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Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome

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Schumann, Alexandra, Sophie Nutten, Dominique Donnicola, Elena M Comelli, Robert Mansourian, Christine Cherbut, Irène Corthesy-Theulaz, and Clara Garcia-Rodenas. Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome. *Physiol Genomics* 23: 235–245, 2005. First published August 30, 2005; doi:10.1152/physiolgenomics.00057.2005.—The postnatal maturation of the gut, partially modulated by bacterial colonization, ends up in the establishment of an efficient barrier to luminal antigens and bacteria. The use of broad-spectrum antibiotics in pediatric practices alters the gut bacterial colonization and, consequently, may impair the maturation of the gut barrier function. To test this hypothesis, suckling Sprague-Dawley rats received a daily intragastric gavage of antibiotic (Clamoxyl; an amoxicillin-based commercial preparation) or saline solution from postnatal *day 7* (d7) until d17 or d21. Luminal microbiota composition and global gene expression profile were analyzed on samples from small intestine and colon of each group. The treatment with Clamoxyl resulted in the almost-complete eradication of *Lactobacillus* in the whole intestine and in a drastic reduction of colonic total aerobic and anaerobic bacteria, in particular *Enterobacteriacae* and *Enterococcus*. The global gene expression analysis revealed that Clamoxyl affects the maturation process of 249 and 149 Affymetrix probe sets in the proximal and distal small intestine, respectively, and 163 probe sets in the colon. The expression of genes coding for Paneth cell products (defensins, matrilysin, and phospholipase A2) was significantly downregulated by the Clamoxyl treatment. A significant downregulation of major histocompatibility complex (MHC) class Ib and II genes, involved in antigen presentation, was also observed. Conversely, mast cell proteases expression was upregulated. These results suggest that early treatment with a large-spectrum antibiotic deeply affects the gut barrier function at the suckling-weaning interface, a period during which the gut is challenged by an array of novel food-borne antigens.

innate immunity; antigen presentation; mast cell

THE GASTROINTESTINAL (GI) mucosa is in constant contact with a luminal environment that contains not only nutrients but also a huge array of potentially harmful microorganisms and toxins. A healthy GI mucosa is able to handle this massive collection of antigens and modulate the immune response according to the level of hazard related to each antigen. This capacity of the GI tract is defined as gut barrier function. In infants, the intestine remains immature and relatively permeable to antigens during the first weeks of life (3, 18, 50). Subsequently, the postnatal maturation of the mucosal barrier reduces drastically such transfer, so that in healthy adults, only small amounts of dietary antigens can reach the circulation (22). The maturation of the intestinal barrier is the consequence of morphological and functional changes of the mucosa, which occur under genetic and endocrine control (6). However, the onset of weaning and the impact of bacterial colonization have been shown to modulate this process (11, 28). The comparison of germ-free vs. conventional microbiota animals has brought interesting clues to further our understanding of the impact of the gut microbiota on the different mechanisms involved in the gut barrier function. In particular, the absence of intestinal microbiota results in decreased mucus layer thickness (56), lower globet cell numbers (32), decreased motility (30), and decreased numbers of IgA-producing cells (40). In addition, the antigen presentation capacity of the intestinal mucosa appears to be affected by the gut microbiota, since germ-free animals do not express major histocompatibility complex (MHC) class II proteins, molecules that are responsible for the presentation of antigenic material to the immune T cells sitting in the lamina propria (54).

The effect of intestinal microbiota perturbations, such as those produced by antibiotic treatment, on the intestinal barrier has been less extensively explored. Therapy with broad-spectrum antibiotics is frequently observed in pediatric practices (43, 49, 51), children within their first year of life being particularly affected (49). One major consequence of such early antibiotherapy is the alteration of the normal colonization process by the gut microbiota. Neonatal antibiotic treatment has been shown to reduce the biodiversity of the fecal microbiota, to delay the colonization by beneficial species such as *Bifidobacteria* or *Lactobacilli*, and to induce colonization by antibiotic-resistant or opportunistic strains (2, 7). The impact of such aberrant colonization on the maturation of the intestinal barrier, particularly in terms of host defense and presentation of the luminal antigens to the immune system, is still unknown.

