

# Genomic Analysis of Intestinal Responses to Gut Flora Altered by Antimicrobials in Human Flora-Associated (HFA) Mice



## ABSTRACT

Genetic expression in colonic epithelial cells, responding to the changes of human intestinal flora altered by exposure to drugs or contaminants, provides valuable health information. Gut flora are important in:

1. Metabolic fermentation and energy salvage.
2. Intestinal cell proliferation and differentiation
3. Development and homoeostasis of immune system and protection against pathogen invasion.

Many drugs orally delivered or present in food may disturb normal flora and cause human health concerns. Here we report a short, but integral method to understand host-microbial relationships in the intestine, as well as to identify the genetic basis for the impact of drugs on intestinal functions. This is achieved by comparing gene expression in colonic epithelial cells between germ-free (GF) mice, human flora-associated (HFA) mice and drug-treated HFA mice using laser capture microdissection, RiboAmp® HS RNA Amplification Kit and cDNA microarrays.

## INTRODUCTION

Many species of bacteria have evolved and adapted to survive and grow in the environment of the human intestine. The bacteria are ten times more abundant than the number of eukaryotic cells in the human body and composed of 300-500 different bacterial species.

## APPLICATION NOTE #10

Most notably, the colon, the major part of the large intestine, contains a complex and dynamic microbial ecosystem of 10<sup>11</sup> or 10<sup>12</sup> bacterial cells per gram of luminal contents and constitutes 60% of faecal solids within the colon<sup>1</sup>. It has been a point of interest to determine how the commensalisms between host human and intestinal microflora have been acquired and maintained<sup>2,3</sup>. Gut flora have important roles in the metabolic fermentation of nondigestible dietary residue and endogenous mucus, salvage of energy as short-chain fatty acids, production of vitamin K, absorption of ions, proliferation and differentiation of intestinal epithelial cells, development and homoeostasis of the immune system and protection against pathogens. The major risk to human health from ingested antimicrobial drugs or residues in food are dysfunction of the above processes due to alteration of gut microflora. It is important to determine the toxic effects of endogenous or exogenous toxicants on the balance of gut flora and the severity of intestinal malfunction. However, the condition of culture and the identification of intestinal flora are too complex to study using conventional microbiological methods. Analysis of the genomic expression profiles in the host, elicited by a given toxicant in a specific cell population, offers extensive information about the interaction between host and toxicants<sup>4,5</sup>. Among intestinal cells, colonic crypt cells are highly responsive to exogenous stress such as change in microflora. Systems for crypt cells possess an exceptionally high proliferation potential and play a central role in intestinal epithelial homeostasis<sup>6</sup>.

Here we describe the study of the effects of changes in gut flora that are disturbed by orally delivered chemicals or contaminants on host intestinal function, using amplified mRNA extracted from laser capture microdissected colonic crypt cells of GF mice, HFA mice or HFA mice exposed orally to antimicrobial drugs.

## REAGENTS USED

- HistoGene® LCM Frozen Section Staining Kit (ThermoFisher; Cat #KIT0401)
- PicoPure® RNA Isolation Kit (ThermoFisher; Cat #KIT0202)
- RNase-Free DNase Set (Qiagen; Cat #79254)
- RNase AWAY® (Invitrogen; Cat #10328-011)
- RiboAmp® HS RNA Amplification Kit (ThermoFisher; Cat #KIT0205)
- Cy3 labeled dUTP (Amersham; Cat #PA53022)
- Cy5 labeled dUTP (Amersham; Cat #PA55022)
- RNasin® Ribonuclease Inhibitor (Promega; Cat #N2515)
- SuperScript II RT and Buffer (Invitrogen; Cat #18064-071)
- RNase H (Invitrogen; Cat #18021-071)
- Random Hexamer (Fermentas; Cat #S0142)
- QiaQuick® PCR Purification Kit (Qiagen; Cat #28106)
- TwinChip™ Mouse-7.4 K cDNA Array (Digital Genomics, Seoul, Korea)
- In situ Hybridization kit-plus (Agilent; Cat #5184-3568)

- Microarray hybridization chamber kit (Agilent; Cat #G2534A)
- 20X Saline-Sodium Citrate (SSC) (Sigma; Cat #S-6639)
- 10% Sodium Dodecyl Sulfate (SDS) (ThermoFisher; Cat #15553-035)
- Tissue Freezing Medium (Leica Instrument GmbH; Cat #020108926)
- iScript™ cDNA synthesis Kit (Bio-Rad; Cat #170-8890)
- iQ SYBR Green Supermix (Bio-Rad; Cat #170-8880)

