

Letter to the Editor

Live and heat-treated probiotics differently modulate *IL10* mRNA stabilization and micro-RNA expression

To the Editor:

For the last century, microorganisms, such as probiotics, have been widely used in food or as food supplement for varied health benefits. In inflammatory diseases and healthy animals, studies have shown a probiotic-induced IL-10 overproduction¹ or an increase in regulatory FoxP3⁺ cell number.² Interestingly, *in vivo* and *in vitro* studies have convincingly demonstrated differential activities of live and heat-treated organisms.³ Yet, little is known about the molecular mechanism involved in this modulation of *IL10* mRNA expression. Several articles have tried to correlate this cytokine expression to Toll-like receptors, pathogen-associated molecular patterns, and nuclear factor kappa b activation, but the results were not always convincing and other modes of activation were frequently suggested. Although probiotics are usually not in direct contact with blood cells, the use of PBMCs constitutes an appropriate *ex vivo* human system to study the intracellular cytokine expression pathways. Microorganisms are naturally present in many raw foods, such as milk-derived

products, and optimal heat treatments are used to prevent them from replicating. Yet, biologically active bacterial components may still persist, be degraded, or be produced, thus modifying the impact of microorganisms on the host immunity. In this study, we hypothesized that heat-treated bacteria may alter the stabilization of *IL10* mRNA via the differential expression of microRNAs (miRNA).

IL-10 is produced and secreted by PBMCs upon stimulation with different live and heat-treated probiotics. Interestingly, heat-treated *Lactobacillus paracasei* Nestlé Culture Collection (NCC) 2461 highly and significantly induced IL-10 secretion in the supernatant (4556 ± 1926 pg/mL; $P < .05$) compared with the live strain (968 ± 505 pg/mL) or the unstimulated control (6.8 ± 1.4 pg/mL) (Fig 1, A). Cellular localization using flow cytometry identified the CD14⁺ cells as the major IL-10 producers in this *in vitro* system (see Fig E1 in this article's Online Repository at www.jacionline.org). These results indicated that live and heat-treated *L. paracasei* NCC 2461 induced differential levels of IL-10 in PBMCs.

The *IL10* mRNA levels were quantified using real-time PCR. A 9.3-fold increase in *IL10* mRNA was observed in PBMCs treated with heat-treated *L. paracasei* NCC 2461, whereas

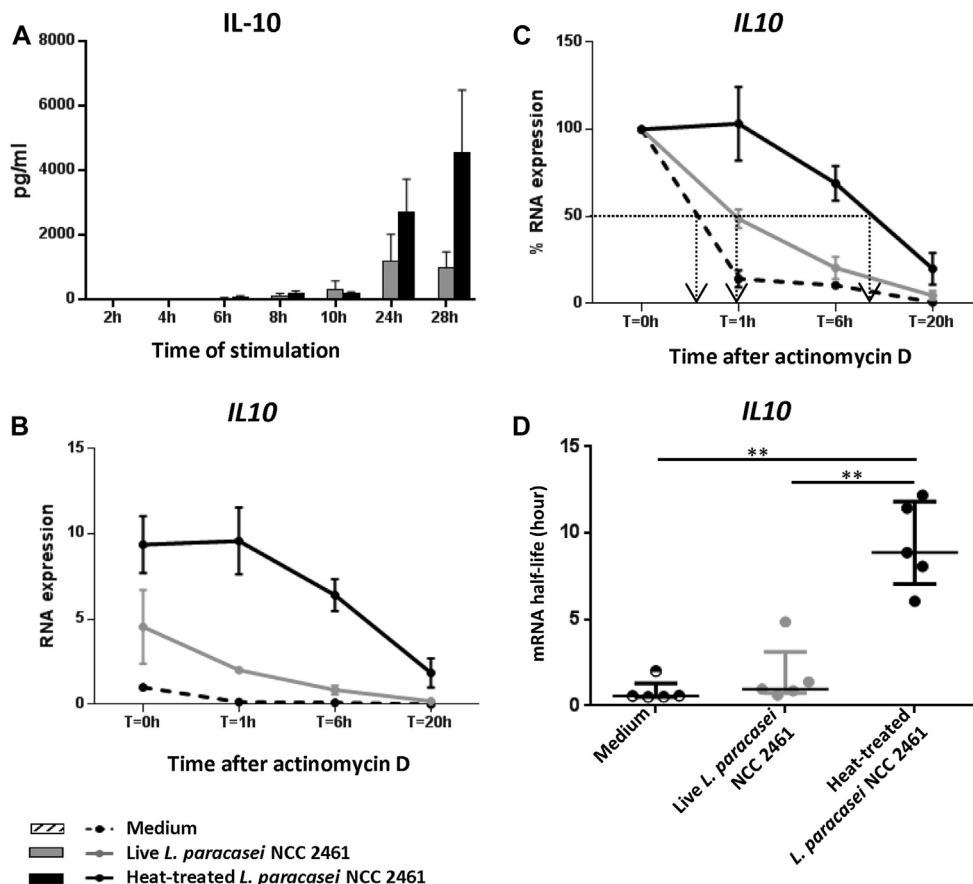


FIG 1. Cytokine expression in PBMCs stimulated with live or heat-treated *L. paracasei* NCC 2461. **A**, IL-10 protein secreted in the supernatant. **B**, *IL10* mRNA expression in PBMCs stimulated with live or heat-treated *L. paracasei* NCC 2461. **C**, *IL10* mRNA half-life in PBMCs. **D**, *IL10* mRNA half-life is shown in 5 PBMC donors. Experiments shown are means of triplicates and representative of 5 independent PBMC donors.