We postulated that the disturbances created by antibiotic administration in early life would affect the postnatal maturation process of the intestine and impair antigen uptake, handling, and/or presentation by the gut at weaning. To assess our hypothesis, we treated suckling rat pups with a commercial preparation of amoxicillin widely employed in pediatric practices (Clamoxyl) and analyzed at the suckling-weaning interface the effect of the treatment on the global gene expression profile in the small intestine and colon.

We observed that several genes related to host defense and antigen presentation were downregulated after the antibiotic treatment, whereas genes related to mast cell activation were upregulated. These results suggest that early antibiotic treatment may disrupt the normal process of antigen presentation

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and handling in a period that is related to a massive penetration of novel food antigens in the gut.

MATERIALS AND METHODS

Animals

Adult male and female Sprague-Dawley rats were obtained from Iffa Credo (Charles River, Lyon, France) and were acclimatized to our internal facility for 2 wk before mating. The pups were born and housed under conventional conditions and were breast-fed until death. All the protocols were approved by the Ethical Committee of the Canton de Vaud (Switzerland).

Experimental Design

At postnatal *day 6* (d6), rat pups were weighed and randomly assigned for fostering to standardized litters of 10 pups. To avoid a dam-related bias, each experimental group was composed of animals from different litters. Pups received by intragastric gavage a daily dose of 1 ml/100 g body wt of either Clamoxyl solution (Clamoxyl, an amoxicillin-based antibiotic preparation; SmithKline Beecham Pharmaceuticals, Toledo, Spain) (10 mg/ml; Clam group, $n = 33$) or saline (Cont group, $n = 33$) from d7 until the day of death. Eleven animals from each group were killed at d15, d17, or d21 by exhaustive bleeding after anesthesia with isoflurane (3.5%). The small intestine and the colon were removed and kept on ice. The small intestine was divided into two parts of identical length, namely proximal and distal parts. After the luminal contents from each of the three segments were flushed with 2 ml of ice-cold sterile saline solution, centered 1-cm-long sections were excised, snap-frozen and stored at −80°C until RNA extraction.

Bacterial Counts of Small Intestine and Colonic Luminal Contents

Fresh luminal contents from the three intestinal segments ($n = 11$/group) were processed within 30 min after collection for the enumeration of the endogenous populations of total aerobic and total anaerobic bacteria and *Lactobacillus* in small intestine and colon, as well as *Enterobacteriaceae* and *Enterococcus* populations in colon. Bacteria were detected on selective or semiselective media. One hundred-fold serial dilutions were performed in prereduced Ringer solution containing 0.5% cysteine. One hundred microliters of diluted samples were plated on Man, Rogosa, and Sharpe (MRS) medium (Difco, Detroit, MI) for *Lactobacillus* and trypticase soy agar plus 5% sheep blood (TSS) medium (Biomerieux SA, Lyon, France) for total anaerobes. The plates were incubated anaerobically at 37°C for 48 h. The anaerobic atmosphere was obtained using Anaerocult A (Merck, Darmstadt, Germany). Dilutions were also applied on bile esculin agar (BEA) (Fluka, Buchs, Switzerland) for *Enterococcus*, Drigalski medium (Bio-Rad, Marnes la Coquette, France) for *Enterobacteriaceae,* and TSS (Biomerieux) for total aerobes. These plates were incubated aerobically at 37°C for 24 h. Bacterial concentration is expressed as colony forming units (cfu) per gram of intestinal contents.

