Bacillus clausii effect on gene expression pattern in small bowel mucosa using DNA microarray analysis

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Background **Probiotics are widely used for the cure or prevention of several clinical conditions. However, clinical decisions need to be substantiated by an analysis of the complex bacteria–host interplay in the intestinal lumen.**

Aims **To identify the gene expression pattern induced by *Bacillus clausii* in the intestinal mucosa of healthy individuals.**

Methods **Six male patients (mean age 38 ± 5 years) affected by endoscopically confirmed mild oesophagitis were treated for one month with esomeprazole, and were randomly selected to receive or not *B. clausii* (groups I and II, respectively). Duodenal biopsies were taken pre and post-treatment to identify the modification of gene expression, using the GeneChip Human U133A array. To validate the microarray analysis, real-time reverse transcriptase–polymerase chain reaction (PCR) of five target genes was performed.**

Results **After *B. clausii* administration, a total of 158 and 265 genes were up and downregulated, respectively. Quantitative PCR confirmed the microarray data. *B. clausii* mainly affected the expression of genes involved in immune response and inflammation, apoptosis and cell growth, cell differentiation, cell–cell signalling, cell adhesion, signal transcription and transduction.**

Conclusions **Our data represent the first global analysis of *B. clausii* effects on the gene expression profile in normal intestine, and provide the basis to identify the mechanisms by which these agents interact with the host and exert their beneficial effects. Future studies are needed to clarify the *B. clausii*-induced gene pattern in the clinical disorders in which probiotics have proved to be effective. *Eur J Gastroenterol Hepatol* 17:951–960**
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Introduction

In the past decade the possibility of modulating the intestinal microflora through the use of probiotics, such as bacilli, *Escherichia coli* spp., enterococci, bifidobacteria, lactic acid bacteria species, and yeasts to improve health has attracted much interest. They have been demonstrated to be effective in preventing and reducing the severity of infectious acute diarrhoea, antibiotics-associated diarrhoea, in relieving symptoms of lactose maldigestion, in an improvement of food allergies and atopy, in the treatment of infections of the urogenital tract, in the maintenance of remission and cure of pouchitis in inflammatory bowel disease (IBD) and in colon cancer prevention. However, potential clinical applications are currently both at the gastrointestinal and extraintestinal stage on the basis of clinical observations [1–3]. Nevertheless, the presence of bacteria in the intestinal lumen has been demonstrated to create an epithelium–bacteria interaction that induces unique host responses [4,5].

Among the different microorganisms, *Bacillus* species (*Bacillus cereus*, *Bacillus clausii*, *Bacillus pumilus*) were characterized for their potential attributes (colonization, immunostimulation, and antimicrobial activity), which could account for their claimed probiotic properties. In particular, *B. clausii* presents several unique properties and a long history of safe use. This probiotic is resistent to commonly used antibiotics, and has been administered to humans for the prevention of gastrointestinal side-effects caused by oral antibiotic chemotherapy [6–8]. *B. clausii* is carried in a commercial probiotic product consisting of bacterial spores, although they are generally absent from the normal microflora of humans. Experimental data also suggest that *B. clausii* spores can adhere to the bowel wall, allowing mucosal colonization [9,10]. Moreover, *B. clausii*

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strains release antimicrobial substances, active against Gram-positive bacteria, in particular against *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile* [11]. Besides, in a study conducted to investigate the modulation of the cytokine pattern after *B. clausii* administration in allergic children with recurrent respiratory infections [12], this probiotic was demonstrated to exert immunomodulatory activities.

Therefore, studies aimed at characterizing the mechanisms responsible for the probiotic beneficial effects of *B. clausii* are rare.

To date, recent advances in complementary DNA microarray technology allow the comparative messenger RNA analysis of thousands of genes in multiple samples simultaneously, enabling the evaluation of a wide pattern of gene expression [13].

In the present study, we investigated the effect of *B. clausii* on the gene expression profile in the small bowel mucosa of healthy individuals using the U133A microarray set by Affymetrix Inc. (Santa Clara, CA, USA).

Materials and methods

Six male patients (mean age 38 ± 5 years) affected by mild oesophagitis (Savary–Miller grade I) and no gastric or duodenal alterations at endoscopy were included in the study. Signed informed consent was obtained from each individual before entering the study.

The patients were then divided into two groups to receive: group I (three patients), *B. clausii*, three vials/day each containing 2×10^9 spores (5 ml) (Sanopy Synthelabo OTC SpA, Sanopy-aventis Group, Milan, Italy) and proton-pump inhibitor (PPI) (esomeprazole 40 mg twice a day) for 30 days; group II (three patients), PPI for 30 days. Two additional patients were enrolled and treated as group I, in order to confirm the microarray data by real-time reverse transcriptase–polymerase chain reaction (RT–PCR) on five patients' samples (before and after treatment). Biopsies from each patient were collected from distal duodenal mucosa pre and post-treatment to assess the modification induced by *B. clausii* on the gene expression pattern.