## EQUIPMENT AND LABWARE

- Fume hood for autopsy
- CM3505 S Cryostat (Leica Microsystems, Germany)
- LCM Slides (ThermoFisher; Cat #KIT0417)
- Laser Capture Microdissection instrument (Laxco, Inc.)
- CapSure® Macro LCM Caps (ThermoFisher; Cat #LCM0201)
- iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA)
- Allegra® 21R Centrifuge (Beckman Coulter, Fullerton, CA)
- Agilent Hybridization Oven G2545A (Agilent Technologies, Palo Alto, CA)
- Affymetrix 418 Array Scanner (Affymetrix, Santa Clara, CA)

- SpectraMax Plus384 Spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA)
- Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany)
- Imagen 5.6 Hybridization Image Processing and Visualization Program (BioDiscovery Inc., El Segundo, CA)
- GeneSpringGX1 Version 7.2 (Agilent Technologies, Santa Clara, CA)
- Cryomold® (Miles Inc., Elkhart, USA, Tissue-Tek 4557)
- Gasket slides (2 microarrays/slide format) (Agilent Technologies, Palo Alto, CA.; Cat #G2534-60002)

## SPECIAL CONSIDERATIONS

1. Precautions should be taken at every step to prevent RNase contamination:
  - Wear disposable gloves and change them frequently.
  - Use RNase-free solutions and plastic labwares.
  - Use RNase AWAY® according to manufacturer's instructions on any surfaces that may come in contact with sample.
2. Fluorescent dyes (Cy5 and Cy3) are unstable under extended exposure to light. Reaction mixture with the probes and purification of the probes should be done in low-light conditions.

## METHOD

1. Animals and Treatment
  - 1.1 Male germ-free ICR mice (4 to 5 weeks, CLEA Inc., Tokyo, Japan) were kept aseptically in 0.2 µm filtered isolator.
  - 1.2 HFA mice were made from GF mice by the oral inoculation of *Bacteroides fragilis* (ATCC 25285) with 0.2 mL/animal (107 cfu/mL) of injected solution. Two days cal samples (diluted 1:99 (w/v) in pre-reduced TGY broth) were administered in a volume of 0.2 mL/animal.
  - 1.3 After confirmation of the stable establishment of human flora in the gut of HFA mice (an average of 12 days after the inoculation of *Bacteroides fragilis*), antimicrobials were administered orally to HFA mice for several days. For this experiment, tetracycline (TC) 200 mg/kg by weight and ciprofloxacin (Cip) 200 mg/kg by weight were injected orally to HFA mice for 4 days.
  - 1.4 Three mice were allocated per animal group (GF, HFA, TC-treated HFA and Cip-treated HFA mice).
2. Colon Tissue preparation
  - 2.1 The colons of each mouse were dissected out and the intestinal contents were washed out by flushing twice with sterilized normal saline.

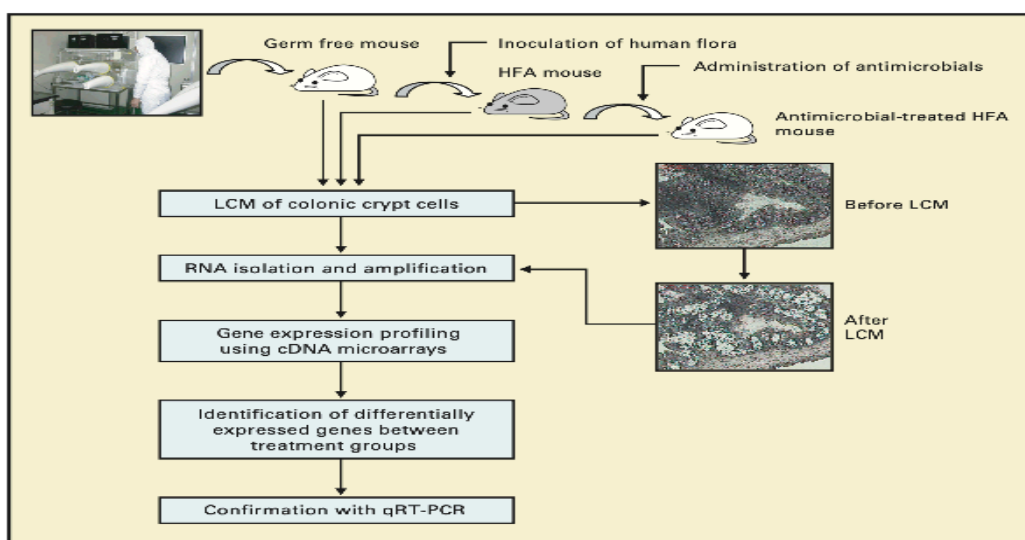


Figure 1. Experimental schematic for gene expression profiling in colonic crypt cells of GF or HFA mice exposed to antimicrobials.

