s_at	*H2-T10,H2-T22,H2-T9*	1·23**	1·47*	belongs to the major histocompatibility complex, class II
92 741_g_at†	*Igh-5*	1·46	1·36	Ig heavy chain of IgD
97 994_at†	*Tcf7*	1·35	1·46	a T-cell-specific transcription factor, which controls thymocyte differentiation
99 945_at†	*Cd19*	1·40	1·45	a transmembrane protein associated with BCR, acts as a adaptor molecule and amplifies BCR signals
103231_at†	*Rhoh*	1·31	1·45	a protein similar to members of the Ras superfamily, expressed in haemopoietic cell lines only
99 510_at†	*Prkcb1*	1·39	1·44	a kinase functionally linked to Bruton kinase in BCR-mediated signal transduction
102851_s_at†	*Ptpn6*	1·30	1·43	a tyrosine protein phsophatase expressed predominantly in haematopoietic cells
96 172_at†	*Gimap4*	1·37	1·42	a protein expressed in B and T cells, function not clear
100377_f_at	*IghmAC 38.205.12*	− 1·01	− 1·69*	Ig heavy chain of IgM
93 638_s_at	*Igl-V1*	− 1·67*	− 1·24	Ig λ chain, variable region
100360_f_at	*Igh-4*	− 1·15	− 1·64*	Ig heavy chain for serum IgG1
97 009_f_at	*Igh-V*	1·00	− 1·61*	Ig heavy chain, variable region
Miscellaneous				
100078_at	*Apoa4*	1·18	1·83*	a satiety signal secreted by the small intestine, implicated in regulation of both short and long-term food intake
95 673_s_at†	*Basp1*	1·20	1·68	expressed in nervous tissue, function largely unknown

Table 2. *Continued*

Probe set ID	Gene symbol	Fold change F19	Fold change NCFB	Potential function
94 540_at	*Cyp2d26*	1·20	1·58*	a member of cytochrome P450 family, specific function poorly understood
94 004_at	*Cnn2*	1·31	1·57*	widely expressed, function largely unknown
101972_at†	*Napsa*	1·37	1·49	an aspartic proteinase, function not clear
93 874_s_at	*Il11ra1, Il11ra2*	1·12	1·48**	a receptor for IL-11, which is a stromal cell-derived cytokine with multiple biological activities
104707_at	*Tm4sf5*	1·06	1·46*	a cell surface protein, function poorly described
92 811_at	*Rbp2*	1·21	1·43*	regulates the uptake and metabolism of vitamin A
160308_at†	*Msn*	1·35	1·45	expressed in different tissues, localizes to membrane protrusions that are important to cell-cell recognition, signalling and cell movement
96 353_at	*Tmem14c*	−2·54*	−1·54	transmembrane protein, function poorly described
93 934_at	*Cldn2*	−1·05	−1·52*	a member of a claudin family of integral membrane proteins localized at tight junctions
100946_at	*Hspa1b*	−1·07	−1·48*	a heat-shock protein, expressed in response to heat shock and a variety of other stress stimuli
98 384_at	*Ptk6*	−1·09	−1·43*	a kinase implicated in cell transformation

Values were significantly different, determined by two-sided Student's *t* test: (*$P \leq 0{\cdot}05$; **$P \leq 0{\cdot}01$).

† Transcripts were identified by similarity search using mean expression profile of *Clu, C3, Bcl6, Ptprc, Serpina1, Laptm5* and *Vcam1* as a template.

‡ Global mRNA expression pattern was characterized in distal ileum using Affymetrix (Santa Clara, CA, USA) gene arrays. The filtering criteria were set to a mean absolute fold change $>1{\cdot}41$ ($\log_2$ fold change $>0{\cdot}5$) and a *P* value $<0{\cdot}05$ in either of the two test groups, compared with the control group. In addition, the mean intensity in the group showing highest expression should be >75.

§ For details of procedures, see Materials and methods.