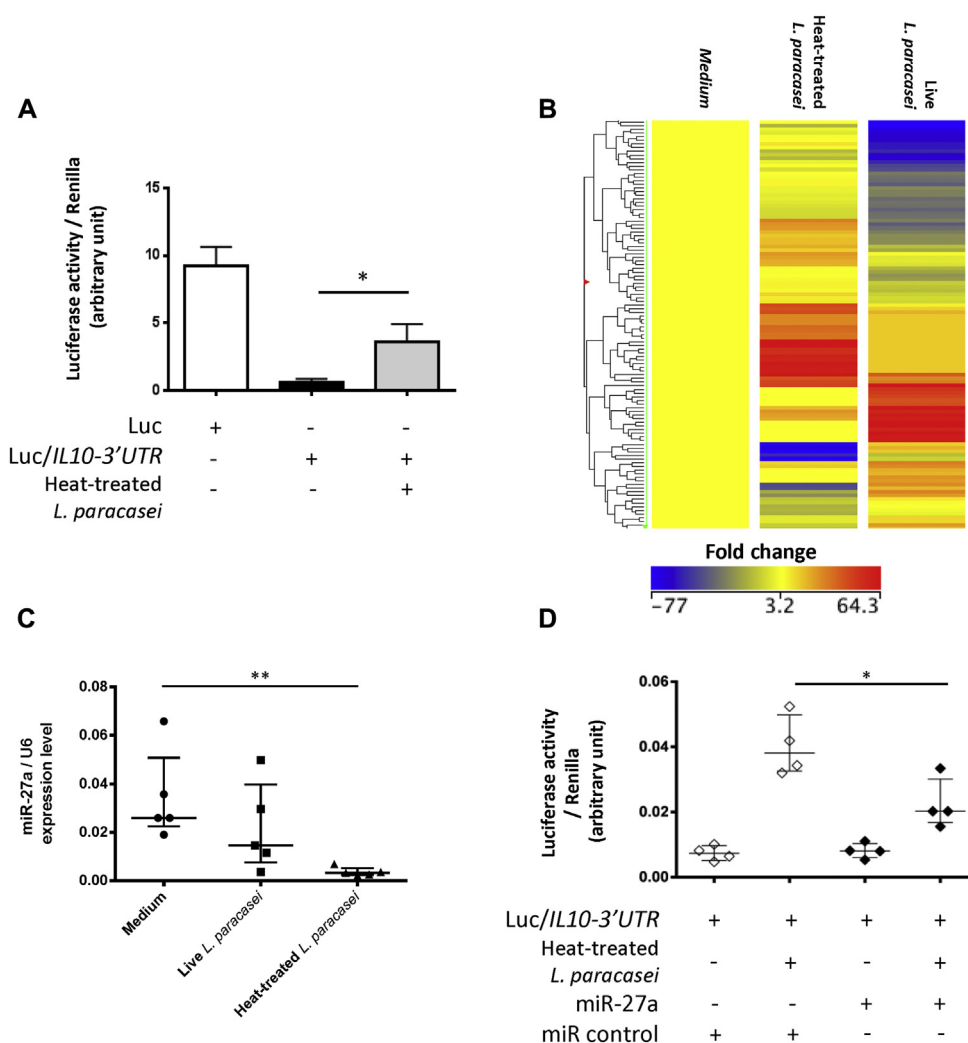


FIG 2. Role of *L. paracasei* NCC 2461 in the 3'UTR-mediated *IL10* mRNA stabilization and in the differential microRNA expression. **A**, Effect of heat-treated *L. paracasei* NCC2461 on *IL10*-3'UTR-dependent luciferase activity. **B**, Differential expression level of microRNA in PBMCs stimulated with live or heat-treated *L. paracasei* NCC 2461. **C**, Expression level of miR-27a in PBMCs. **D**, Effect of miR-27a on the *IL10*-3'UTR-dependent luciferase activity.

stimulation with live *L. paracasei* NCC 2461 induced only a 4.5-fold increase in *IL10* mRNA expression compared with unstimulated PBMCs. Interestingly, the disconnect between the 2- to 3-log induction in IL-10 protein level (4556 ± 1926 pg/mL with the heat-treated probiotic strain compared with 6.8 ± 1.4 pg/mL in unstimulated PBMCs; Fig 1, A) and the only 9.3-fold induction of *IL10* mRNA expression (Fig 1, B) is striking and suggests that the *IL10* mRNA might have an excellent translation efficiency under stimulation with heat-treated *L. paracasei* NCC 2461. Numerous mechanisms explaining a differential mRNA/protein ratio exist; here, we hypothesized that it was possibly due to a stabilization of the mRNA. Indeed, treatment of *L. paracasei*-stimulated PBMCs with actinomycin D revealed that *IL10* mRNA (but not *IFNG*; see Fig E2 in this article's Online Repository at www.jacionline.org) was specifically and significantly stabilized by the heat-treated *L. paracasei* NCC 2461 (half-life of 9.33 ± 2.51 hours; $P < .05$) compared with its live counterpart (half-life of 1.74 ± 1.76 hours) or the unstimulated control (half-life of 0.84 ± 0.66 hours) (Fig 1, C). This

mRNA stabilization was observed in all 5 PBMC donors tested (Fig 1, D). All together, these results suggest that heat-treated *L. paracasei* NCC 2461 stabilizes *IL10* mRNA.

There are several AU-rich elements in the 3' untranslated region (UTR) of *IL10* mRNA that have been shown to regulate *IL10* gene expression level.⁴ PBMCs, transfected with a plasmid containing the *IL10*-3'UTR downstream of the luciferase (Luc) gene (Luc/*IL10*-3'UTR) and stimulated by heat-treated *L. paracasei* NCC 2461 produced a significantly higher level of luciferase activity (3.6 ± 0.6 vs 0.6 ± 0.11 ; $P < .05$) than did transfected unstimulated cells (Fig 2, A), suggesting a role for *IL10*-3'UTR in heat-treated *L. paracasei* NCC 2461-induced *IL10* mRNA stabilization. We then investigated whether microRNA could be involved in this mechanism. We identified that live and heat-treated *L. paracasei* NCC 2461 differently influenced microRNA expression (Fig 2, B). These results suggest that the physiological stage of the studied probiotic strain (live or heat-treated) may differently affect the host immunity, underlining the need to properly control probiotics cultures. Numerous microRNAs were

specifically dysregulated in PBMCs stimulated by heat-treated *L. paracasei* (Table E1), but surprisingly only few microRNAs specific for *IL10* were downregulated when compared with live *L. paracasei* NCC 2461-treated or unstimulated cells (Fig E2). MiR-27a, previously shown to regulate *IL10* mRNA level,^{5,6} was significantly ($P < .005$) decreased by the heat-treated probiotic (Fig 2, C). Transfection of the miR-27a in cells was able to decrease *IL10*-3'UTR-dependent luciferase activity in PBMCs stimulated with the heat-treated probiotic (Fig 2, D). These results suggest that miR-27a may play a role in heat-treated probiotic-induced IL-10 expression, but other pathways are certainly also involved. We are currently considering the possibility that specific probiotic sequences may regulate the host immune function by a direct modulation of mRNA stabilization or indirectly through regulation of microRNA function. Probiotic strains may contain free RNA sequences that could be released on bacterial heat treatment because of partial membrane disruption. Multiple copies of sequences with more than 70% similarity with miR-27a have been identified in several probiotic strains (Fig E4). This concept of cross-kingdom regulation by microRNAs was recently described for rice, which could regulate mammalian host cell gene expression via the plant MIR168a.⁷

In conclusion, we identified that heat-treated *L. paracasei* NCC 2461 modulated IL-10 expression level by specifically stabilizing *IL10* mRNA. In our study, the bacterial heat treatment led to a differential regulation of microRNAs. This illustrates that depending on probiotics' physiological state, they may differently stabilize specific host cell mRNAs, thus potentially leading to varying effects. Importantly, we demonstrated that beyond classical molecular pattern recognition by dedicated receptors, epigenetic mechanisms may also play a role in host-microbe interaction. Finally, in this study, we illustrate how miRNA regulation by probiotics may lead to enhanced IL-10 production and partly explain the anti-inflammatory effects observed in clinical trials with specific probiotic strains.

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Audrey Demont, BS
Ferial Hacini-Rachinel, PhD
Rémi Doucet-Ladevèze, MS
Catherine Ngom-Bru, PhD
Annick Mercenier, PhD
Guénolée Prioult, PhD
Carine Blanchard, PhD

From Nutrition and Health Research, Nestlé Research Center, Lausanne, Switzerland.
E-mail: Carine.Blanchard@rdls.nestle.com.