- 2.2** The colon tissue was cut into small pieces (three to four pieces) and all pieces were placed longitudinally in cold cryomolds on dry ice, half-filled with OCT.
- 2.3** OCT was then added to each cold cryomold until specimens were covered.
- 2.4** Frozen specimens were stored in the cryomold at  $-80^{\circ}\text{C}$ .

### **3. Cryosection and Staining**

- 3.1** Using the cryostat,  $8\mu\text{m}$  sections were cut from the frozen tissue blocks, each block containing 3-4 pieces of colon.
- 3.2** Sections (3-5) were prepared per colon sample and mounted onto room temperature glass LCM slides. Slide sections were immediately placed into a microslide box and placed on dry ice.
- 3.3** Immediately following slide preparation, sections were stained following the HistoGene LCM Frozen Section Staining Kit user manual. One section was stained and used for the following experiment, and the remaining sections were stored unstained at  $-80^{\circ}\text{C}$  for up to two months.

### **4. Laser Capture Microdissection**

- 4.1** The infrared laser spot size was adjusted to  $15\mu\text{m}$  diameter.
- 4.2** Default laser power and duration settings were maintained.
- 4.3** Colonic crypt cells were visually selected from prepared colon sections from GF mice, and HFA mice treated with and without antimicrobials.
- 4.4** Selected crypt cells were microdissected using the CapSure Macro LCM Caps.
- 4.5** 100-200 crypt cells were captured from one section of colon tissue per animal.

### **5. RNA Extraction and Isolation**

- 5.1** CapSure Macro LCM Caps containing the captured colonic crypt cells (one cap per animal) were placed into a nuclease-free  $0.5\text{ mL}$  micro- centrifuge tube containing  $50\mu\text{L}$  of extraction buffer.
- 5.2** The CapSure Macro LCM Cap microcentrifuge tube assembly was inverted and incubated for 30 minutes at  $42^{\circ}\text{C}$ .
- 5.3** The cap-tube assembly was centrifuged at  $800 \times g$  for 2 minutes to collect the cell extract into microcentrifuge tube.
- 5.4** RNA isolation proceeded following the PicoPure RNA Isolation Kit user manual for one extract per animal.
- 5.5** DNase treatment was performed on column to remove any contaminating DNA following the protocol in the kit user manual.
- 5.6** Total RNA was eluted in  $11\mu\text{L}$  elution buffer provided with the kit.

### **6. RNA Amplification**

- 6.1** The mRNA fraction of total cellular RNA (entire eluted volume of  $11\mu\text{L}$ ), as isolated from one slide of colonic crypt cells ( $100\text{-}200$  cells) per animal, was amplified through two rounds of linear amplification following the protocol described in the RiboAmp HS RNA Amplification Kit user manual.
- 6.2** The amplified antisense RNA (aRNA) was purified using the MiraCol™ Purification Columns, following the kit user manual.
- 6.3** The purified aRNA was eluted in  $30\mu\text{L}$  of elution buffer provided with the kit.

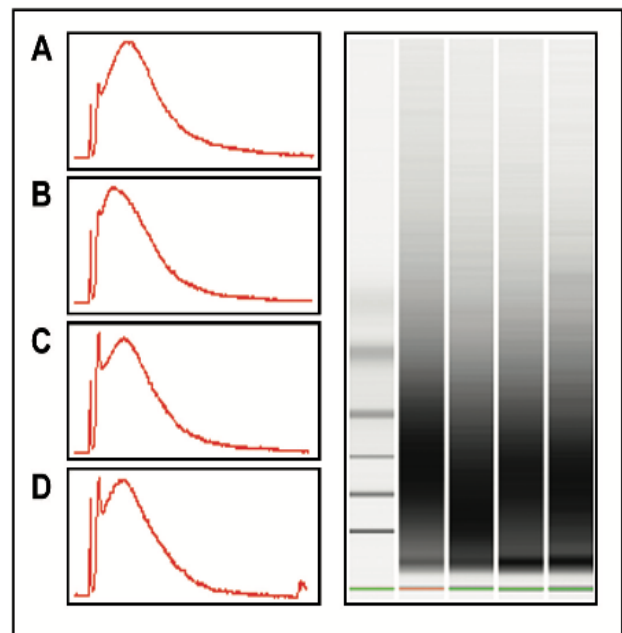
## 7. aRNA Quality and Quantity Assessment