Microarray Analysis of Gene Expression

Global gene expression analysis was performed on the proximal and distal small intestine and on the colon of six rats per group (Clam and Cont) killed at d17 and d21, leading to three biological replicates for each condition. Total RNA was extracted from each sample independently, as previously described (12), and then DNase I treated and purified using the Nucleospin II kit (Machery-Nagel AG, Oensingen, Switzerland) according to the manufacturer's instructions. Sample monitoring by the Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Rotkreuz, Germany) consistently demonstrated high-quality RNA (28S-to-18S ratio from 1.9 to 2.2 in proximal small intestine and from 1.5 to 1.9 in distal small intestine and colon) for all samples but one (colon, Cont group, d17), which was excluded from further analysis. First- and second-strand cDNA syntheses were performed using the SuperScript double-stranded cDNA synthesis kit (Invitrogen, Basel, Switzerland), using an oligo(dT) primer containing T7 RNA polymerase binding site. Labeled cRNA was prepared with the RNA transcript labeling kit (Enzo Biochem). Twenty micrograms of cRNA were fragmented and hybridized to the Affymetrix RAE230A probe array cartridge (Affymetrix, High Wycombe, UK), which contains 15,866 probe sets, following the manufacturer's instructions. Each sample was hybridized to one array, leading to a total of 35 chips used. The arrays were scanned at 488 nm using an Argon-ion laser (Agilent), and hybridization signals were generated with Affymetrix MicroArray Suite 5.0 (MAS 5.0). The entire data set is publicly available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (**http://www.ncbi.nlm.nih.gov/geo/**) through the accession number GSE2354. Differentially expressed genes were identified using the Global Error Assessment (GEA) method (37) at a test rejection level of 0.001. This method consists in first calculating the within-group mean square error (MSE) of the four studied groups for each probe set. Because of the small sample size, the MSE of a given gene is replaced by the median MSE of 201 genes with similar level of expression. This statistic was used for all statistical analyses of the microarray data. The data sets corresponding to the three intestinal parts were treated independently. To determine the effect of Clamoxyl at each age, we compared, using a Fisher's least significant differences (LSD) *t*-test, the control and antibiotic groups at d17 and d21. The effect of Clamoxyl on the genes changing with the maturation was analyzed by two consecutive LSD *t*-tests: the first one allowed the selection of a subset of the genes differentially expressed between d17 and d21 in the control group. The second *t*-test selected, within these genes, those showing a significant interaction between age and antibiotic treatment (i.e., by comparing the difference between d21 and d17 in the controls vs. antibiotic group); a comparison-wise error rate of 0.0005 was employed as a cutoff in both tests to obtain an experiment-wise error rate of 0.001. Results were visualized using the Eisen's software (19). Selected probe sets were annotated using NetAffx (**http://www.affymetrix.com/analysis/index.affx**). When the same gene was represented by more than one probe set, only one was selected based on the following criteria: *1*) unique chromosomal localization (**http://www.ensembl.org/**) and *2*) specificity of hybridization, as indicated by the probe set suffix (Affymetrix GeneChip Rat Expression Set 230 technical note). To identify the biological processes modulated by the antibiotic, the selected probe sets were clustered using the Biological Process Gene Ontology (GO) database (**http://www.geneontology.org**) as previously described (41), but using the NetAffx instead of the Ensembl annotation. Indeed, only 13% of the probe sets contained in the RAE230A chip have an Ensembl (v29 release of this database) annotation compared with 32% in NetAffx. Further searches with Hammer (17) against Pfam (**http://www.sanger.ac.uk/Software/Pfam/**) and with PSI Blast (1) for murine and human homologs did not yield any additional information.

Taqman Real-Time PCR Analysis of Matrilysin and Mast Cell Protease 4

RNA was prepared as described above from the proximal and distal small intestine and from the colon of 11 rats per group (Clam and Cont) at each age (d17 and d21), including the 3 rats that had been used in the microarray analysis. Two micrograms of total RNA from each sample were reverse transcribed with the first-strand cDNA synthesis kit for RT-PCR (Catalys, Wallisellen, Switzerland) using an oligo(dT)$_{15}$ primer. Ribonucleoprotein F was used as a housekeeping