The colours represent the differential expression pattern. Green indicates down-regulation; red indicates up-regulation with an absolute $\log_2$ fold change $>0{\cdot}5$. BCR, B cell receptor.

several genes involved in regulation of energy homeostasis (*Adipoq, Retnlb, Scd1*) were represented and called Present on these arrays; however, none of these transcripts was regulated in response to *Bacterioides* in the manner similar to *Lactobacillus*. On the contrary, two probe sets supported an up-regulation of *Retnlb* by *Bacteroides*, while this gene was down-regulated in response to *Lactobacillus*. Interestingly, *Rbp2*, encoding a protein shown to participate in uptake and metabolism of vitamin A in the small intestine (Levin, 1993; Lissoos *et al.* 1995), was up-regulated in response to *Bacteroides* as well as *Lactobacillus*. Similar to *Lactobacillus*, administration of *Bacteroides* increased expression of genes implicated in host defence, including small proline like proteins (*Sprr2a* up-regulated by *Bacteroides* and *Sprr1a* by *Lactobacillus*) and *Pla2g2a* (data not shown).

Discussion

In the present study, we investigated the molecular effects of the two *Lactobacillus* strains – *L.* F19 and *L. acidophilus* NCFB 1748 – on global gene expression profile in the distal small intestine using the Affymetrix gene arrays. The changes in transcriptional profile caused by oral administration of *Lactobacillus* bacteria in NMF mice were compared with the effects evoked by administration of these strains in GF mice, with the GF mice representing a simplified model of interactions between gut commensals and their host.

Lactobacilli have long been acknowledged to promote the intestine's immunological barrier, particularly through enhancement of humoral immune responses, induction of germinal centre formation, activation of the phagocytosis by macrophages and alleviation of intestinal inflammatory responses (Wagner *et al.* 1997; Perdigon *et al.* 1999; Shu & Gill, 2002; Ibnou-Zekri *et al.* 2003; Galdeano & Perdigon, 2006). Recent data indicate that differences may exist in the immune stimulatory effects of specific strains of probiotic bacteria (Perdigon *et al.* 1999; Ibnou-Zekri *et al.* 2003). In line with the previous reports, the present study demonstrated that the administration of two *Lactobacillus* strains to NMF mice caused concerted increases in a cluster of genes involved in immune response (Table 2, Fig. 2(A)). Several components of B cell receptor-signalling were up-regulated (*Ighs*, *Cd79a*, *Cd79b*, *Ptprc* (alias *Cd45*), *Cd19, Blk, Lck, Prkcb1*), suggesting mobilization of B-lymphocytes. Also, transcripts implicated in phagocytosis (*Mfge8*, *Coro1a*), complement function (*C3*, *Clu*) and architectural organization of B cells within lymphoid structures (*Ltb, Cxcl13, Vcam1* and *Bcl6)* were increased in response to *Lactobacillus* administration. Previously, Di Caro *et al.* (2005) demonstrated by global gene expression profiling in duodenal mucosa that administration of *L. rhamnosus* to human subjects induced expression of a number of genes involved in immune response, including *Ltb, Cxcl13*, *C3* and *Ms4a1*, which were also up-regulated by the two strains used in the present study. We did not detect any qualitative differences comparing the effect of *L.* F19 *v. L. acidophilus* NCFB 1748 on the expression profile of immune response-related genes. However, the mean fold change of the increased signal for this group of transcripts was higher in mice receiving *L. acidophilus* NCFB 1748. Notably, immune stimulatory effect in response to *Lactobacillus* administration was not observed in mice raised under GF conditions. The intestinal microflora has a large impact on the development of gut-associated lymphoid tissue. Also, the response to probiotic bacteria depends on the immunological state of the host (Falk *et al.* 1998). Therefore, we speculate that under the conditions used, the intestinal tissue of gnotobiotic mice might have been incompetent to respond to the stimulation by lactic acid bacteria as observed in NMF mice.