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METHODS

Bacterial strains

The bacterial strain *Lactobacillus paracasei* NCC 2461 (CNCM I-2116) from the Nestlé Culture Collection was grown in a 15-L bioreactor (new MBR, Zürich, Switzerland) using industrial media (fermentation composition media provided by Nestlé PTC, Konolfingen, Switzerland). Biomass was harvested when entering the stationary phase. Bacterial cells were then spun down at 5000g for 20 minutes and resuspended in PBS until heat treatment. Glycerol 15% (v/v) was added to live samples, and the exact number of colony-forming units (CFUs) was determined by plating serial dilutions on agar plates.

Bacteria were heat-treated at 140°C for 15 seconds by a plate heat exchanger (Alfa Laval RF 372, Alfa Laval Mid Europe AG, Dietlikon, Switzerland). Heat treatment resulted in no residual CFU in the preparation. All samples were kept frozen at -80°C until use.

Isolation and stimulation of human PBMCs

Human PBMCs were isolated from filters obtained from the transfusion center of the Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland). Ethical approval was obtained to perform this study. The cells trapped in the filters were flushed back and diluted 1:2 with HBSS (Sigma, Lachen, Switzerland). After a Histopaque gradient centrifugation (Sigma), mononuclear cells were collected at the interface and washed twice with 40 mL HBSS according to the manufacturer's protocol. Cells were then resuspended in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% heat-inactivated FCS (Bioconcept, Paris, France), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), and 0.1% gentamycin (Sigma). PBMCs (1.5×10^6 cells/well) were then incubated with live and heat-treated bacterial strains (1.5×10^7 equivalent CFU/well) in 24-well plates. The effects of live and heat-treated bacteria were tested in triplicate on PBMCs (ratio 1:8) isolated from 5 individual donors.

An inhibitor of transcription (actinomycin D, 10 µg/mL) was added to PBMCs to evaluate the RNA stability of PBMCs. PBMCs were stimulated with probiotics for 24-hour incubation and subsequently cultured with actinomycin D for 1, 6, and 20 hours.

To assess whether intermediate protein synthesis was involved, before adding live or heat-treated bacterial strains PBMCs were incubated with or without 20 µg/mL cycloheximide (inhibitor of protein synthesis) for 30 minutes to inhibit the protein synthesis. Then strains were added (7×10^6 equivalent CFU/well) and incubated for 18 hours at 37°C in 5% CO₂.

Cytokine measurement

The levels of cytokines (IFN-γ; IL-10) were determined in the cell culture supernatants after 2- to 28-hour incubation using electrochemiluminescence-based multiplex assay (Meso Scale Discovery, Gaithersburg, Md) following the manufacturer's instructions. Results are expressed as means (pg/mL) ± SEM of 4 donors per bacterial preparation.

RNA extraction

RNA was extracted with TRIzol reagent following the manufacturer's instruction (Invitrogen, 15596-018, Life Technologies Europe B.V., Zug, Switzerland).

Briefly, supernatants were removed and kept at -20°C until cytokine measurement and cells were lysed directly by adding TRIzol. PBMCs and TRIzol were scraped with cell-scrapers. The homogenized samples were incubated for 5 minutes at room temperature. One volume of chloroform was added to 5 volumes of TRIzol reagent for 2 minutes at room temperature and centrifuged at 12,000g for 15 minutes at 4°C. Two volumes of the upper aqueous phase were transferred and mixed with 1 volume of isopropanol. Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed, and the pellet was washed with 75% ethanol and centrifuged at 8000g for 5 minutes at 4°C. Ethanol was removed, and the pellet was dried for 1 hour. The pellet was

dissolved in RNase-free water. The concentration was measured at an OD of 260 nm (NanoDrop), and the purity was determined by measuring the ratio A260/A280.

cDNA synthesis and TaqMan real-time PCR

Reverse transcription was performed on 750 ng of total RNA using qScript cDNA Synthesis Kit (Quanta BioSciences, #95047-025; VWR International, Dietikon, Switzerland) following the manufacturer's instruction. Reverse transcription was performed on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Rotkreuz, Switzerland) using the following cycle program: 5 minutes at 22°C, 30 minutes at 42°C, and 5 minutes at 85°C to finish at 4°C. Quantitative PCR was performed with the synthesized cDNA using TaqMan PCR. Each reaction mixture contained TaqMan Universal Master Mix 1×, primers *IL10* and *IFNG* (Hs00174086_m1, Hs99999041_m1, Applied Biosystems), 50 ng cDNA, and water. After 1 cycle of 50°C for 2 minutes and 95°C for 10 minutes to activate the polymerase, real-time PCR was carried out for 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute by using ABI-Prism 7900HT (Applied Biosystems). The mRNA were quantified and normalized with a house-keeping gene (HPRT). The mRNA expression was determined for each gene (*IL10* and *IFNG*) on the basis of average Ct value obtained. The results were calculated by using the formula $2^{-\Delta Ct}$. Three replicates of all samples were included, and experiments were performed on 5 donors. To control for possible DNA contamination in the RNA samples, "non-RT" controls were included in all experiments and no amplification above background levels was observed. Water controls were also included for each gene in each run, and no amplification above background levels was observed.

Flow cytometry analysis

After 18 hours of culture, BD GolgiStop (BD Biosciences, Allschwil, Switzerland) was added for 4 hours to PBMCs. Cells were harvested, washed once in ice-cold PBS, and suspended in a small amount of PBS. Cells were then incubated with 10% human serum for 15 minutes at 4°C to block Fc receptors. Specific fluorochrome-conjugated antibodies (CD3-phycoerythrin, CD14-fluorescein isothiocyanate, CD4-Allophycocyanin) were incubated for 30 minutes at 4°C. Cells were washed, fixed and permeabilized with Cytotfix/Cytoperm solution (BD Bioscience) for 20 minutes at 4°C, and pelleted cells were stained for intracellular cytokines using phycoerythrin-conjugated antibodies against human IL-10 or IFN-γ for 30 minutes at 4°C in the dark. Phycoerythrin-conjugated isotype controls were used in parallel. Finally, cells were washed and resuspended in PBS-BSA for flow cytometry analysis using FACS Fortessa (BD Biosciences) and FlowJO software version 7.6.5 (Tree Star, Ashland, Ore).