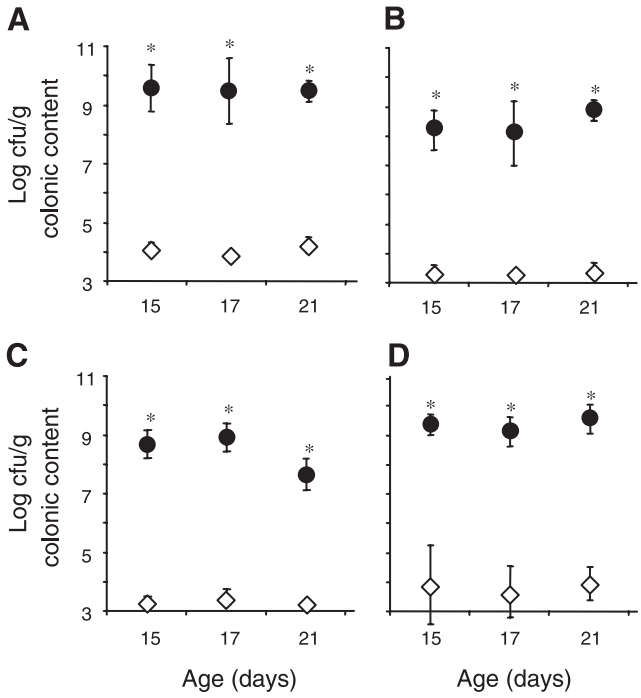


Fig. 1. Live bacteria concentrations in the colonic contents of Clamoxyl-treated (Clam; ◇) and control (Cont; ●) groups at postnatal *day 15* (d15), d17, and d21. Data are expressed as $\log_{10}$ colony forming units (cfu)/g colonic content (means ± SD, $n = 11$). *A*: total aerobic bacteria. *B*: *Lactobacillus*. *C*: *Enterococcus*. *D*: *Enterobacteriaceae*. *Significant difference between Clam and Cont groups ($P < 0.05$). Detection limit = 3.3 log cfu/g luminal content.

gene, as the microarray data showed this gene not to be affected by the age or the treatment. The primers and TaqMan probe sets for matrilysin, mast cell protease 4, and ribonucleoprotein F were synthesized by Applied Biosystems (Foster City, CA) through their Assays-on-Demand service (assays Rn00563467_m1, Rn00820964_g1, and Rn00821053_g1, respectively). Real-time PCR amplification was then performed using an ABI 7900HT Thermocycle (Applied Biosystems) with the following conditions: 2 min at 50°C and 10 min at 95°C for the initial set-up, followed by 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and elongation). All the samples were analyzed in triplicate. Cycle-to-cycle fluorescence emission was monitored and quantified using the GeneAmp SDS 2.1 software (Applied Biosystems). Relative gene expression was calculated after normalization to ribonucleoprotein F. Statistical analysis of gene expression differences was carried out as further explained.

Statistical Analysis of Microbiota and Real-Time PCR Analyses

When data followed normal distribution, analysis of variance (ANOVA) at a significance level of 5% was performed. LSD was subsequently calculated to determine the differences between pairs of samples. When data did not follow a normal distribution, nonparametric Kruskal-Wallis ANOVA on ranks and multiple-comparison *Z*-value test were performed. Differences were considered significant when $P < 0.05$. Results are expressed as means ± SD.

RESULTS

Clamoxyl Drastically Altered the Intestinal Microbiota Composition

The impact of Clamoxyl treatment on the process of intestinal colonization by the resident microbiota was monitored at the suckling-weaning interface by quantifying total aerobic and anaerobic living bacteria, as well as some of the predominant species in the suckling rat intestine, namely *Lactobacillus, Enterobacteriaceae*, and *Enterococcus* (16, 42). Figures 1 and 2 show, respectively, the concentration of the intestinal microbiota in the colon and small intestine of control (Cont) and Clamoxyl-treated (Clam) groups. In colon, total aerobic (Fig. 1*A*) and anaerobic (data not shown) counts were ~10^5 cfu/g lower in the Clam than in the Cont group at d15, d17, and d21 ($P < 0.05$). Similarly, colonic *Lactobacillus* (Fig. 1*B*), *Enterococcus* (Fig. 1*C*), and *Enterobacteriaceae* (Fig. 1*D*) were drastically depleted after the antibiotic administration. In the small intestine, total aerobic (Fig. 2*A*) and anaerobic (data not shown) populations were not significantly different between the Clam and Cont groups ($P > 0.1$). However, the *Lactobacillus* species were almost eradicated in the Clam group (Fig. 2*B*).