- 7.1** The optical density (OD) of each aRNA isolate was measured at both A260 and A280 using the Spectra- Max Plus384 Spectrophotometer (Molecular Devices, Sunnyvale, CA)
- 7.2** The aRNA quantity was determined by multiplying the OD at A260 by the dilution factor of 10 and the RNA constant of 40 [(A260)(dilution factor) (40) = ng/μL RNA].
- 7.3** In this experiment, aRNA yield was between 18 – 23 μg (Table 1).
- 7.4** The ratio of A260/A280 was evaluated as an initial indicator of aRNA quality. Very pure aRNA should have an A260/A280 ratio between 2.0-2.6.
- 7.5** aRNA quality was also assessed by running the samples on the 2100 Bioanalyzer (Agilent, Waldbrook, Germany) to verify the size distribution of amplified aRNA. (Figure 2).
- 8.** Fluorescent cDNA synthesis Protocol #3<sup>7</sup>
- 8.1** cDNA was synthesized from 10 μg of amplified aRNA (volume was adjusted to 22 μL with nuclease-free water) by mixing with 2 μL of 5 mg/ml of random hexamer and heating at 70°C for 10 minutes.
- 8.2** To generate cDNA containing Cy3 and Cy5 dUTPs (1mM for each dye), the First Strand Master Mix solution was first prepared for each dye (see Protocol #3 for master mix components).
- 8.3** 26 μL of the First Strand Master Mix was added to each aRNA obtained from Step 6 and incubated at 27°C for 10 minutes, followed by 37°C for 2 hours.
- 8.4** 2 units of RNase H were added to each reaction tube and incubated for 20 minutes at 37°C.
- 8.5** Cy3- and Cy5-labeled cDNA targets (50 μL each) were combined into one tube. In this experiment Cy3- and Cy5-labeled cDNA targets were prepared as follows:

| Experiments            | Cy3 labeled aRNA | Cy5 labeled aRNA                     | Replicates |
|------------------------|------------------|--------------------------------------|------------|
| HFA/GF comparison      | GF mouse         | HFA mouse                            | 3          |
| HFA-TC/HFA comparison  | HFA mouse        | HFA mouse treated with tetracycline  | 3          |
| HFA-Cip/HFA comparison | HFA mouse        | HFA mouse treated with ciprofloxacin | 3          |

| Group           | Animal No. | Number of cells captured | Absorbance |        |             | Dilution Factor | aRNA Quantity (ng/μL) | Total amount of aRNA (μg) |
|-----------------|------------|--------------------------|------------|--------|-------------|-----------------|-----------------------|---------------------------|
|                 |            |                          | A260       | A280   | A260 / A280 |                 |                       |                           |
| GF              | 1          | 100-200                  | 1.4852     | 0.5729 | 2.59        | 10              | 594.08                | 17.82                     |
|                 | 2          | 100-200                  | 1.8775     | 0.7668 | 2.45        | 10              | 751.00                | 22.53                     |
|                 | 3          | 100-200                  | 1.8383     | 0.7423 | 2.48        | 10              | 735.32                | 22.06                     |
| HFA             | 4          | 100-200                  | 1.7735     | 0.7087 | 2.50        | 10              | 709.40                | 21.28                     |
|                 | 5          | 100-200                  | 1.8433     | 0.7236 | 2.55        | 10              | 737.32                | 22.12                     |
|                 | 6          | 100-200                  | 1.8226     | 0.7229 | 2.52        | 10              | 729.04                | 21.87                     |
| TC-treated HFA  | 7          | 100-200                  | 1.6916     | 0.6696 | 2.53        | 10              | 676.64                | 20.30                     |
|                 | 8          | 100-200                  | 1.8335     | 0.7258 | 2.53        | 10              | 733.40                | 22.00                     |
|                 | 9          | 100-200                  | 1.8388     | 0.7335 | 2.51        | 10              | 735.52                | 22.07                     |
| Cip-treated HFA | 10         | 100-200                  | 1.6035     | 0.5920 | 2.71        | 10              | 641.40                | 19.24                     |
|                 | 11         | 100-200                  | 1.6011     | 0.6193 | 2.59        | 10              | 640.44                | 19.21                     |
|                 | 12         | 100-200                  | 1.8961     | 0.7535 | 2.52        | 10              | 758.44                | 22.75                     |

**Table 1.** aRNA quantity after amplification of RNA collected from LCM captured colonic crypt cells. The values represent the range of aRNA yields obtained from all samples.