Total RNA extractions and TaqMan human MicroRNA array

The RNA extracted from PBMCs (Trizol, Invitrogen) stimulated with live or heat-treated bacteria and unstimulated PBMCs were pooled from 3 donors. Reverse transcription (RT) was performed on 500 ng total RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, #4366596). The total RNA was mixed with 100 mM deoxyribonucleotide triphosphates, 50 U/µL Multiscribe RT enzyme, 10× RT buffer, 20 U/µL RNase inhibitor, and 3 µL Megaplex Primer Pool A or B (Applied Biosystems, #4444750) in a final volume of 15 µL. Reverse transcription was performed using GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) with the following cycle program: 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 5°C to finish at 4°C. TaqMan Array Human MicroRNA Set Cards v2.0 from Applied Biosystems (#4400238) was loaded and run according to the manufacturer's instruction on a quantitative ABI-Prism 7900HT (Applied Biosystems). The results were normalized to 3 endogenous controls. In exploratory experiment, no false-discovery rate correction was applied to conserve all potential microRNA targeting *IL10*. In the experiment aiming at confirming miR-27a expression, Dunn's multiple comparisons test correction

was used. The list of the dysregulated microRNA is provided in Table E1. The results were calculated by using the formula $2^{-\Delta\text{ct}}$. Fold increase result expression was normalized to expression levels in the unstimulated group. Results were transferred and analyzed in the GeneSpring Software (Agilent Technologies, Santa Clara, Calif) and data were analyzed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, Calif; www.qiagen.com/ingenuity).

Transfection of PBMCs

PBMCs were left to adhere and then transiently transfected with lipofectamin iRNAiMAX (Invitrogen, Life Technologies) and 0.01 nM miR-27a mimic or inhibitor or the respective negative controls for 6 hours. The supernatant were collected for cytokine quantification, and cells were lysed for RNA quantification. When cells were transfected with plasmids,

cells were left to adhere and then transiently cotransfected with lipofectamin 2000 (Life Technologies) and 1 μ g PGL3-promoter vector containing the *Firefly* luciferase gene followed by the 3'UTR of the *IL10* mRNA, and 50 ng of pRL-TK vector (*Renilla Luciferase*). Cells were then washed and stimulated with the probiotics for 18 hours (overnight). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis). For the transfection of microRNA, *mirVana* miRNA control and mimic were obtained from Ambion (Life Technologies) and transfected in a 24-well plate with 2 μ L Lipofectamine RNAiMax Reagent (Invitrogen) per well in OPTI-MEM. The effects of live and heat-treated bacteria were studied on PBMCs (ratio 1:8). Transfection experiments are representative of at least 2 experiments tested in quadruplicates. Transfection efficacy was approximately 20% based on flow cytometry analyses of cells transfected with Cy3 Dye-Labeled Anti-miR Negative Control #1 from Ambion (Life Technologies).

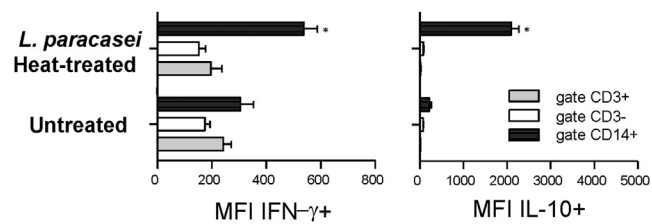


FIG E1. Intracellular IL-10 or IFN- γ cytokine expressions in PBMCs. Intracellular IL-10 or IFN- γ cytokine expressions were determined in CD3⁺ CD3⁺ and CD14⁺ cell subpopulations. Geometric means of fluorescence intensity (MFI) are shown. * $P < .05$ compared with unstimulated in the corresponding population subset using the Mann-Whitney U test.

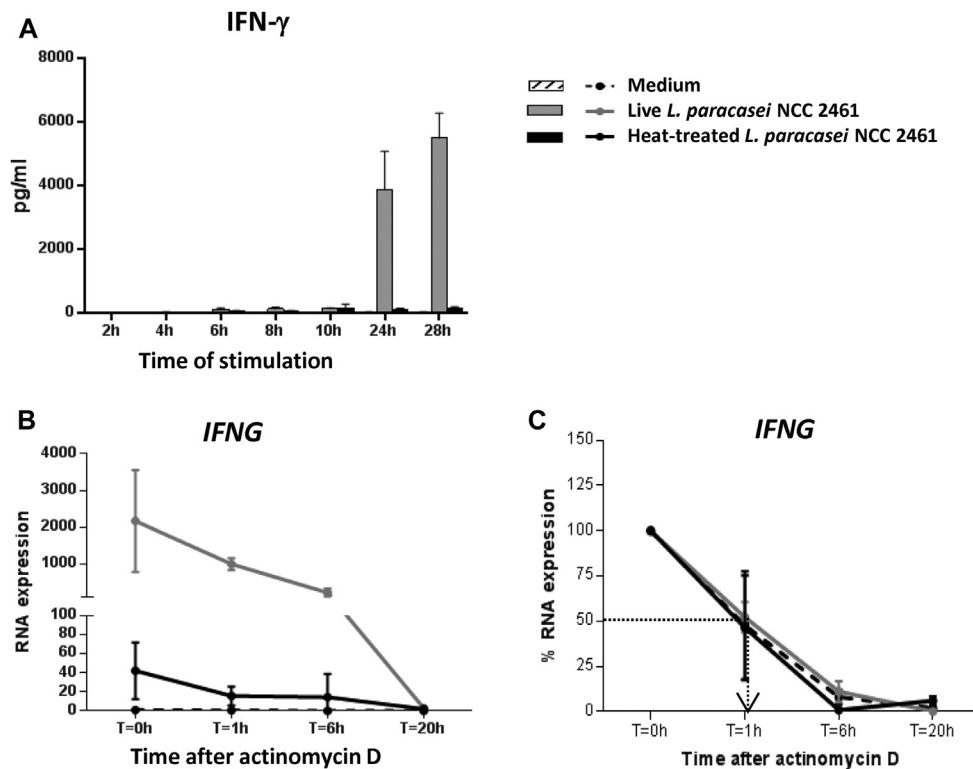
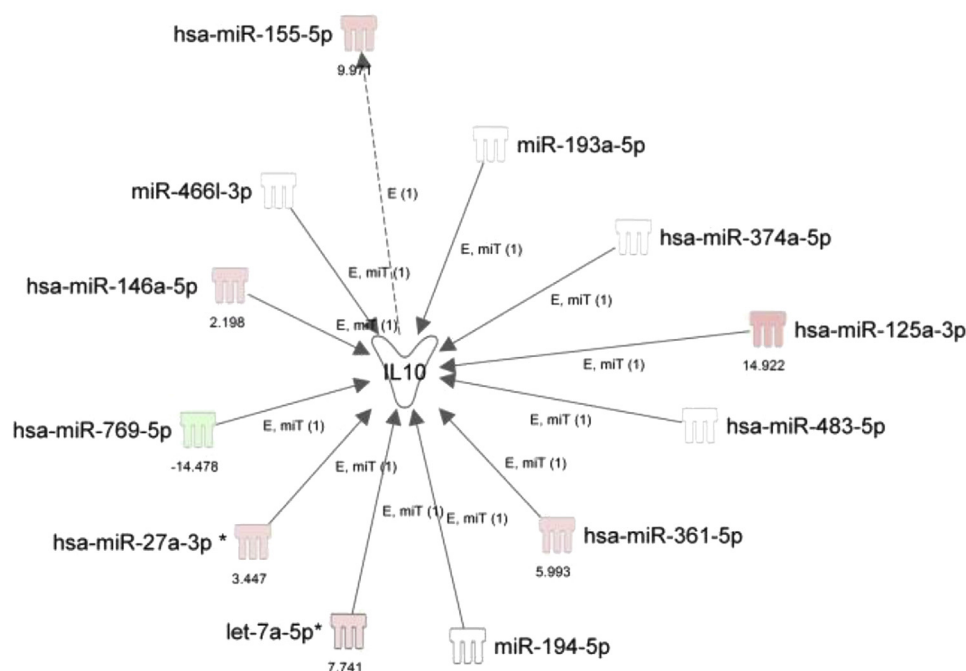