Table 3. Differentially expressed genes in germ-free mice receiving *Lactobacillus paracasei* ssp. *paracasei* F19 (F19) or *Lactobacillus acidophilus* NCFB 1748 (NCFB) compared with the control group of mice receiving placebo product†‡

Probe set ID	Gene symbol	Fold change F19	Fold change NCFB	Potential function
Energy homeostasis				
99 671_at	*Cfd*	1·59	2·35*	regulates insulin sensitivity
99 104_at	*Adipoq*	1·76	2·26*	regulates insulin sensitivity
160375_at	*Car3*	1·70	2·17*	implicated in fatty acid metabolism
94 057_g_at	*Scd1*	1·78	1·95*	catalyses a rate-limiting step in the synthesis of unsaturated fatty acids
94 056_at	*Scd1*	1·63	1·80*	
160306_at	*Thrsp*	1·55*	1·25**	implicated in lipogenesis
93 755_at	*Retnlb*	−29·33*	−25·14*	antagonizes insulin action
Host defence				
92 917_at	*Mmp7*	2·26*	1·53	implicated in regulation of intestinal mucosal defence
101753_s_at	*Lyzs, Lzp-s*	2·20*	1·60	catalyses the hydrolysis of certain mucopolysaccharides of bacterial cell walls
100611_at	*Lyzs*	1·91*	1·48	
92 735_at	*Pla2g2a*	2·02*	1·58	phospholipase, may contribute to the gastric response to bacterial infection
101752_f_at	*Igh-6*	1·05	1·74*	Ig heavy chain of IgM
160909_at	*Sprr1a*	1·15	1·45*	implicated in fortifying the intestinal epithelial barrier in response to bacterial colonization
Miscellaneous				
93 142_at	*Bach1*	1·75***	1·50*	forms heterodimers with MafK and coordinates transcription activation and repression by this factor
92 811_at	*Rbp2*	1·47	1·57*	regulates the uptake and metabolism of vitamin A
104155_f_at	*Atf3*	1·46*	1·55*	a member of the mammalian activation transcription factor/CREB protein family of transcription factors
94 910_at	*Nde1*	2·13**	−1·06	function in the intestine poorly understood
160906_i_at	–	1·71*	1·28	–
162190_r_at	*Lmbr1l*	1·65*	1·40	function poorly understood
96 679_at	*Dnajb9*	1·52*	1·27	function poorly understood
160829_at	*Phlda1*	1·37*	1·49*	function poorly understood
92 470_f_at	*LOC546230*	1·20	1·49*	–
101704_at	*Hnf4g*	1·47*	1·40	transcription factor involved in divergent functions
93 975_at	*Errfi1*	1·15	1·45*	function poorly understood
102208_at	*St3gal6*	−1·91*	−1·35	catalyses the transfer of sialic acid to terminal positions on the carbohydrate groups of glycoproteins and glycolipids
95 586_at	*P2rx4*	−1·51*	−1·41*	ligand-gated ion channel
95 518_at	*1810015C04Rik*	−1·50*	−1·31	–
93 372_at	*Anp32a*	−1·47*	−1·29*	a putative HLA class II-associated protein
96 656_at	*Wdr48*	−1·43*	−1·33	function poorly understood
96 088_at	*Ndrg2*	−1·42*	−1·25	function in the intestine poorly understood

Values are significantly different, determined by two-sided Student's *t* test ($^*P \leq 0{\cdot}05$; $^{**}P \leq 0{\cdot}01$; $^{***}P \leq 0{\cdot}005$).

† Global mRNA expression pattern was characterized in distal ileum using Affymetrix (Santa Clara, CA, USA) gene arrays. The filtering criteria were set to a mean absolute fold change $>1{\cdot}41$ ($\log_2$ fold change $>0{\cdot}5$) and a *P* value $<0{\cdot}05$ in either of the two test groups, compared with the control group. In addition, the mean intensity in the group showing highest expression should be >75.

‡ For details of procedures, see Materials and methods.

The colours represent the differential expression pattern. Green indicates down-regulation; red indicates up-regulation with an absolute $\log_2$ fold change $>0{\cdot}5$. CREB, cyclic AMP-response element-binding; HLA, histocompatibility locus antigen.