DNA microarray analysis

Each biopsy has been measured separately. Total RNA was extracted from each sample using Trizol lysis buffer (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was synthesized from 5 μg of total RNA, using the Superscript Choice system (Invitrogen). After cDNA purification, labelling of complementary RNA was achieved using the ENZO Bioarray High Yield RNA Transcript Labeling kit (ENZO Biochem, New York, NY, USA). *In vitro* transcription (IVT) reactions were carried out at 37°C for 4.5 h. The purified (RNeasy Mini Kit) cRNA was fragmented (buffer: 200 mmol/l Tris-acetate pH 8.1; 500 mmol/l potassium acetate; 150 mmol/l magnesium acetate) at 95°C for 35 min. The biotinylate targets (11 μg/probe array) were hybridized to the Human Genome U133A array set (Affymetrix), which contains 22 215 human gene cDNA probes, in a hybridization mixture containing probe array controls, bovine serum albumin and herring sperm DNA. Hybridization was allowed to continue for 16 h at 45°C. After washing and staining, the arrays were scanned twice using an HP GeneArray Scanner (Hewlett-Packard, Palo Alto, CA, USA), and the average of the intensities was used to evaluate the signal value. GeneChip expression software (Affymetrix) was used to determine the absolute analysis metrics. A set of 'housekeeping genes', such as *GAPDH* and *beta-Actin*, was used for normalization.

The gene expression profiles were tested in all biopsies collected for each patient before and after *B. clausii* administration. For statistical analysis, we first evaluated the differences in gene expression comparing the pre and post-treatment data of the patients treated with *B. clausii* and PPI (group I). Then, to differentiate the genes modified by the probiotic from those modified by PPI, group I and group II were compared. To be considered a 'significantly changed gene', a gene had to satisfy the following criteria: absolute expression level (to eliminate background); difference in the expression level of each gene across samples (before versus after treatment for each patient; at least 200); fold change for each gene in each patient (before versus after treatment), or whether gene expression has been up or downregulated (at least twofold change); reproducibility of the measurements, or whether samples with similar characteristics had similar amounts of gene transcript [14].

Significant differentially expressed genes were also grouped on the basis of molecular function (tasks performed by the individual gene product) and biological processes (broad biological goals accomplished by ordered assemblies of molecular functions) using the DAVID database [15].

Real-time SYBR-green dye I real-time polymerase chain reaction analysis

Five target genes were subjected to confirmation by SYBR-green dye I real-time RT–PCR. For polymerase chain reaction (PCR) analysis we enrolled two additional patients following the inclusion criteria and the treatment protocol of group I. The selected genes plus *GAPDH* and the primers designed on the basis of the GeneBank sequences using Primers3 software are reported in Table 1.

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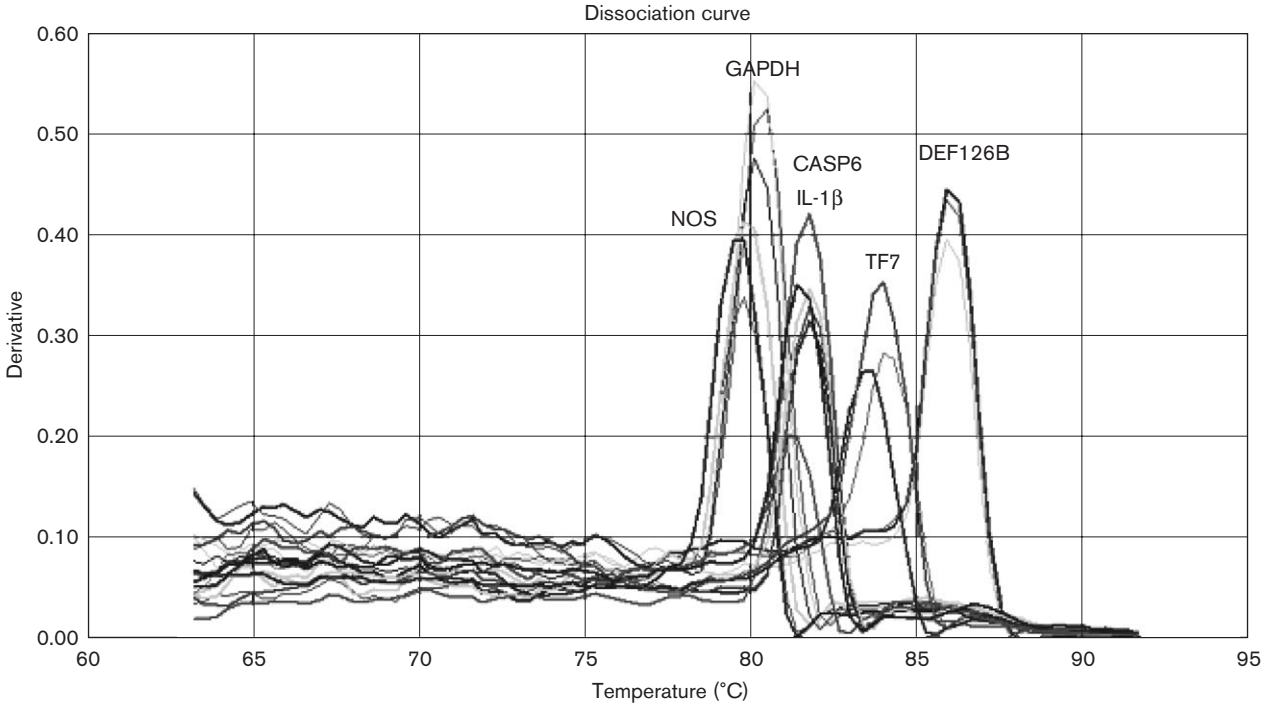
Table 1 Gene-specific primers for the real-time SYBR-green analysis selected according to the manufacturer's recommendations (Applied Biosystems)