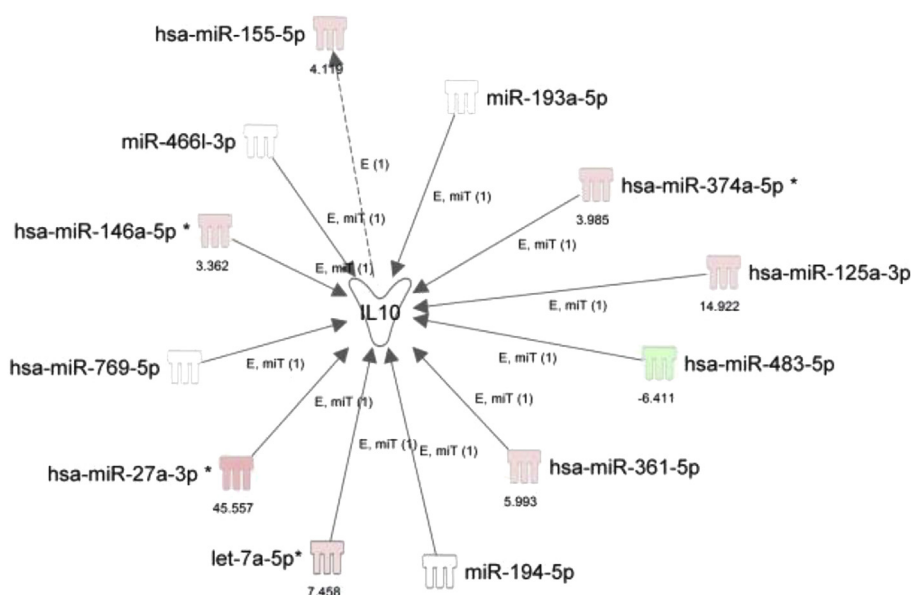
FIG E2. IFN- γ expression in PBMCs stimulated with live or heat-treated *L. paracasei* NCC 2461. **A**, IFN- γ protein secreted in the supernatant. **B**, *IFNG* mRNA expression in PBMCs stimulated with live or heat-treated *L. paracasei* NCC 2461. **C**, *IFNG* mRNA half-life in PBMCs. Experiments shown are means of triplicates and representative of 5 independent PBMC donors.

Medium vs. Heat-treated



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Heat-treated vs. Live



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FIG E3. Comparative expression of the microRNA targeting *IL10* mRNA. Fold changes in microRNA expression in PBMCs stimulated with *L. paracasei* (live or heat-treated) compared with unstimulated cells were plotted using Ingenuity Pathway Analysis. *Only 1 of the microRNAs is shown while several are in the list. Here, miR-27a is downregulated, miR-27b is upregulated. Only miR-27b upregulation is shown here. See [Table E1](#) for exact fold changes of individual microRNAs.

Pre-miR-27a sequences tested:

>hsa-mir-27a MI0000085

CUGAGGAGCA**GGGCUUAGCUGCUUGUGAGCA**GGGUCCACACCAAGUGUG**UUCACAUGGCCUAAGUCCG**CCCCCAG**Control sequence****Sequence miR-27a**

	Control sequence in miR-27a			Sequence miR-27a		
Number of mismatches	4	5	6	4	5	6
<i>L. paracasei</i> NCC 2461	0	0	0	4	14	131
<i>B. longum</i> NCC 3001	0	0	0	2	17	88
<i>B. lactis</i> NCC 2818	0	0	0	0	8	71
<i>L. johnsonii</i> NCC 533	0	0	0	1	4	58
<i>L. rhamnosus</i> NCC 4007	0	0	0	2	22	153
<i>B. longum</i> NCC 2705	0	0	0	1	17	86

FIG E4. Number of sequences with at least 70% similarity to miR-27a and control sequence in the genome of 6 probiotic strains. A control sequence present in the pre-miR-27a sequence and the miR-27a sequence were compared with the chromosomes of 6 probiotic strains. The 2 sequences of 20-21 nucleotides were compared after reverse translation U->T against the genome of 6 strains using FUZZNUC (EMBOSS package) allowing for 4 to 6 mismatches (80% to 70% similarity).