**Figure 2.** Bioanalyzer profiles of aRNA samples from colonic crypt cells. A. GF mouse, B. HFA mouse, C. HFA-TC mouse, D. HFA-Cip mouse. All anti-sense aRNA ranged 300-500 bp and no 28s or 18S peaks were visible.

**8.6** The Cy3- and Cy5-labeled cDNA targets were purified using QiaQuick PCR Purification Kit, following the manufacturer's recommended protocol, as detailed below:

- The columns were pre-treated by incubating with 100  $\mu$ L of QiaQuick PB buffer for 5 minutes, and then centrifuging at 13200 rpm for 1 minute.
- 210  $\mu$ L of QiaQuick PB buffer was added to the sample tube, mixed and then loaded onto the pre-treated columns.
- The columns containing the samples were washed with 750  $\mu$ L QiaQuick PE buffer by centrifugation at 13200 rpm for 1 minute. Flowthrough was discarded and the tubes were centrifuged for an additional 2 minutes at 13200 rpm to remove residual wash solution.
- The columns were placed into new 2-mL microcentrifuge tubes.
- 50  $\mu$ L of nuclease-free water, pH 8.5, was added directly onto the column membrane and incubated for 5 minutes, and then centrifuged at maximum speed for 1 minute to elute the sample.
- The elution step was repeated with 30  $\mu$ L of nuclease-free water, pH 8.5.
- A total of 80  $\mu$ L of combined cDNA targets were prepared.

## **9. Hybridization of combined Cy3- and Cy5-labeled samples to DNA micro array chip**

- 9.1** The combined cDNA targets (80  $\mu$ L) were heat denatured for 3 minutes at 98°C and then cooled to room temperature. Hybridization reactions were performed for each sample and triplicate hybridization experiments were accomplished per each group.
- 9.2** 25  $\mu$ L of 10X control targets and 105  $\mu$ L 2X hybridization buffer (Agilent In Situ Hybridization Kit) were added to the 80  $\mu$ L combined cDNA targets.
- 9.3** For the hybridization of the combined cDNA targets to the TwinChip™ Mouse-7.4 K cDNA array (Digital

Genomics, Seoul, Korea), a clean Gasket slide was loaded into the hybridization chamber base and then slowly the entire amount (~200  $\mu$ L) of hybridization solution was drawn up into one of two block wells. More hybridization solution was drawn up into another separate block well of the twin Gasket slide.

- 9.4** The TwinChip™ Mouse-7.4 K cDNA array slides were carefully placed, facing the Gasket slides to complete the sandwiched slide pairs.
- 9.5** The chamber assemblies were placed into the hybridization rotator rack with the rotator speed set at 4 rpm. The slides were then incubated at 60°C for 17 hours in hybridization oven.
- 9.6** After incubation, the hybridization chamber was disassembled and the Gasket slides were removed by immersing the slides in 2X SSC/0.1% SDS at 42°C for 5 minutes.
- 9.7** The slides were transferred to 0.1X SSC/0.1% SDS and incubated for 10 minutes at room temperature.
- 9.8** The slides were transferred to 0.1X SSC and incubated for 1 minute at room temperature. This final wash was repeated four times.
- 9.9** The slides were dried by centrifugation at 650 rpm for 5 minutes.

## **10. Image scanning and analyzing**

- 10.1** The hybridized cDNA arrays were scanned using the Affymetrix array scanner and the fluorescence intensity was analyzed using a data analysis program (Image 5.6).
- 10.2** The values were normalized by global normalization and the ratios of Cy3 and Cy5 signal intensity were calculated.
- 10.3** Gene expression profiles in colonic crypt cells were compared between GF and HFA mouse and between HFA and antimicrobial-treated HFA mouse (Figure 3).
- 10.4** GeneSpring GX1 (Version 7.2, Agilent Tech.) was used to determine confidence in array calls. Hierarchical clustering analysis was performed between treatment sets using genes expressing

more than two fold differences in at least one assay among triplicate assays. Genes showing missing data in more than 50% of the assays were rejected. Differential gene expression was tested for statistical significance ( $p \leq 0.05$ ).