Gene	Forward primer	Reverse primer
GAPDH	5′–ACGGATTTGGTCGTATTGGGC–3′	5′–TTGACGGTGCCATGGAATTTG–3′
NOS	5′–TCAATGACCTATGGGGGAAG–3′	5′–CCACCTCAGTCTCCCAGTTC–3′
DEFB126	5′–CCTACTGTTCACCCTTGCAGT–3′	5′–CACAGCAGTCCCTTTGTTTG–3′
IL-1β	5′–GCATCCAGCTACGAATCTCC–3′	5′–TCGTTATCCCATGTGTCGAA–3′
TF7	5′–CTCAAGTCGTCGCTCGTG–3′	5′–GGGCTCTGGAAGTTTGTCC–3′
CASP6	5′–ACAAGTGTCACAGCCTGGTT–3′	5′–ACATGAGGAAGTCAGCTCCA–3′

Fig. 1



Melting curve analysis validates the specificity of products of SYBR-green real-time polymerase chain reaction.

The total RNA (200 ng) retrieved from each biopsy of group I and the total RNA extracted from the two additional patients enrolled was used. The cDNA (for each sample) was synthesized using a Superscript First Strand cDNA synthesis kit (Invitrogen). Each PCR system (25 µl) contained 2 µl diluted cDNA, 12.5 µl 2 × SYBR-green PCR Master Mix, 0.5 µmol/l of each specific gene primer and water and 1.25 U AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA). Real-time PCR was performed and analysed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All the samples were run in triplicate in the amplification programme, and the average amount was used as the final expression value. A negative control without the cDNA template was run to assess the overall specificity. Melting curves for each PCR were generated to ensure the purity of the amplification product (Fig. 1). Products were double-checked by run on an ethidium bromide staining in agarose gel. A standard curve method (generated preparing serial dilutions of total cDNA) was used to calculate the relative amounts of target gene expression. All the samples were run in triplicate, and the average was considered to be the final value. Quantities of each target gene were normalized to the corresponding *GAPDH*.

Results

DNA microarray analysis

After *B. clausii* administration, 92 and 265 genes showed greater than twofold up and down changes, respectively.

B. clausii mainly affected genes involved in immune response and inflammation, apoptosis and cell growth,

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Table 2 Representative subset of differentially expressed (up and downregulated) genes after *Bacillus clausii* administration