TABLE E1. MicroRNA expression in probiotic-stimulated PBMCs

Fold change	ID	Notes	Symbol	Entrez gene name
Heat-treated NCC 2461 vs medium				
53.047	hsa-miR-144		miR-144-3p	(miRNAs w/seed ACAGUAU)
50.723	hsa-miR-9		miR-9-5p	(and other miRNAs w/seed CUUUGGU)
49.290	hsa-miR-152	D	miR-148a-3p	(and other miRNAs w/seed CAGUGCA)
34.995	hsa-miR-100	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
34.944	hsa-miR-99a	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
26.359	hsa-miR-523		miR-523-3p	(miRNAs w/seed AACGCGC)
22.424	hsa-miR-642		miR-642a-5p	(miRNAs w/seed UCCCUCU)
19.960	hsa-miR-652		miR-652-3p	(miRNAs w/seed AUGGCGC)
16.956	hsa-miR-18a	D	miR-18a-5p	(and other miRNAs w/seed AAGGUGC)
14.922	hsa-miR-125a-3p		miR-125a-3p	(miRNAs w/seed CAGGUGA)
14.447	hsa-miR-146b-3p		miR-1231-3p	(and other miRNAs w/seed GCCCUGU)
12.654	hsa-miR-22		miR-22-3p	(miRNAs w/seed AGCUGCC)
10.560	hsa-miR-222	D	miR-221-3p	(and other miRNAs w/seed GCUACAU)
10.288	hsa-miR-330		miR-330-3p	(and other miRNAs w/seed CAAAGCA)
9.971	hsa-miR-155		miR-155-5p	(miRNAs w/seed UAAUGCU)
9.444	hsa-miR-411		miR-411-5p	(and other miRNAs w/seed AGUAGAC)
9.171	hsa-miR-486-3p		miR-486-3p	(and other miRNAs w/seed GGGGCAG)
8.313	hsa-miR-133a		miR-133a-3p	(and other miRNAs w/seed UUGGUCC)
8.257	hsa-miR-942		miR-7028-3p	(and other miRNAs w/seed CUUCUCU)
7.741	hsa-let-7c	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
5.993	hsa-miR-361		miR-361-5p	(miRNAs w/seed UAUCAGA)
5.895	hsa-miR-15a	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
5.099	hsa-miR-21		miR-21-5p	(and other miRNAs w/seed AGCUUAU)
4.475	hsa-miR-526b		miR-526b-5p	(miRNAs w/seed UCUUGAG)
3.997	hsa-miR-1		miR-1-3p	(and other miRNAs w/seed GGAUUGU)
3.964	hsa-miR-200b	D	miR-200b-3p	(and other miRNAs w/seed AAUACUG)
3.947	hsa-miR-32	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
3.447	hsa-miR-27b	D	miR-27a-3p	(and other miRNAs w/seed UCACAGU)
3.419	hsa-miR-130a	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
3.394	hsa-miR-636		miR-636	(miRNAs w/seed GUGCUUG)
3.220	hsa-miR-362		miR-362-5p	(and other miRNAs w/seed AUCCUUG)
3.042	mmu-miR-187		miR-187-3p	(miRNAs w/seed CGUGUCU)
2.926	hsa-miR-148b	D	miR-148a-3p	(and other miRNAs w/seed CAGUGCA)
2.896	hsa-let-7f	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
2.847	hsa-miR-199a-3p		miR-199a-3p	(and other miRNAs w/seed CAGUAGU)
2.740	hsa-miR-1290		miR-1290	(miRNAs w/seed GGAUUUU)
2.671	hsa-miR-29a	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
2.534	hsa-miR-15b	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
2.526	hsa-miR-28	D	miR-708-5p	(and other miRNAs w/seed AGGAGCU)
2.503	hsa-miR-454	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
2.491	hsa-miR-1305		miR-1305	(miRNAs w/seed UUUCAAC)
2.455	hsa-miR-221	D	miR-221-3p	(and other miRNAs w/seed GCUACAU)
2.430	hsa-miR-132	D	miR-132-3p	(and other miRNAs w/seed AACAGUC)
2.364	hsa-miR-1271		miR-96-5p	(and other miRNAs w/seed UUGGCAC)
2.359	hsa-miR-664		miR-664-3p	(and other miRNAs w/seed AUUCAUU)
2.313	hsa-miR-210		miR-210-3p	(miRNAs w/seed UGUGCGU)
2.198	hsa-miR-146a		miR-146a-5p	(and other miRNAs w/seed GAGAACU)
2.194	hsa-miR-363	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
2.083	hsa-miR-190b		miR-190a-5p	(and other miRNAs w/seed GAUAUGU)
2.060	hsa-miR-519b-3p		miR-519a-3p	(and other miRNAs w/seed AAGUGCA)
2.060	hsa-miR-212	D	miR-132-3p	(and other miRNAs w/seed AACAGUC)
-2.007	hsa-miR-197		miR-197-3p	(and other miRNAs w/seed UCACCAC)
-2.057	hsa-miR-18a	D	miR-18a-5p	(and other miRNAs w/seed AAGGUGC)
-2.059	hsa-miR-30a-5p		miR-30c-5p	(and other miRNAs w/seed GUAAACA)
-2.070	hsa-miR-328		miR-328-3p	(and other miRNAs w/seed UGGCCCU)
-2.109	hsa-miR-193b		miR-193a-3p	(and other miRNAs w/seed ACUGGCC)
-2.370	hsa-miR-1275		miR-1275	(and other miRNAs w/seed UGGGGGA)
-2.402	hsa-miR-148a	D	miR-148a-3p	(and other miRNAs w/seed CAGUGCA)
-2.508	hsa-miR-31		miR-31-5p	(and other miRNAs w/seed GGCAAGA)
-2.586	hsa-miR-95		miR-95-3p	(miRNAs w/seed UCAACGG)
-2.712	hsa-miR-429	D	miR-200b-3p	(and other miRNAs w/seed AAUACUG)

(Continued)

TABLE E1. (Continued)

Fold change	ID	Notes	Symbol	Entrez gene name
-2.752	hsa-miR-432		miR-432	(and other miRNAs w/seed CUUGGAG)
-2.993	hsa-miR-34a	D	miR-34a-5p	(and other miRNAs w/seed GGCAGUG)
-3.004	hsa-miR-638		miR-638	(miRNAs w/seed GGAUCCG)
-3.650	hsa-miR-509-5p		miR-509-5p	(and other miRNAs w/seed ACUGCAG)
-3.671	hsa-miR-545		miR-545-3p	(miRNAs w/seed CAGCAAA)
-3.984	hsa-miR-26b		miR-26a-5p	(and other miRNAs w/seed UCAAGUA)
-6.601	hsa-miR-27a	D	miR-27a-3p	(and other miRNAs w/seed UCACAGU)
-7.205	hsa-miR-577		miR-577	(miRNAs w/seed AGAUAAA)
-8.346	hsa-miR-1249		miR-1249-3p	(and other miRNAs w/seed CGCCCUU)
-8.353	hsa-miR-505		miR-505-3p	(miRNAs w/seed GUCAACA)
-8.359	mmu-miR-496		miR-503-3p	(and other miRNAs w/seed GAGUAUU)
-8.765	mmu-miR-134		miR-3118	(and other miRNAs w/seed GUGACUG)
-11.042	hsa-miR-1244		miR-1244	(miRNAs w/seed AGUAGUU)
-11.467	hsa-miR-571		miR-571	(miRNAs w/seed GAGUUGG)
-12.837	hsa-miR-127		miR-127-3p	(miRNAs w/seed CGGAUCC)
-12.892	hsa-miR-539		miR-539-5p	(miRNAs w/seed GAGAAAU)
-14.478	hsa-miR-769-5p		miR-769-5p	(miRNAs w/seed GAGACCU)
-15.122	rno-miR-29c	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
-19.458	hsa-miR-449b	D	miR-34a-5p	(and other miRNAs w/seed GGCAGUG)
-22.429	hsa-miR-598		miR-598-3p	(miRNAs w/seed ACGUCAU)
-25.956	hsa-miR-107		miR-103-3p	(and other miRNAs w/seed GCAGCAU)
-26.273	hsa-miR-449	D	miR-34a-5p	(and other miRNAs w/seed GGCAGUG)
-28.571	hsa-miR-125b		miR-125b-5p	(and other miRNAs w/seed CCCUGAG)
-30.393	hsa-miR-15a	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
-31.668	hsa-miR-130b	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
-32.432	hsa-miR-433		miR-433-3p	(miRNAs w/seed UCAUGAU)
-33.203	hsa-miR-485-3p		miR-485-3p	(and other miRNAs w/seed UCAUACA)
-53.414	hsa-miR-30a-3p		miR-30a-3p	(and other miRNAs w/seed UUUCAGU)
-57.982	hsa-miR-450a		miR-450a-5p	(and other miRNAs w/seed UUUGCGA)
-60.486	hsa-miR-708	D	miR-708-5p	(and other miRNAs w/seed AGGAGCU)
-70.142	hsa-miR-99b	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
-75.581	hsa-miR-324-5p		miR-324-5p	(miRNAs w/seed GCAUCCC)
-95.606	hsa-miR-138		miR-138-5p	(miRNAs w/seed GCUGGUG)
Heat-treated NCC2461 vs live NCC2461				
150.942	hsa-miR-30e-3p	D	miR-30a-3p	(and other miRNAs w/seed UUUCAGU)
118.263	mmu-miR-491		miR-491-5p	(and other miRNAs w/seed GUGGGGA)
50.723	hsa-miR-9		miR-9-5p	(and other miRNAs w/seed CUUUGGU)
45.557	hsa-miR-27b	D	miR-27a-3p	(and other miRNAs w/seed UCACAGU)
40.176	hsa-miR-139-5p		miR-139-5p	(miRNAs w/seed CUACAGU)
39.009	hsa-miR-101		miR-101-3p	(and other miRNAs w/seed ACAGUAC)
37.788	hsa-miR-526b		miR-526b-5p	(miRNAs w/seed UCUUGAG)
34.995	hsa-miR-100	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
33.864	hsa-miR-362		miR-362-5p	(and other miRNAs w/seed AUCCUUG)
33.566	hsa-miR-103		miR-103-3p	(and other miRNAs w/seed GCAGCAU)
30.687	hsa-miR-339-5p		miR-339-5p	(and other miRNAs w/seed CCCUGAG)
29.653	hsa-miR-31	D	miR-31-5p	(and other miRNAs w/seed GGCAAGC)
27.195	hsa-miR-532		miR-532-5p	(and other miRNAs w/seed AUGCCUU)
26.359	hsa-miR-523		miR-523-3p	(miRNAs w/seed AACGCGC)
22.424	hsa-miR-642		miR-642a-5p	(miRNAs w/seed UCCCUCU)
19.960	hsa-miR-652		miR-652-3p	(miRNAs w/seed AUGGCGC)
19.887	hsa-miR-210		miR-210-3p	(miRNAs w/seed UGUGCGU)
19.633	hsa-miR-423-5p		miR-423-5p	(and other miRNAs w/seed GAGGGGC)
17.681	hsa-miR-502-3p		miR-501-3p	(and other miRNAs w/seed AUGACCC)
16.478	hsa-miR-148a	D	miR-148a-3p	(and other miRNAs w/seed CAGUGCA)
15.813	hsa-miR-671-3p		miR-671-3p	(and other miRNAs w/seed CCGGUUC)
14.970	hsa-miR-212	D	miR-132-3p	(and other miRNAs w/seed AACAGUC)
14.922	hsa-miR-125a-3p		miR-125a-3p	(miRNAs w/seed CAGGUGA)
14.724	hsa-miR-190b		miR-190a-5p	(and other miRNAs w/seed GAUAUGU)
14.456	hsa-miR-411		miR-411-5p	(and other miRNAs w/seed AGUAGAC)
14.447	hsa-miR-146b-3p		miR-1231-3p	(and other miRNAs w/seed GCCCUGU)
14.371	hsa-miR-410		miR-344d-3p	(and other miRNAs w/seed AUAUAAC)
12.970	mmu-miR-495		miR-495-3p	(and other miRNAs w/seed AACAAAC)