Lactobacillus bacteria have been shown to improve clinical symptoms of inflammatory bowel disease, a major clinical problem in the field of gastroenterology (Matsumoto *et al.* 2001; Saggioro, 2004). In this context, it is interesting to note that *Serpina1*, encoding α1-antitrypsin, was up-regulated in the NMF mice in response to *Lactobacillus* administration (Table 2). The concentration of α1-antitrypsin is increased in the intestine in connection with inflammatory bowel disease, where its release is thought to protect the mucosa from proteolytic damage (Faust *et al.* 2002). We hypothesize that increase in *Serpina1* transcription may be part of the mechanism for *Lactobacillus* bacteria to reduce the severity of inflammatory bowel disease, which remains to be addressed in further studies.

Recently, the role of factors secreted from the gut in regulation of food intake and whole body energy partitioning has been acknowledged (Bloom *et al.* 2005). Also, connection between gut microflora and energy homeostasis of the host organism has become recognized. Colonization of GF mice with normal gut microflora was shown to increase body fat and cause insulin resistance (Backhed *et al.* 2004). Conversely, obesity affects the composition of the gut microbiota in mice (Ley *et al.* 2005). Interestingly, consumption of dairy products supplemented with *Lactobacillus* bacteria has been shown to decrease serum cholesterol and LDL-cholesterol (Akalin *et al.* 1997). However, the mechanisms responsible for directing these changes remain largely unknown. In this context, one of the most interesting findings of the present study was the coordinated differential regulation of transcripts for several secreted factors controlling whole body lipid and glucose metabolism, in response to the administration of *Lactobacillus* in GF mice. Adiponectin (*Adipoq*) and adipsin

(*Cfd*), both up-regulated in the test groups of mice, are known to be decreased in overweight human subjects and/or in animal models of obesity (Flier *et al.* 1987; Lowell *et al.* 1990; Shillabeer *et al.* 1992; Hu *et al.* 1996; Yamauchi *et al.* 2001). Interestingly, pharmacological adiponectin treatment in rodents has been shown to increase insulin sensitivity (Pajvani & Scherer, 2003). Resistin like β (*Retnlb*), on the other hand, was down-regulated in the test groups of mice. Recently, elevated serum levels of resistin like β, attributable to increased mRNA and protein production in intestine and bone marrow, were reported in mice fed a high-fat diet as well as in animal models of type 2 diabetes (Shojima *et al.* 2005). Infusion of resistin like β has been shown to induce insulin resistance (Rajala *et al.* 2003). In the light of this knowledge, an increased signal for adiponectin and adipsin in combination with reduced expression of resistin like β, observed in GF mice in response to the delivery of *Lactobacillus* (Table 3), suggests the possibility for improved insulin sensitivity of the host organism. The present report does not answer the question if similar host responses are elicited by other components of the gut microflora. Notably, colonization of GF mice with normal gut microflora has been shown to decrease, rather than increase, insulin sensitivity (Backhed *et al.* 2004). One factor suppressed in the ileum in response to conventionalization of gnotobiotic mice, implicated in the promotion of adiposity and insulin resistance, is Fiaf (Backhed *et al.* 2004). Probe sets for Fiaf were represented on the Affymetrix microarray used in this study. However, the mRNA level for this gene was unaltered in response to *Lactobacillus* administration (data not shown). Interestingly, mono-colonization of GF mice with common gut bacterium *Bacteroides thetaiotaomicron* (Hooper *et al.* 2001) did not evoke effects similar to *Lactobacillus* on the expression of genes involved in regulation of energy homeostasis. This suggests that the gut microbiota influence the expression of genes important for energy metabolism differently depending on the microbial composition and that *Lactobacilli* may influence transcription of genes involved in regulation of energy homeostasis in a favourable way. However, notice should be taken that different strains of GF mice have been used in the reports compared (C57BL/6J, NMRI/KI or Swiss Webster strain was used by Backhed *et al.* 2004, Hooper *et al.* 2001 or in the present study, respectively) and, therefore, we cannot exclude the possibility that the differences in the transcriptional changes described in response to the different bacterial strains are at least partly related to the variation in the genetic background of the host.