Gene name	Function	Ratio	Gene name	Function	Ratio
Decay accelerating factor for complement (CD55) (**DAF**)	Inhibits the amplification of the complement cascade	3.6	Defensin, beta 126 (**DEFB126**)	Protease inhibitor activity; proteolysis and peptidolysis; xenobiotic metabolism	4.3
Ankyrin repeat and MYND domain containing 1 (**ANKMY1**)	Mediate protein–protein interactions; contains four ankyrin (Ank) repeats and two MORN motifs	6.5	MAX dimerization protein 1 (**MAD**)	Cell proliferation; transcription factor activity	3.9
Myeloid cell leukemia sequence 1 (BCL2-related) (**MCL1**)	Apoptosis; development; heat shock response; similar to BCL2	2	Endothelin receptor type B (**ENDRB**)	Endothelin receptor type B is a G protein-coupled receptor that activates a phosphatidylinositol-calcium second messenger system	2.4
v-Jun sarcoma virus 17 oncogene homologue (**JUN**)	RNA polymerase II transcription factor activity; cell growth	2.5	Prostaglandin E receptor 2 (subtype EP2), 53 kDa (**PTGER2**)	G-protein coupled receptor protein signalling pathway; prostaglandin E receptor activity; rhodopsin-like receptor activity; thromboxane receptor activity	2.2
Cytochrome c oxidase subunit Vb (**COX5B**)	Cytochrome c oxidase activity	4.8	Nitric oxide synthase 1 (**NOS1**)	Cell–cell signalling; electron transport; intracellular signalling cascade; nitric oxide biosynthesis; nitric oxide synthase activity; oxidoreductase activity	2.5
Adrenergic, alpha-1A-receptor (**ADRA1A**)	Member of the G protein-coupled receptor superfamily; regulation of cells growth and proliferation	2.2	Protocadherin 12 (**PCDH12**)	Calcium ion binding; cell adhesion molecule activity; cytoskeleton; homophilic cell adhesion; neuronal cell recognition	6.4
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 34 (**DDX34**) and box polypeptide 9 (**DDX9**)	RNA helicase; implicated in a translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly	4 13.2	IL-1, beta (**IL-1β**) and IL-1 receptor-like 1 ligand (**IL1RL1LG**)	Apoptosis; cell proliferation; cell–cell signalling; immune response; inflammatory response; regulation of cell cycle; signal transducer activity; signal transduction	4.2 2.2
MHC class II transactivator (**MHC2TA**)	MHC2TA encodes a non-DNA binding transactivator that functions in MHC class II expression	4.2	Replication factor C (activator 1) 1, 145 kDa (**RFC1**)	DNA-dependent ATPase required for eukaryotic DNA replication and repair	2.3
Matrix metalloproteinase 8 (**MMP8**)	Collagen catabolism; extracellular matrix; hydrolase activity; metalloendopeptidase activity; neutrophil collagenase activity; zinc ion binding	3.2	Chloride channel 6 (**CLCN6**)	Chloride transport; ion transport; regulation of cell volume; signal transduction; small molecule transport; voltage-gated chloride channel activity	3.9
Sialophorin (**SPN**)	Cellular defence response; chemotaxis; establishment and/or maintenance of cell polarity; negative regulation of cell adhesion; signal transduction; transmembrane receptor activity	2.7	Mitogen-activated protein kinase kinase kinase 3 (**MAP3K3**)	Protein that directly regulates the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by activating SEK and MEK1/2, respectively; it does not regulate the p38 pathway	2.4
CD79B antigen (immunoglobulin-associated beta) (**CD79B**)	Cell surface receptor linked signal transduction; immune response; transmembrane receptor activity	3.3	Intestinal cell kinase (**ICK**)	Protein amino acid phosphorylation; protein serine/threonine kinase activity; signal transduction	2.8
Protein tyrosine phosphatase type IVA, member 1 (**PTP4A1**)	Oncogenesis; phrenylated protein tyrosine phosphatase activity; protein amino acid dephosphorylation	2.2	DnaJ (Hsp40) homologue, subfamily C, member 3 (**DNAJC3**)	Defence response; protein kinase inhibitor activity; regulation of translation	2.7
HUS1 checkpoint homologue (**HUS1**)	DNA damage response, signal transduction resulting in cell cycle arrest; DNA repair	2.9	Vitronectin (somatomedin B, complement S-protein) (**VTN**)	Cell adhesion; heparin binding; immune response	2.5
Growth differentiation factor 3 (**GDF3**)	Cell growth and/or maintenance; cytokine activity; growth factor activity; member of the TGF-β superfamily	2.3	Fibroblast growth factor 16 (**FGF16**)	Cell–cell signalling; growth factor activity; histogenesis and organogenesis; metabolism; signal transduction	8.1
Caspase 5, apoptosis-related cysteine protease (**CASP5**)	Apoptosis regulator activity; caspase activity; cysteine-type peptidase activity; hydrolase activity; intracellular proteolysis and peptidolysis	5.1	RYK receptor-like tyrosine kinase (**RYK**)	ATP binding; protein amino acid phosphorylation; receptor activity; signal transduction; transferase activity; transmembrane receptor protein tyrosine kinase activity	2.1
Cytochrome P450, family 11, subfamily B, polypeptide 1 (**CYP11B**)	C21-steroid hormone biosynthesis; electron transport; monooxygenase activity; steroid 11-beta-monooxygenase activity	2.3	Fc fragment of IgG, low affinity IIb, receptor for (CD32) (**FCGR2B**)	IgG binding; cell growth and/or maintenance; immune response; internalization receptor activity; signal transduction	4.1