(Continued)

TABLE E1. (Continued)

Fold change	ID	Notes	Symbol	Entrez gene name
12.394	hsa-miR-223	D	miR-223-3p	(miRNAs w/seed GUCAGUU)
10.291	hsa-miR-15b	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
10.049	hsa-miR-1275		miR-1275	(and other miRNAs w/seed UGGGGGA)
9.942	hsa-miR-99b	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
9.034	hsa-miR-574-3p		miR-574-3p	(miRNAs w/seed ACGCUCA)
8.954	hsa-miR-185		miR-185-5p	(and other miRNAs w/seed GGAGAGA)
8.909	hsa-miR-106b	D	miR-17-5p	(and other miRNAs w/seed AAAGUGC)
8.782	hsa-miR-99a	D	miR-100-5p	(and other miRNAs w/seed ACCCGUA)
8.725	hsa-miR-942		miR-7028-3p	(and other miRNAs w/seed CUUCUCU)
8.424	hsa-miR-339-3p		miR-339-3p	(miRNAs w/seed GAGCGCC)
7.484	hsa-miR-29b	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
7.458	hsa-let-7f	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
7.105	hsa-miR-125a-5p		miR-125b-5p	(and other miRNAs w/seed CCCUGAG)
6.934	hsa-miR-486-3p		miR-486-3p	(and other miRNAs w/seed GGGGCAG)
6.715	hsa-miR-425-5p		miR-425-5p	(and other miRNAs w/seed AUGACAC)
6.712	hsa-miR-660		miR-660-5p	(and other miRNAs w/seed ACCCAUU)
6.704	hsa-miR-132	D	miR-132-3p	(and other miRNAs w/seed AACAGUC)
6.622	hsa-miR-192		miR-192-5p	(and other miRNAs w/seed UGACCUA)
6.616	hsa-let-7b	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
6.434	hsa-miR-28		miR-708-5p	(and other miRNAs w/seed AGGAGCU)
6.260	hsa-miR-296		miR-296-5p	(miRNAs w/seed GGGCCCC)
6.070	hsa-let-7a	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
6.034	hsa-miR-142-5p		miR-142-5p	(and other miRNAs w/seed AUAAAGU)
5.993	hsa-miR-361		miR-361-5p	(miRNAs w/seed UAUCAGA)
5.960	hsa-miR-532-3p		miR-532-3p	(miRNAs w/seed CUCCAC)
5.895	hsa-miR-15a	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
5.324	hsa-miR-518f	D	miR-518a-3p	(and other miRNAs w/seed AAAGCGC)
5.099	hsa-miR-21	D	miR-21-5p	(and other miRNAs w/seed AGCUUUA)
4.931	hsa-miR-625		miR-625-5p	(and other miRNAs w/seed GGGGGAA)
4.518	hsa-miR-454	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
4.446	hsa-miR-1260		miR-1260a	(and other miRNAs w/seed UCCACC)
4.146	hsa-let-7e	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
4.119	hsa-miR-155		miR-155-5p	(miRNAs w/seed UAAUGCU)
4.042	hsa-miR-223	D	miR-223-3p	(miRNAs w/seed GUCAGUU)
3.997	hsa-miR-1		miR-1-3p	(and other miRNAs w/seed GGA AUGU)
3.985	mmu-miR-374-5p	D	miR-374b-5p	(and other miRNAs w/seed UAUAUA)
3.964	hsa-miR-200b	D	miR-200b-3p	(and other miRNAs w/seed AAUACUG)
3.947	hsa-miR-32	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
3.728	hsa-miR-16	D	miR-16-5p	(and other miRNAs w/seed AGCAGCA)
3.592	hsa-miR-19a	D	miR-19b-3p	(and other miRNAs w/seed GUGCAAA)
3.583	hsa-miR-454	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
3.394	hsa-miR-636		miR-636	(miRNAs w/seed GUGCUUG)
3.385	hsa-miR-140-3p		miR-140-3p	(and other miRNAs w/seed ACCACAG)
3.362	hsa-miR-146a	D	miR-146a-5p	(and other miRNAs w/seed GAGAACU)
3.291	hsa-miR-30b	D	miR-30c-5p	(and other miRNAs w/seed GUAAACA)
3.127	hsa-miR-422a		miR-378a-3p	(and other miRNAs w/seed CUGGACU)
3.103	hsa-miR-20b	D	miR-17-5p	(and other miRNAs w/seed AAAGUGC)
3.099	hsa-miR-197		miR-197-3p	(and other miRNAs w/seed UCACCAC)
3.049	rno-miR-7		miR-7a-5p	(and other miRNAs w/seed GGAAGAC)
3.042	mmu-miR-187		miR-187-3p	(miRNAs w/seed CGUGUCU)
3.020	hsa-miR-374	D	miR-374b-5p	(and other miRNAs w/seed UAUAUA)
2.970	hsa-miR-484		miR-344a-5p	(and other miRNAs w/seed CAGGCUC)
2.913	hsa-miR-376a		miR-376a-3p	(and other miRNAs w/seed UCAUAGA)
2.897	hsa-miR-24		miR-24-3p	(and other miRNAs w/seed GGCUCAG)
2.842	hsa-miR-30c	D	miR-30c-5p	(and other miRNAs w/seed GUAAACA)
2.839	hsa-miR-31	D	miR-31-5p	(and other miRNAs w/seed GGCAAGA)
2.832	hsa-miR-590-3P		miR-590-3p	(miRNAs w/seed AAUUUUA)
2.830	hsa-miR-301	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
2.766	hsa-miR-191		miR-191-5p	(and other miRNAs w/seed AACGGAA)
2.747	hsa-miR-1254		miR-1254	(and other miRNAs w/seed GCCUGGA)
2.739	hsa-miR-133a		miR-133a-3p	(and other miRNAs w/seed UUGGUCC)
2.636	hsa-miR-20a	D	miR-17-5p	(and other miRNAs w/seed AAAGUGC)