## **11. Confirmation of microarray data with quantitative RT-PCR analysis**

**11.1** Optimal primer sets (forward and reverse primers) were designed for selected genes that represented a more than two-fold difference in gene expression.

**11.2** cDNA was synthesized by mixing iScript Reaction Mix and reverse transcriptase (BioRad, Hercules, CA) with 2  $\mu$ L of purified RNA (obtained at step 5). Complete reaction mixtures were incubated according to the following conditions: 25°C for 5 minutes 42°C for 30 minutes 85°C for 5 minutes Held at 4°C or stored at -80°C until ready for real-time PCR

**11.3** Real-time PCR was performed in triplicate for each by mixing 1  $\mu$ L of synthesized cDNA from each sample with 12.5  $\mu$ L of iQ SYBR Green Supermix, 0.5  $\mu$ L each of forward and reverse primer (10 pmol/ $\mu$ L) and 10.5  $\mu$ L of sterile water. Repeat cycle number was set to 40.

**11.4** The cycle number reaching threshold for each sample after normalization was compared with that of the internal control gene (Rp119). Rp119 was chosen as the internal control because its expression level showed no change between treatment groups in QRT-PCR assays (**Figure 4**).

## **RESULTS**

We colonized germ free mice with normal human gut flora and induced disruption of the colonization by the injection of antimicrobials. We analyzed gene expression in colonic crypt cells using cDNA microarray chips in response to intestinal flora colonization as well as in response to the disruption of this colonization due to the induction of antimicrobials.

RNA amplification yielded 18 - 20  $\mu$ g aRNA from 100-200 colonic crypt epithelial cells, after two rounds of amplification with RiboAmp HS RNA amplification kit. Fifty-one genes relating to cell proliferation and cycling (Tegt, Tnfrsf11b, Rpa1, Rpl27, Ccnd2, etc.), immune response (Ilgj, Saa3, Daf1, etc.) and solute carrier (Sprr2a, Slc26a1, Mpp1, Ddx1, etc.) were induced, while 58 genes including Eif2ak, Dap3, Casp14, Ang, Il6, Mt-1, Cxcl7, etc. were re-duced by human flora colonization, among 7,400 mouse genes selected on microarray (Table 2). Differential gene expression was observed between HFA mice with and without antimicrobial injection.

The gene expression pattern observed from cDNA microarray chip analysis of the colonic crypt cell cDNA was confirmed by QRT-PCR showing high correlation between the results ( $r=0.856$ ) (Figure 5).

## **DISCUSSION**

Colonic epithelial cells originate from crypt cells and are composed of villi and crypt epithelial cells. The crypt epithelial cells react sensitively to gut flora for crypt cells, possess high proliferation potential and maintain homeostasis of intestinal epithelia. Gut flora build up commensalisms with the host animal, offering a barrier of protection and fermented nutrients to the host.

In the present study, colonic epithelial cells of antimicrobial (tetracycline and ciprofloxacin)-treated HFA mice exhibited gene expression patterns similar to each other and to germ-free mice, when compared to HFA mice. Ninety-two genes including Tegt, Rpa1 and Ccnd of germ-free and antimicrobial-treated HFA mice demonstrated significant differential expression from those of HFA mice. Real-time quantitative RT-PCR analysis confirmed the findings observed in cDNA array expression analysis with Pearson's correlation coefficient ( $r$ ) of 0.856.

Genes that induce cell proliferation such as *Tegt*, *Rpa1*, *Ccnd2* and *Rpl27* were induced when the human flora formed a colonization barrier on the colonic epithelium (HFA mice), while genes inducing cell death, or apoptosis, and immune reaction, such as *Dap3*, *Casp14*, *Gmnn*, *Il6* and *Tnfsf13b* were repressed under the same conditions. In addition, solute and nutrient carrier genes, such as *Slc26a1*, *Ddx1*, *Sprr2a* and *Slc22a11* were induced by human flora. Those results represent that human flora stimulate cell proliferation and solute or nutrient carrier genes. Further, these function stimulated by gut flora are inhibited by antimicrobials through the disruption of intestinal flora. It can be inferred that the genes sensitive to gut flora can be used to understand the relationship of microflora and host intestine and gastro-intestinal toxicity of chemicals that disrupt intestinal microflora.