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Cytochrome P450, family 11, subfamily B, polypeptide 2 (**CYP11B2**)		4.1			
Epidermal growth factor receptor pathway substrate 8-like 1 (**EPS8L1**)	Protein related to epidermal growth factor receptor pathway substrate 8 (EPS8)	3	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (**YWHAZ**)	Member of the 14-3-3 family of proteins that mediate signal transduction by binding to phosphoserine-containing proteins	2.2
Programmed cell death 6 interacting protein (**PDCD6IP**)	Signal transduction	2	Cadherin 6, type 2, K-cadherin (**CDH6**)	Calcium ion binding; cell adhesion; cell adhesion molecule activity; homophilic cell adhesion	4.5
Putative c-Myc-responsive (**RCL**)	Cell proliferation	2.6	Nuclear factor related to kappa B binding protein (**NFRKB**)	Inflammatory response; specific RNA polymerase II transcription factor activity	2.2
Heat shock 105 kDa/110 kDa protein 1 (**HSPH1**)	ATP binding; cytoplasm; heat shock protein activity	3.5	Cysteine-rich protein 1 (intestinal) (**CRIP1**)	Antimicrobial humoral response; cell proliferation; zinc ion binding	2
RAB6A	Member RAS oncogene family	2.8	Villin 2 (ezrin) (**VIL2**)	Cytoskeletal anchoring; microvillus	2.3
8D6 antigen (**8D6A**)	Signal transduction; cell–cell signalling; humoral immune response; regulation of cell proliferation	0.3	Calcium/calmodulin-dependent protein kinase (CaM kinase) II beta (**CAM2B**)	Serine/threonine kinase activity; signal transduction; transferase activity	0.2
Cyclin D1 (**CCND1**)	Cell cycle	0.3	Cyclin E1 (**CCNE1**)	Cell cycle	0.2
Colon cancer antigen 16 (**SDCCAG16**)	Tumour antigen	0.07	BCL2-antagonist of cell death (**BAD**)	Induction of apoptosis; mitochondrial outer membrane	0.3
Insulin-like growth factor 1 (**IGF1**)	Insulin-like growth factor I (somatomedin C); activates cell proliferation and differentiation	0.3	Mitogen-activated protein kinase kinase kinase kinase 4 (**MAP4K4**)	Serine/threonine protein kinase; activation of MAPK8/JNK	0.4
T-cell leukemia/lymphoma 1B (**TCL1B**)	T-cell leukemia/lymphoma 1B, a member of the TCL1 family	0.3	IL-15 receptor, alpha (**IL15RA**)	Cell proliferation; signal transduction	0.2
Transmembrane 4 superfamily member tetraspan NET-7 (**NET7**)	Regulation of cell development, activation, growth and motility	0.4	TNF superfamily, member 17 (**TNFRSF17**)	Antimicrobial humoral response; cell proliferation; immune response; signal transduction	0.3
Mitogen-activated protein kinase 8 interacting protein 1 (**MAPK8IP1**)	MAP-kinase scaffold protein activity; kinesin binding; protein kinase binding; protein kinase inhibitor activity; regulation of JNK cascade; vesicle-mediated transport	0.4	IL-13 (**IL13**)	Cell proliferation; cell–cell signalling; immune response; inflammatory response; signal transduction	0.3
Lymphocyte antigen 64 homologue (**LY64**)	Immune response; inflammatory response; receptor activity	0.4	Fibroblast growth factor receptor 1 (**FGFR1**)	FGF receptor signalling pathway; MAPKKK cascade	0.3
G protein-coupled receptor 44 (**GPR44**)	G-protein coupled receptor protein signalling pathway; N-formyl peptide receptor activity; immune response	0.3	Cathepsin S (**CTSS**)	Cathepsin S activity; hydrolase activity; immune response; proteolysis and peptidolysis	0.3
IL-6 receptor (**IL6R**)	Cell proliferation; signal transduction; immune response; haematopoietin/interferon-class; cytokine receptor activity	0.4	Latent transforming growth factor beta binding protein 4 (**LTBP4**)	Calcium ion binding	0.4
Myeloperoxidase (**MPO**)	Anti-apoptosis; defence response; eosinophil peroxidase, lactoperoxidase, myeloperoxidase, oxidoreductase activity; response to oxidative stress	0.3	Jagged 2 (**JAG2**)	Notch binding; T-cell differentiation; cell cycle; cell differentiation and proliferation; cell fate determination; cell–cell signalling; growth factor activity; regulation of cell migration	0.1
BCL2/adenovirus E1B 19 kDa interacting protein 1 (**BNIP1**)	Anti-apoptosis	0.1	Caspase 6, apoptosis-related cysteine protease (**CASP6**)	Caspase activity; cysteine-type peptidase activity; hydrolase activity; induction of apoptosis; proteolysis and peptidolysis	0.3
Calcium/calmodulin-dependent protein kinase kinase 2, beta (**CAMKK2**)	Calcium/calmodulin-dependent (CaM) kinase cascade	0.4	Transmembrane 4 superfamily member 7 (**TM4SF7**)	Protein complex assembly	0.3
Interferon-induced protein with tetratricopeptide repeats 4 (**IFIT4**)	Immune response	0.4	Lymphocyte-specific protein 1 (**LSP1**)	Cell motility; cellular defence response; signal transducer activity; signal transduction	0.4
Transcription factor 7 (T-cell specific, HMG-box) (**TCF7**)	RNA polymerase II transcription factor activity; immune response	0.4	Superkiller viralicidic activity 2-like (**SKIV2L**)	ATP binding; ATP dependent RNA helicase activity; RNA binding; oncogenesis; regulation of translation	0.4
Insulin-like growth factor 2 (**IGF2**)	Cell proliferation; insulin receptor signalling pathway; insulin-like growth factor receptor binding; regulation of cell cycle	0.4	Chemokine-like receptor 1 (**CMKLR1**)	G-protein coupled receptor protein signalling pathway; chemokine receptor activity; chemotaxis; immune response; rhodopsin-like receptor activity	0.2
Hepatocyte growth factor (**HGF**)	Cell proliferation	0.4	H factor 1 (complement) (**HF1**)	Complement activation, alternative pathway	0.4

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cell adhesion, transcription, cell communication, defence response and cell cycle, as shown in Table 2.