(Continued)

TABLE E1. (Continued)

Fold change	ID	Notes	Symbol	Entrez gene name
2.627	hsa-miR-331		miR-331-3p	(miRNAs w/seed CCCCUGG)
2.600	hsa-miR-19b	D	miR-19b-3p	(and other miRNAs w/seed GUGCAAA)
2.599	hsa-miR-26b	D	miR-26a-5p	(and other miRNAs w/seed UCAAGUA)
2.585	hsa-miR-92a	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
2.554	hsa-miR-29a	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
2.547	hsa-miR-126		miR-126a-3p	(and other miRNAs w/seed CGUACCG)
2.539	hsa-miR-342-3p		miR-342-3p	(miRNAs w/seed CUCACAC)
2.533	hsa-miR-144		miR-144-3p	(miRNAs w/seed ACAGUAU)
2.531	hsa-miR-199a-3p		miR-199a-3p	(and other miRNAs w/seed CAGUAGU)
2.485	mmu-miR-93	D	miR-17-5p	(and other miRNAs w/seed AAAGUGC)
2.479	hsa-miR-376c		miR-376c-3p	(and other miRNAs w/seed ACAUAGA)
2.386	hsa-miR-25	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
2.381	hsa-miR-26a	D	miR-26a-5p	(and other miRNAs w/seed UCAAGUA)
2.368	hsa-let-7c	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
2.359	hsa-miR-186		miR-186-5p	(miRNAs w/seed AAAGAAU)
2.359	hsa-miR-664		miR-664-3p	(and other miRNAs w/seed AUUCAUU)
2.306	hsa-miR-21	D	miR-21-5p	(and other miRNAs w/seed AGCUUAU)
2.276	hsa-miR-146b	D	miR-146a-5p	(and other miRNAs w/seed GAGAACU)
2.257	hsa-miR-200c	D	miR-200b-3p	(and other miRNAs w/seed AAUACUG)
2.234	hsa-miR-106a	D	miR-17-5p	(and other miRNAs w/seed AAAGUGC)
2.177	hsa-miR-222	D	miR-221-3p	(and other miRNAs w/seed GCUACAU)
2.149	hsa-miR-221	D	miR-221-3p	(and other miRNAs w/seed GCUACAU)
2.130	hsa-let-7d	D	let-7a-5p	(and other miRNAs w/seed GAGGUAG)
2.089	hsa-miR-335		miR-335-5p	(and other miRNAs w/seed CAAGAGC)
2.084	hsa-miR-29c	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
2.080	hsa-miR-150		miR-150-5p	(and other miRNAs w/seed CUCCCAA)
2.060	hsa-miR-519b-3p		miR-519a-3p	(and other miRNAs w/seed AAGUGCA)
2.022	hsa-miR-1305		miR-1305	(miRNAs w/seed UUUCAAC)
2.008	hsa-miR-590-5p	D	miR-21-5p	(and other miRNAs w/seed AGCUUAU)
-2.275	hsa-miR-18a		miR-18a-5p	(and other miRNAs w/seed AAGGUGC)
-2.868	hsa-miR-130a	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
-3.145	hsa-miR-539		miR-539-5p	(miRNAs w/seed GAGAAAU)
-4.294	hsa-miR-29a	D	miR-29b-3p	(and other miRNAs w/seed AGCACCA)
-4.442	hsa-miR-501		miR-501-5p	(miRNAs w/seed AUCCUUU)
-4.580	hsa-miR-148b	D	miR-148a-3p	(and other miRNAs w/seed CAGUGCA)
-4.725	hsa-miR-342-5p		miR-342-5p	(and other miRNAs w/seed GGGGUGC)
-5.500	hsa-miR-1227		miR-1227-3p	(miRNAs w/seed GUGCCAC)
-6.308	hsa-miR-222	D	miR-221-3p	(and other miRNAs w/seed GCUACAU)
-6.411	hsa-miR-483-5p		miR-483-5p	(miRNAs w/seed AGACGGG)
-6.427	hsa-miR-432		miR-432	(and other miRNAs w/seed CUUGGAG)
-6.929	hsa-miR-26b	D	miR-26a-5p	(and other miRNAs w/seed UCAAGUA)
-6.992	hsa-miR-363	D	miR-92a-3p	(and other miRNAs w/seed AUUGCAC)
-7.362	hsa-miR-518b	D	miR-518a-3p	(and other miRNAs w/seed AAAGCGC)
-9.878	hsa-miR-27a	D	miR-27a-3p	(and other miRNAs w/seed UCACAGU)
-10.100	hsa-miR-505		miR-505-3p	(miRNAs w/seed GUCAACA)
-12.811	hsa-miR-30d	D	miR-30c-5p	(and other miRNAs w/seed GUAACA)
-18.215	mmu-miR-134		miR-3118	(and other miRNAs w/seed GUGACUG)
-18.842	hsa-miR-502		miR-502-5p	(and other miRNAs w/seed UCCUUGC)
-20.700	hsa-miR-627		miR-627-5p	(miRNAs w/seed UGAGUCU)
-28.711	hsa-miR-598		miR-598-3p	(miRNAs w/seed ACGUCAU)
-31.629	hsa-miR-130b	D	miR-130a-3p	(and other miRNAs w/seed AGUGCAA)
-51.340	hsa-miR-30a-3p	D	miR-30a-3p	(and other miRNAs w/seed UUUCAGU)