Adiponectin, adipsin and resistin like β were differentially regulated in response to *Lactobacillus* administration in GF but not in NMF mice. The gut microbiota are known to

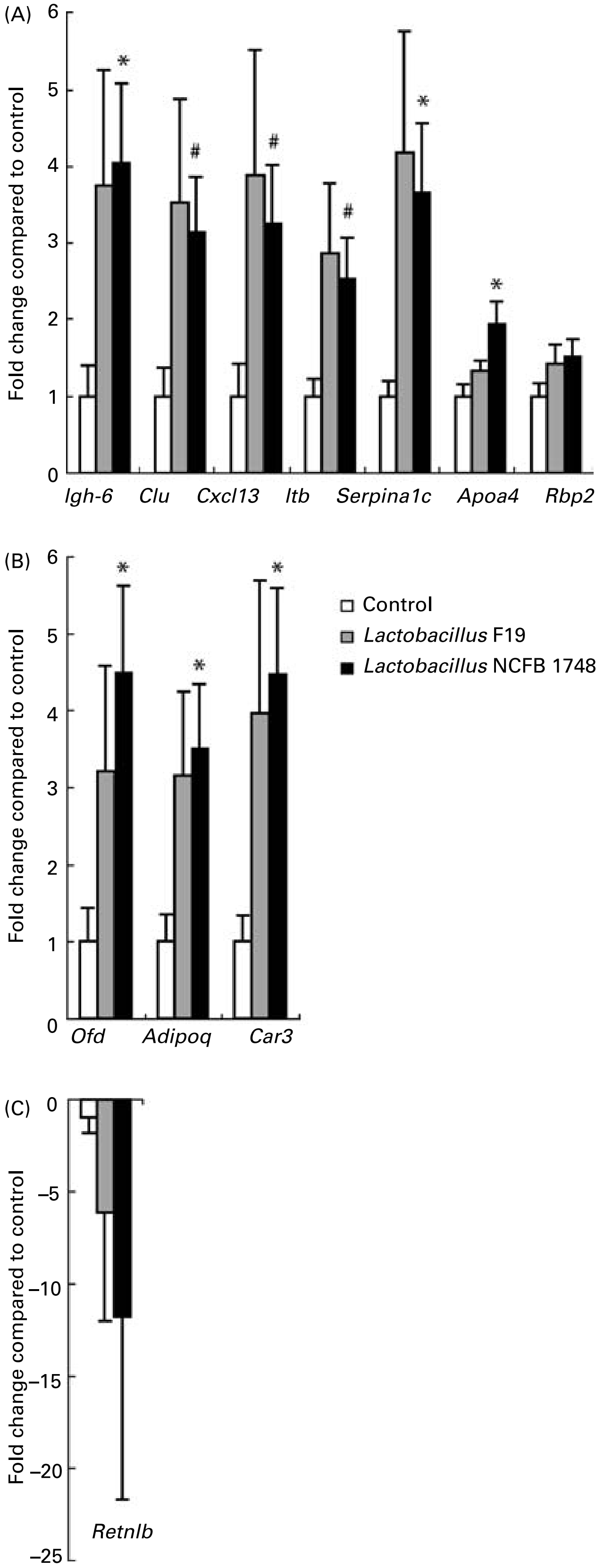

Fig. 1. Expression analysis by quantitative real-time PCR in (A) normal microflora and (B), (C) germ-free mice receiving *Lactobacillus paracasei* ssp. F19 (*L.* F19; ▨) or *Lactobacillus acidophilus* NCFB 1748 (*L.* NCFB 1748; ■) compared with the control group of mice (☐) receiving placebo product.