Those genes were grouped, on the basis of the molecular and biological functions exerted, as genes working in a similar context (Table 3). The differentially expressed genes were thus categorized on the basis of their known function. As an example, a group of 31 modified genes are related to transcription. Some of them are also involved in cell proliferation, cell growth and cell adhesion, such as cadherin 6, MAX dimerization protein 1 and v-jun. Another category with a large number of changed genes included those involved in immune regulation and inflammatory response, such as IL-1β, Hsp40, proteoglycan 2, sialophorin, cathepsin S, defensin 126, cysteine-rich protein 1, MPA4K4, H factor 1, TNF receptor superfamily, member 17, IL-1 and IL-1 receptor, IL-6 receptor, IL-15 receptor α, IL-13, somatomedin C, and plasminogen activator. Moreover, several altered genes play a role in cell growth, apoptosis, and cell cycle, such as growth differentiation factor 3, RAS oncogene family member RAB6A, jagged 2, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF)-1, cyclin E1 and D1, somatostatin, HUS1 checkpoint homolog, NIMA (never in mitosis gene a)-related kinase 9, MAX dimerization protein 1,

Table 3 Classification by function of genes with altered expression after *Bacillus clausii* administration using the DAVID database

Downregulation	Genes, *n*	Upregulation	Genes, *n*
Biological processes		**Biological processes**	
Signal transduction	39	Cell communication	39
Nucleobase, nucleotide, RNA, DNA metabolism	29	Cell growth and maintenance	35
Cell proliferation	27	Signal transduction	32
Protein metabolism	26	Nucleobase, nucleotide, RNA, DNA metabolism	19
Transport	25	Protein metabolism	17
Response to biotic stimuli	21	Transport	13
Transcription	20	Cell proliferation	13
Defence response	20	Cell surface receptor	12
Biosynthesis	11	Biosynthesis	11
Response to pathogens	10	Transcription	11
Phosphorus metabolism	10	Intracellular signalling cascade	11
Programmed cell death	9	Cell adhesion	10
Regulation of cell proliferation	9	Cell–cell signalling	9
Cell–cell signalling	9	Response to biotic stimulus	9
Cell death	9	Catabolism	8
Antiapoptosis	9	Immune response	8
G-protein coupled receptor	9	Lipid metabolism	7
Lipid metabolism	8	Intracellular transport	7
Response to abiotic stimuli	5	G-protein coupled receptor signalling pathways	7
Cell adhesion	4	Response to abiotic stimulus	6
RNA metabolism	4	Phosphorus metabolism	6
Inflammatory response	3	Carbohydrate metabolism	6
		Cell death	4
Molecular functions		Response to pathogens	4
Purine nucleotide binding	23	Macromolecule biosynthesis	4
DNA binding	19	Apoptosis	4
Transferase	12	Regulation of cell proliferation	4
Protein kinase activity	10	Programmed cell death	4
RNA binding	9	Chemotaxis	3
Protein serine/threonine kinases	7	Response to DNA damage stimulus	3
Transcription factor	7	DNA repair	2
G-protein coupled receptor	5		
Protein tyrosine kinase	5	**Molecular functions**	
Transcription factors binding	4	Signal transducer activity	26
Cytokine binding	3	Transporter activity	16
Cell adhesion	3	DNA binding	15
Cytokines	2	Transcription regulator activity	12
Growth factor activity	2	Purine nucleotide binding	12
IL receptor activity	2	Transmembrane receptor	12
Defence activity	2	Transcription factor	10
		Cell adhesion	9
		Transferase	8
		G-protein coupled receptor	8
		Cytokines	7
		Kinase activity	5
		RNA binding	5
		Peptidase	4
		Protein kinase	4
		Alpha type channel	3
		Tyrosine kinase	3
		Cytochrome P450 activity	2
		Serine/threonine kinase	2

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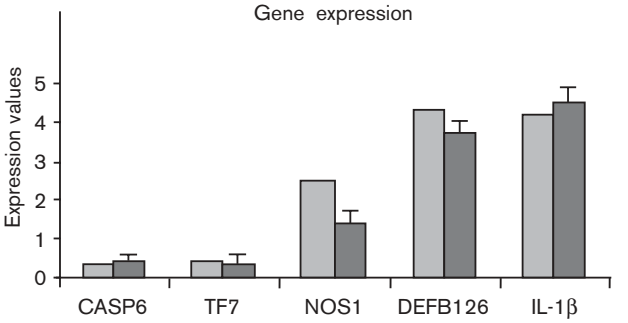
myeloperoxidase, BCL2-antagonist of cell death, caspase 5 and 6, and programmed cell death 6 interacting protein.

Fifteen and 12 genes followed the same pattern of up and downregulation, respectively, in both group I and group II. However, as those genes are involved in multiple cellular functions and do not appear to affect the unique cellular pathways preferentially, they were excluded from the analysis.

Real-time SYBR-green analysis

Quantitative real-time RT–PCR confirmed the reliability of the microarray analysis. As shown in Figure 2, there was a very high level of agreement between the two methodologies used, and the expression changes were qualitatively similar, supporting the validity of our results. However, the magnitude of the gene expression alterations differed between the two analytical methods.

Fig. 2



The relative changes in expression levels for the genes evaluated were qualitatively similar by real-time reverse transcriptase–polymerase chain reaction and microarray analysis, although the magnitude of the modification differed among the two analytical methods. The values 0.5 and 2 represent the cut-off for down and upregulation (fold change) after *Bacillus clausii* administration in the microarray analysis, whereas the value 1 is the cut-off for up and downregulation (fold change) after *B. clausii* administration in polymerase chain reaction (PCR) analysis. For PCR analysis, means and SEM are represented. ▢ Microarray analysis; ▢ SYBR-green PCR analysis.