The combined use of technologies in this application; laser captured microdissection for obtaining a specific region of tissue, RNA amplification, QRT-PCR and DNA microarray analysis, make it possible to understand the impact of human intestinal flora and oral antimicrobials on host animal health.

## REFERENCES

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7. Protocol #3. Generating Cy3 or Cy5 labeled cDNA probe from amplified aRNA for microarray hybridizations using a direct incorporation method.

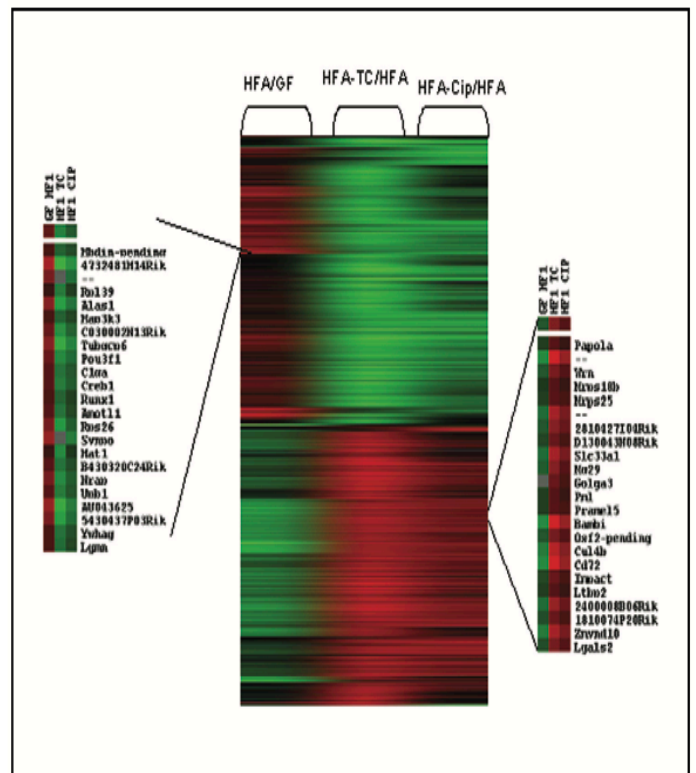


Figure 3. Gene expression profiles in colonic epithelial cells of GF, HFA, tetaracycline treated HFA (HFA-TC) and ciprofloxacin-treated HFA (HFA-Cip) mice.



| Group                         | Gene      | Fold induction*<br>(Global M, 2 <sup>0</sup> ) |               |                 | Gene function   |
|-------------------------------|-----------|--|---------------|-----------------|---|
|                               |           | HFA/GF   | HFA-TC<br>HFA | HFA-Cip/<br>HFA |   |
| Cell proliferation or cycling | Tegf      | 1.30   | -2.17         | -2.45           | Testis enhanced gene transcript                       |
|                               | Tnfrsf11b | 0.91   | -1.63         | -1.16           | Tumor necrosis factor (anti-apoptosis)                |
|                               | Rpa1      | 1.28   | -0.97         | -0.58           | Replication protein A1                                |
|                               | Rpl27     | 1.08   | -2.24         | -1.29           | Ribosomal protein L27                                 |
|                               | Cond2     | 1.13   | -1.82         | -1.19           | Cyclin D2   |
|                               | Calm2     | 0.90   | -1.85         | -1.46           | Calmodulin 2  |
|                               | Trp63     | 1.18   | -1.11         | -0.13           | Transformation related protein 63                     |
|                               | Brip31    | 1.02   | -1.54         | -1.57           | BCL2/adenovirus E1B 19kDa-interacting protein 3-like  |
|                               | Elf2ak3   | -1.27  | 1.73          | 1.42            | Eukaryotic translation initiation factor              |
|                               | Cdc451    | -1.39  | 1.56          | 1.42            | Cell division cycle 45 homolog-like                   |
| Cell death or apoptosis       | Dap3      | -0.93  | 1.55          | 1.32            | Death associated protein 3                            |
|                               | Casp14    | -1.23  | 1.69          | 1.40            | Caspase14   |
|                               | Gmnn      | -1.53  | 1.43          | 1.31            | Geminin (inhibitor of DNA replication)                |
|                               | Ang       | -0.55  | 1.49          | 1.49            | Angiogenin (inhibitor of neovascularization)          |
| Immune response               | Igj       | 2.36   | -2.96         | -4.40           | Immunoglobulin joining chain                          |
|                               | Saa3      | 1.95   | -2.62         | -2.23           | Serum amyloid A3                                      |
|                               | Daf1      | 1.74   | -1.57         | -1.20           | Decay accelerating factor 1                           |
|                               | Tnfrsf13b | -1.44  | 1.49          | 1.29            | Tumor necrosis factor                                 |
|                               | Cxc17     | -1.79  | 1.67          | 1.59            | Chemokine (C-X-C motif) ligand 7                      |
|                               | Il6       | -1.85  | 1.71          | 1.53            | Interleukin 6   |
| Solute Carrier                | Slc26a1   | 2.00   | -2.46         | -2.69           | Solute carrier family 26 (sulfate transporter)        |
|                               | Mpp1      | 1.73   | -1.94         | -2.17           | Membrane protein palmitoylated mRNA                   |
|                               | Ddx1      | 1.54   | -2.64         | -2.17           | (Asp-Glu-Ala-Asp) dox polypeptide 1                   |
|                               | Spr2a     | 1.47   | -3.37         | -2.36           | Small proline-rich protein 2A                         |
|                               | Caacb3    | 2.43   | -1.40         | -1.33           | Ca channel, voltage-dependent, beta3                  |
|                               | Slc22a11  | 1.14   | -2.24         | -2.12           | Solute carrier family 22 (organic cation transporter) |
|                               | Mt-1      | -1.65  | 2.18          | 2.05            | Metallothionein 1                                     |