The relative quantities of different mRNA transcripts were calculated after normalization of the data against an endogenous control – acidic ribosomal phosphoprotein P0 (*Arbp*). The results are shown as the fold change of expression in two test groups relative to the corresponding control group, with the expression level in the control groups set to 1 (A),(B) or −1 (C). Quantitative real-time PCR analysis was performed on the same set of samples that was used in the gene array experiment. Statistical differences were determined by two-sided Student's *t* test: $^{*}P \leq 0.05$; $\#P < 0.06$. For details of procedures, see Materials and methods.

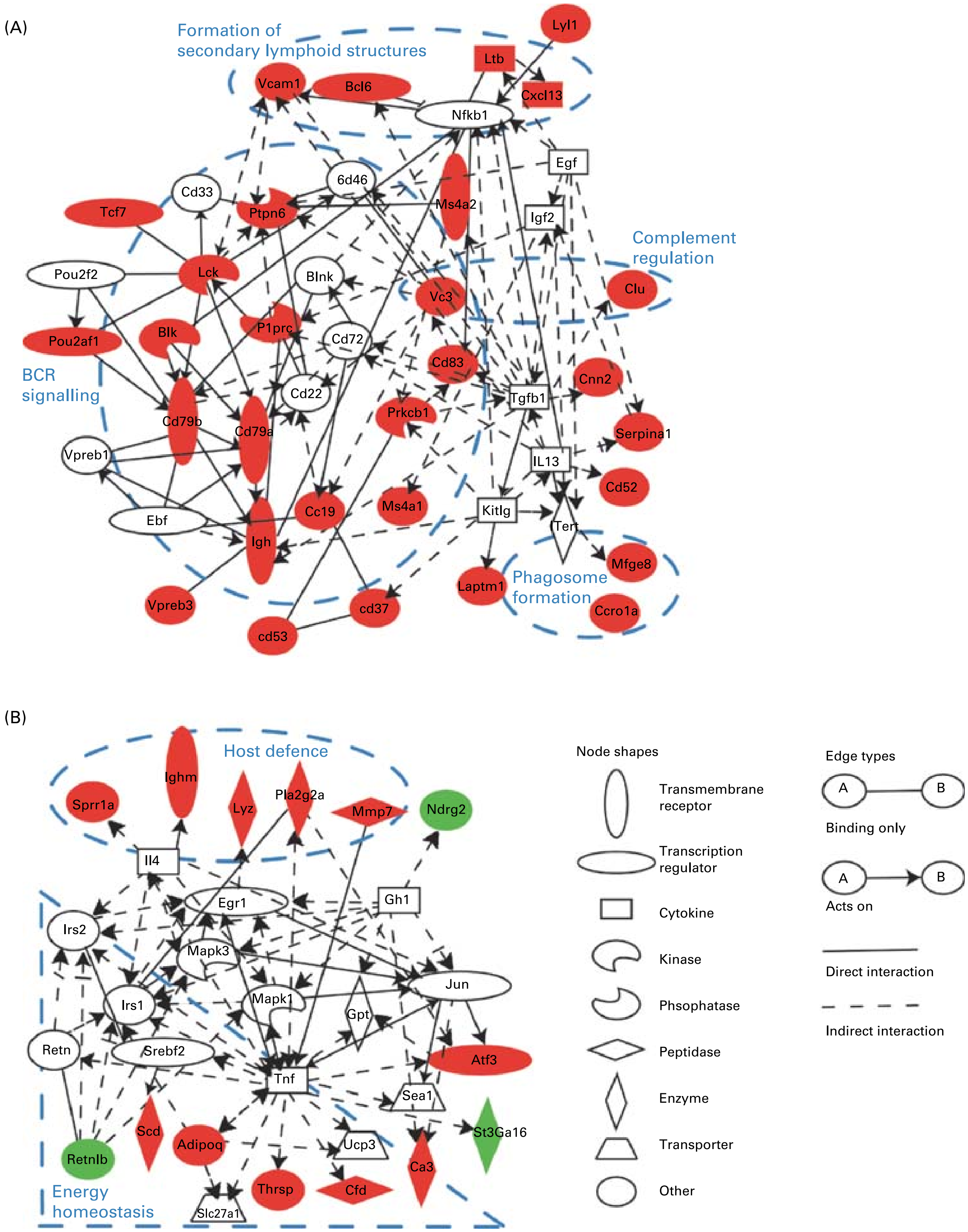

Fig. 2. The Affymetrix Probe Set IDs (Santa Clara, CA, USA) for genes listed in Tables 2 and 3 were imported into the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA), to generate interactome and transcriptional networks connecting the query genes and all other gene products. The networks generated by the Ingenuity Pathway Analysis tool are ranked by score reflecting on how relevant they are to the genes within the dataset. For normal microflora dataset, the two networks with the highest score contained a number of common focus genes and were merged into a single network (A). For germ-free dataset, the network with the highest score containing fourteen focus genes is presented (B). Red and green nodes are input genes with red indicating up-regulation and green indicating down-regulation in mice receiving *Lactobacillus* compared with the control group of mice receiving placebo product (Tables 2 and 3). The white nodes indicate genes not part of the dataset file. The common biological function for selected groups of nodes is shown. Some peripheral nodes and connection genes were removed for simplicity. The displayed network features only genes with functional interactions. BCR, B cell receptor. For details of procedures, see Materials and methods.

regulate the intestinal motility, differentiation, assembly of gut-associated lymphoid tissue, etc., resulting in substantial differences comparing the morphology and function of gnotobiotic *v.* NMF intestine (Falk *et al.* 1998). Additionally, *Lactobacillus* bacteria have been shown to colonize the intestine of both GF and NMF mice (Bateup *et al.* 1995; Filho-Lima *et al.* 2000). However, the colonization efficiency might differ comparing the gnotobiotic *v.* NMF gut. Therefore, it is not surprising that the changes in gene expression pattern caused by *Lactobacillus* administration in GF mice differ from the responses in NMF mice. Further studies are required to clarify if the differential expression of *Adipoq*, *Cfd* and *Retnlb* observed in GF mice can be evoked in NMF conditions applying different product administration regimes and/or dose of probiotics. Also, the influence of genetic background and/or species differences of the host on the effect of *Lactobacillus* administration remains to be addressed.