Clamoxyl Affected the Maturational Changes of Gene Transcription

A GeneArray analysis was performed on the proximal and distal small intestine and on the colon of Cont and Clam rats at d17 and d21 to determine how the Clamoxyl treatment affected the expression of genes during the maturation process. Figures 3–5 show the impact of the treatment on the age-related changes in gene expression. Probe sets whose expression was affected by age in the control group ($P < 0.0005$) are shown in the first column of the heat maps, in which the fold change between d21 and d17 defines the maturation-associated profile of each gene. The changes related to maturation were quantitatively more important in the small intestine, where 9 and 12% of the probe sets showed modulation in the proximal and distal parts, respectively (Figs. 3 and 4), than in the colon (3% of the probe sets; Fig. 5). The second column of the heat maps represents the age-related fold change for the selected genes in the antibiotic-treated animals and was used to calculate the interaction of age and treatment (i.e., third column of the heat maps). Among the genes affected by age in the control group, the proportion of probe sets in which the maturation profile was modulated by the Clamoxyl treatment was higher in the colon (34%) than in the small intestine (17% in the proximal part and

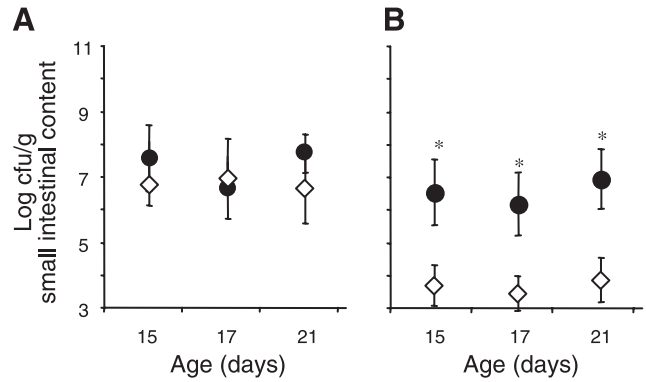
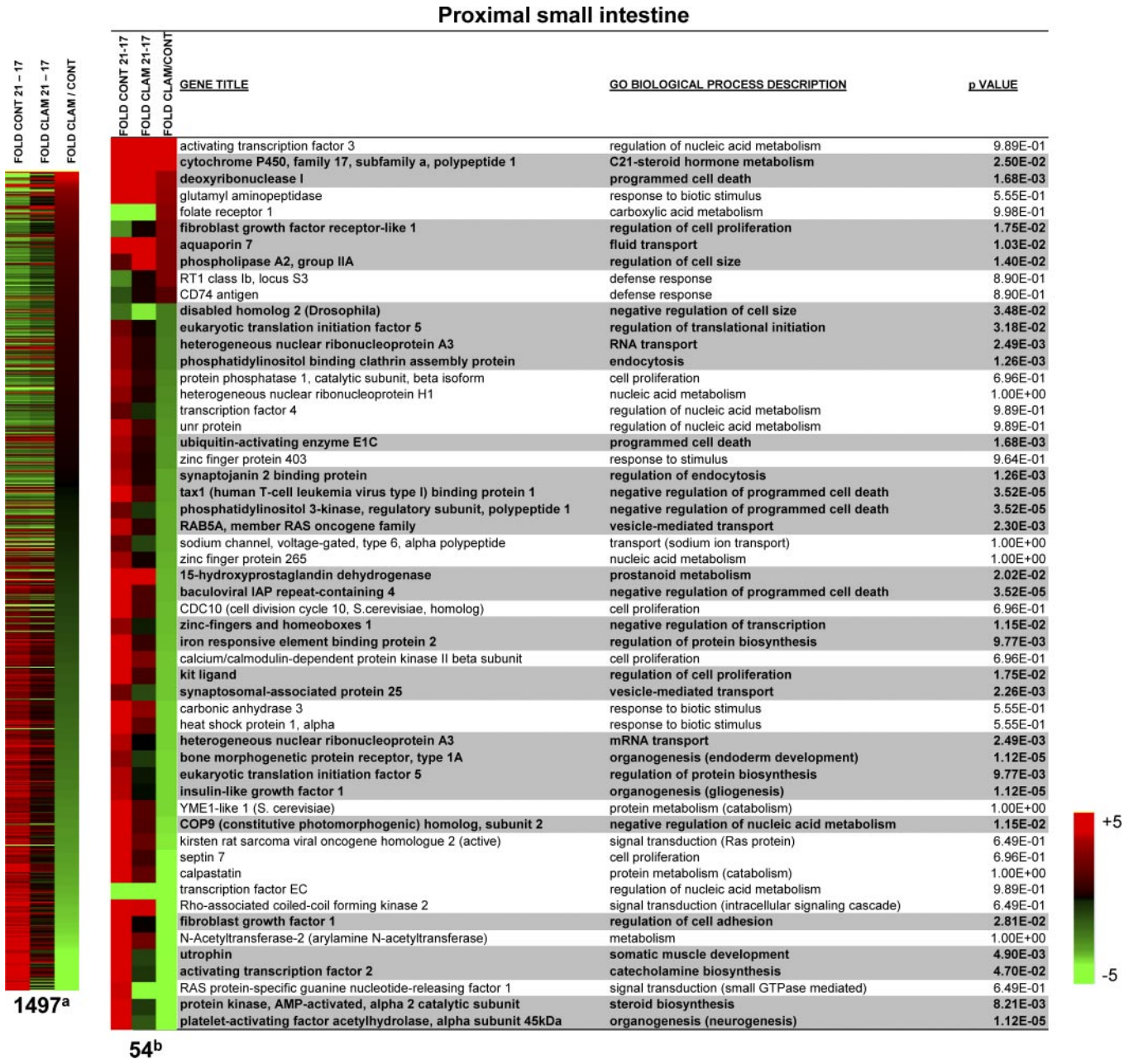