Discussion

Intestinal mucosa homeostasis mainly depends on the interaction among the resident flora, the epithelium and the immune system. Interest in nutritional supplements and functional foods has increased significantly in recent years, based on the hypothesis that probiotic bacteria in particular may modulate the intestinal flora and the mucosal immune response. Probiotics are defined as live microorganisms that confer a health benefit to the host [16]. The beneficial effects attributed to probiotics undoubtedly require a biochemical communication between the microorganisms and the enterocytes [17,18]. Therefore, a basic understanding of the consequences of probiotic–epithelial cross-talk is necessary to delineate the role of probiotic agents in health as well as in disease to support clinical decisions scientifically.

Among the most common probiotics used, *B. clausii* has a long history of use, with over 40 years of clinical application and excellent tolerability. However, the mechanisms of action of *B. clausii* and how it affects the intestinal mucosa need to be clarified.

The present study shows for the first time the distinctive pattern of gene expression that characterizes the intestinal mucosa after *B. clausii* administration. The clinical benefits observed with probiotic use are mainly attributed to the antimicrobial substances produced by probiotic strains and to their immunomodulatory effect. The last is based on a bacterial–mucosal interaction. The GeneChip Human U133A microarray set from Affymetrix, which represents approximately 22 000 transcribed genes, was used to identify target genes of *B. clausii* activity, comparing the gene expression profiles in the small bowel mucosa of three healthy individuals before and after probiotic treatment. Real-time PCR was performed for five target genes and confirmed the reliability of microarray data.

The expression of genes involved in widely diverse functions was modified by *B. clausii* treatment. Interestingly, genes involved in inflammation, immune response, defence response, intestinal permeability, cell adhesion, cell growth, cell differentiation, cell–cell signalling, apoptosis, signal transcription and transduction, were affected by probiotics.

For example, among genes implicated in the defence response, defensin 126 beta expression was induced after treatment. Defensins are thought to play a major role in the defence of small intestinal crypts against colonization by potential pathogens [19]. Moreover, a lack of the induction of inducible beta-defensins has been indicated in Crohn's disease [20]. Another upregulated gene was leukosialin, a sialoglycoprotein on the surface of human T lymphocytes, monocytes, granulocytes, and some B lymphocytes, which appears to be important for immune function and may be part of a physiological ligand-receptor complex involved in T-cell activation [21].

Several studies have reported that probiotics are able to regulate anti and pro-inflammatory cytokine production. Moreover, data in the literature report that some strains of probiotic may act differently as immunomodulatory agents pro-T helper type 2 or pro-T helper type 1 type immune response [22]. The importance of this modulation is suggested by the fact that, for example, Crohn's disease is associated with T helper type 1 cytokine

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production, such as IFN-γ, TNF-α and IL-12, whereas ulcerative colitis is linked to a cytokine pattern that is less clear but seems to be a modified T helper type 2 response, with the production of IL-5, IL-10 and IL-13 [18]. Moreover, atopic diseases such as asthma are characterized by a T helper type 2 response [23]. Our results suggest that unique immunomodulatory properties of each probiotic strain should be characterized when developing clinical applications for extended target populations. In particular, *B. clausii* appears to have a trend for the stimulation of a pro-T helper type 2 pattern of genes. This result seems in contrast with a previous study present in literature, in which the immunomodulatory effects of *B. clausii* were tested in the nasal mucosa of allergic children [12]. Within the cytokine pattern, IL-6 and IL-15 receptors (downregulated) and IL-13, IL-1β and its receptor (upregulated) were affected by *B. clausii*. IL-6 is a potent pro-inflammatory cytokine that regulates cell growth and differentiation and plays an important role in the immune response [24]. IL-15 receptor alpha is reported to enhance cell proliferation and the expression of apoptosis inhibitors BCL2L1/BCL2-XL and BCL2 [25]. Abnormalities of IL-15 expression have been described in patients with rheumatoid arthritis or IBD [26]. IL-13, instead, is involved in several stages of B-cell maturation and differentiation and downregulates macrophage activity, thereby inhibiting the production of pro-inflammatory cytokines and chemokines [27]. The last cytokine affected, IL-1, is produced by activated macrophages and is an important mediator of the inflammatory response, involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [28].

Most of the previously described genes play a crucial role in inflammatory pathways. Inflammation is a key process involved in the response to pathogens in the bowel. However, it has been demonstrated that chronic or recurrent mucosal inflammation may initiate and promote the development of colon cancer, such as in IBD. Homeostasis in the immune system depends on a balance between the responses that control infection and tumour growth and the reciprocal responses that prevent inflammation and autoimmune diseases.

Other genes affected by *B. clausii*, involved in the inflammatory cascade, are family members of nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK), nitric oxide synthase (NOS) and TNF. The NF-κB transcription factor is crucial in the induction of pro-inflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors and enzymes [29,30]. This factor has been recognized to be an important regulator in IBD [31]. The overall role that nitric oxide plays in intestinal inflammation, instead, is still unclear [32]. However, recent data demonstrated that NOS plays an important role in limiting injury to the intestine during experimental colitis [33]. The MAPK family of signal transduction proteins is an important intracellular mediator of inflammation, and recently a MAPK inhibitor was successfully used for the treatment of patients with Crohn's disease [34].