Gene array approaches are limited by multiple comparison caveats and, therefore, a certain number of false–positive results can be expected. Also, several of the alterations in gene expression that we describe are of relatively low magnitude and of marginal significance. However, the biological relevance of the differentially regulated targets highlighted in the present report is supported by the fact that a number of genes from the same biological pathway are coordinately changed as illustrated by Ingenuity Pathway Analysis (Fig. 2). Furthermore, the two genetically close *Lactobacillus* strains used in the study evoke similar changes in the expression profile of the genes belonging to these functional groups, which further emphasizes the biological interpretation of the data. Finally, the differential expression of selected targets was confirmed by an alternative approach, quantitative real-time PCR, which largely supported the conclusions from the gene array experiments (Fig. 1).

In summary, the present study characterizes global transcriptional responses to administration of two probiotic strains – *L.* F19 and *L. acidophilus* NCFB 1748 – *in vivo*. The results reveal that *Lactobacillus* bacteria modulate expression of host genes participating in different fundamental physiological processes in the intestine. Several changes in gene expression that we describe were expected, being consistent with the earlier publications in the field, e.g. stimulation of the immune system in NMF mice and increased expression of host defence markers in GF mice. Additionally, we report differential expression of several genes previously not known to be regulated by *Lactobacillus*, such as transcripts involved in regulation of energy homeostasis. The differential response in NMF *v.* GF mice that we describe underlines the fact that host–bacterial interactions are both complex and dynamic, and any impact of *Lactobacillus* feeding is likely to be affected by factors such as age, sex, health status, already existing gut microflora, etc. Consequently, additional molecular and physiological studies are required to characterize the functional impact of the changes in transcriptional profile presented in the present report.

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