Table 2. DNA chip analysis of gene expression in colonic crypt cells of GF, HFA, HFA-TC and HFA-Cip mice. \* Fold change statistical significance for all samples at  $\leq 0.05$ .

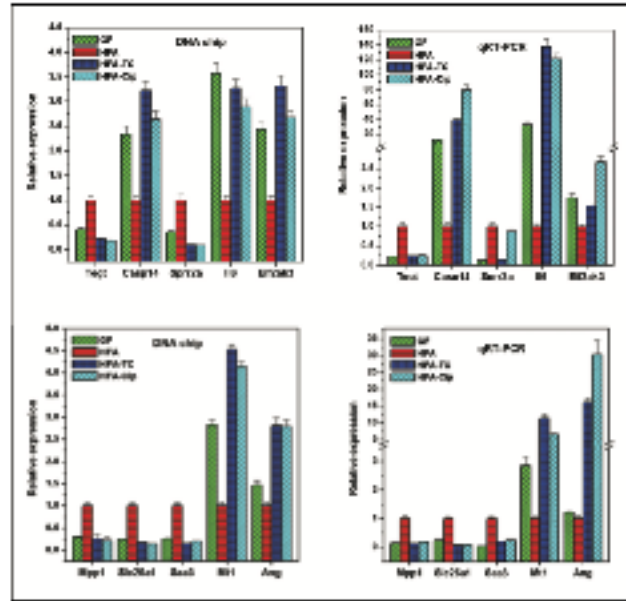


Figure 4. Comparison of microarray and RT-PCR analysis for 10 selected genes. Microarray data are global normalized value and mean±SE of three hybridizations. RT-PCR was performed in triplicate and the results were normalized with internal control gene (Rpl19) of the same sample.

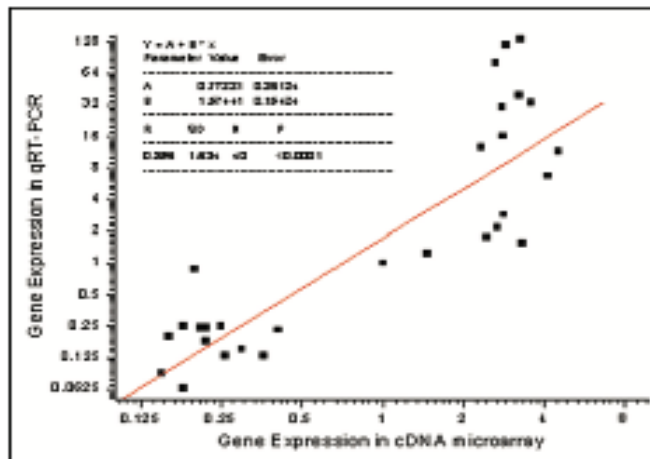


Figure 5. Correlation of gene expression patterns analyzed by DNA microarray and real-time RT-PCR for 10 selected genes in colonic crypt cells of GF, HFA, HFA-antimicrobials mice. Gene expression data are normalized by global normalization in DNA microarray and with control gene (Rpl19) in RT-PCR analysis.



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