Another gene enhanced by *B. clausii* administration is JUN, which interacts directly with specific target DNA sequences to regulate gene expression [35].

Furthermore, in post-treatment specimens we found a remarkable variation in the expression of a group of genes regulating cell fate. Interestingly, the dysregulation of cell cycle progression, angiogenesis and apoptosis (programmed cell-death) processes are involved in tumorigenesis. *B. clausii* seems to favour cell proliferation and has an anti-apoptotic trend. Among the upregulated genes, MAD competes with MYC for binding to MAX to form a sequence-specific DNA-binding complex, and is a candidate tumour suppressor gene [36]. Also the HUS1 gene was upregulated and is involved in cell cycle arrest in response to DNA damage [37].

Several growth factors were enhanced by *B. clausii*, such as growth differentiation factor 3, a member of the transforming growth factor (TGF)-β superfamily, IGF-I, FGF, and HGF. All of them are potent mitogens that have been demonstrated to stimulate the proliferation of epithelial and non-epithelial cells, to promote intestinal wound healing and to exert trophic effects within the intestine [38–40]. Interestingly, the administration of HGF ameliorated many of the features of bowel disease in a rat model [41]. Moreover, the interplay between serum IGF-1 and IL-1β and IL-6 in IBD, all genes affected by *B. clausii*, has been studied by Street *et al.* [42], who demonstrated a relationship between IGF-1 levels and interleukin levels, disease activity and anatomical distribution, consistent with active inflammation. Conversely, the expression of somatostatin, a negative regulator of cell proliferation, was decreased by *B. clausii*.

Other genes involved in the regulation of the cell cycle, but downregulated after treatment, are Jagged 2, cyclin D1 and E1, caspase 6 and BAD. Jagged 2 is a member of the Notch gene family that are critical for various cell fate decisions, including cell growth, cell differentiation, cell–cell signalling, cell proliferation and migration [43]. The BAD gene, instead, which is a member of the BCL-2 family, positively regulates cell apoptosis by forming heterodimers with BCL-XL and BCL-2, and reversing their death repressor activity [44]. Moreover, the caspase 6 gene, a member of the caspase family that plays a central role in initiating, amplifying, executing apoptosis and in the immunological response was repressed by the

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probiotics, whereas caspase 5 expression was enhanced [45]. The other downregulated genes belong to the highly conserved cyclin family, such as cyclin D1 and E1. Both of them are required for cell cycle G1/S transition. Mutations, amplification and overexpression of these cyclins, which alter cell cycle progression, are observed frequently in a variety of tumours and may contribute to tumorigenesis, including the multistep process of gastrointestinal carcinogenesis [46].

Nevertheless, *B. clausii* also affected genes involved in signal transduction, such as calcium/calmodulin family members CAMK2B and CAMKK2, which are serine/threonine protein kinases, and regulators of cell adhesion and migration, such as cadherin 6, fibronectin and villin 2, which play a key role in cell surface structure adhesion, migration, and organization. The cell–cell signalling pathway, which includes a series of surface events, and the activation of distinct signal transduction pathways, is crucial for regulating intestinal homeostasis. In the human small intestine, the proliferation, migration, differentiation and death of epithelial cells are influenced by extracellular matrix proteins [47]. Interestingly, microtubule-associated protein, first identified by its binding to the APC protein that is often mutated in colorectal cancer, has been found to respond to treatment [48].

The purpose of this study was to generate a highly reliable pool of genes whose expression is closely associated with *B. clausii* administration, and consequently, with *B. clausii* effects on the host. Therefore, we are now faced with the challenge of understanding how variations in gene expression after probiotic administration contribute to clinical effects and the mechanisms by which the probiotics affect the genes involved in various biological functions.

Furthermore, we evaluated the effects of a specific probiotic strain in healthy subjects, and it is reasonable to suppose that different probiotic preparations, different tracts of intestine, different conditions, i.e. inflammation or atopy, and other factors may contribute to the gene expression profile, with the subsequent activation of specific cellular pathways and unique clinical consequences.

However, the small number of samples analysed represents the major limitation of the study. For this reason a gene has to satisfy four selection criteria (not only the fold change value) to be considered as 'significantly changed' after treatment.

Our data provide for the first time the panel of genes modified in the intestinal mucosa after *B. clausii* exposure, and represent the first methodological approach to basic research on the consequences of probiotics addressing various biological functions, in order to lay the basis for the future comprehension of mechanisms of action and to support clinical decisions.

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Conflict of interest

None declared.

Authors' contributions

Conception and design – SDC, ARS, AG, Analysis and interpretation of the data – SDC, HT, ARS, AG, Collection and assembly of the data – SDC, AG, EF, FF, Drafting of article – SDC, Critical Revision – SDC, ARS, AG, GG. Final approval – SDC, ARS, AG, GG, MAZ.

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