Fig. 2. Live bacteria concentrations in the small intestinal contents of Clam (◇) and Cont (●) groups at d15, d17, and d21. Data are expressed as $\log_{10}$ cfu/g luminal content (means ± SD, $n = 11$). *A*: total aerobic bacteria. *B*: *Lactobacillus*. *Significant difference between Clam and Cont groups ($P < 0.05$). Detection limit = 3.3 log cfu/g luminal content.

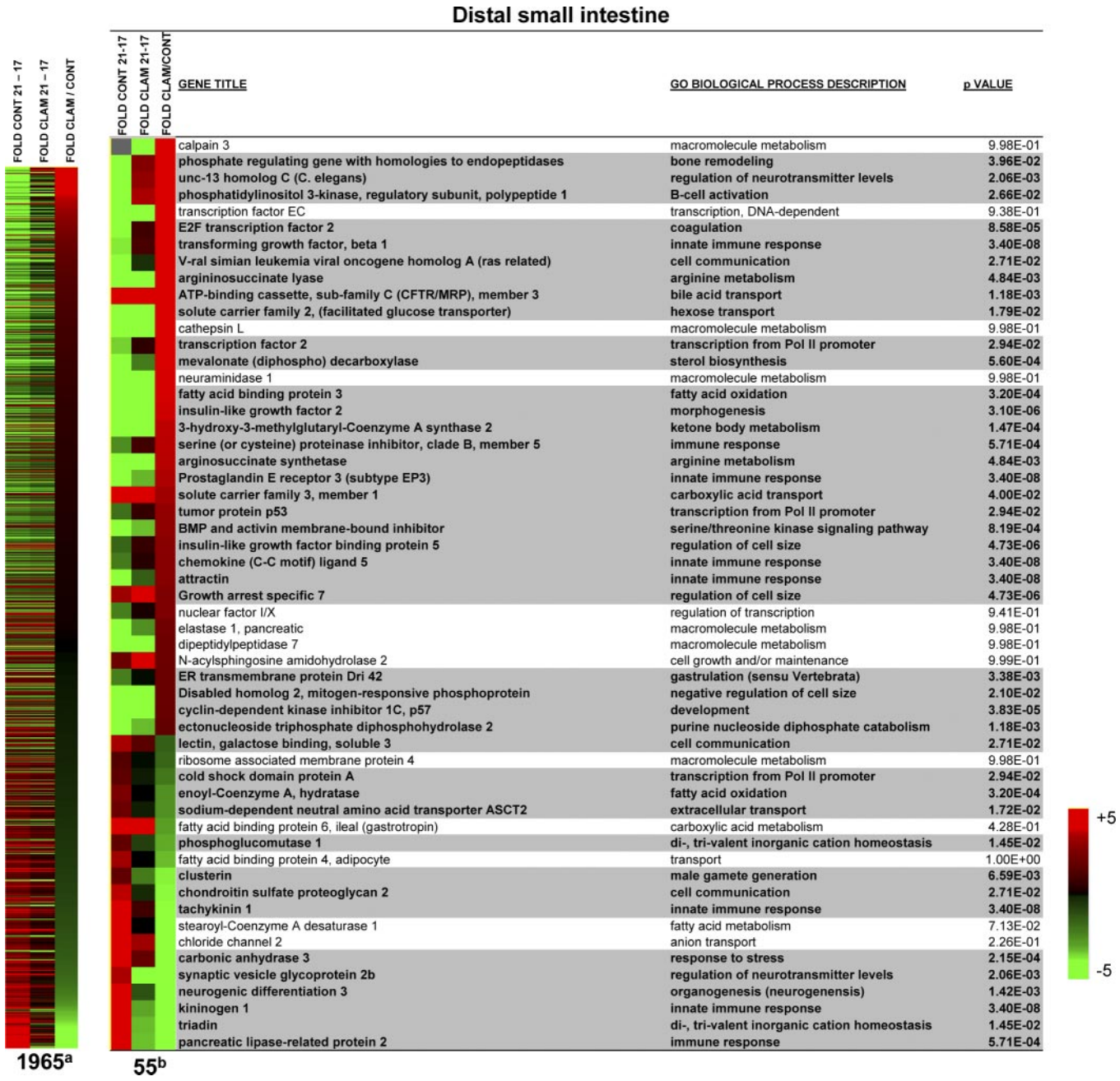


FOLD CONT 21-17	FOLD CLAM 21-17	FOLD CLAM/CONT	GENE TITLE	GO BIOLOGICAL PROCESS DESCRIPTION	p VALUE
			activating transcription factor 3	regulation of nucleic acid metabolism	9.89E-01
			cytochrome P450, family 17, subfamily a, polypeptide 1	**C21-steroid hormone metabolism**	**2.50E-02**
			deoxyribonuclease I	**programmed cell death**	**1.68E-03**
			glutamyl aminopeptidase	response to biotic stimulus	5.55E-01
			folate receptor 1	carboxylic acid metabolism	9.98E-01
			fibroblast growth factor receptor-like 1	**regulation of cell proliferation**	**1.75E-02**
			aquaporin 7	**fluid transport**	**1.03E-02**
			phospholipase A2, group IIA	**regulation of cell size**	**1.40E-02**
			RT1 class Ib, locus S3	defense response	8.90E-01
			CD74 antigen	defense response	8.90E-01
			disabled homolog 2 (Drosophila)	**negative regulation of cell size**	**3.48E-02**
			eukaryotic translation initiation factor 5	**regulation of translational initiation**	**3.18E-02**
			heterogeneous nuclear ribonucleoprotein A3	**RNA transport**	**2.49E-03**
			phosphatidylinositol binding clathrin assembly protein	**endocytosis**	**1.26E-03**
			protein phosphatase 1, catalytic subunit, beta isoform	cell proliferation	6.96E-01
			heterogeneous nuclear ribonucleoprotein H1	nucleic acid metabolism	1.00E+00
			transcription factor 4	regulation of nucleic acid metabolism	9.89E-01
			unr protein	regulation of nucleic acid metabolism	9.89E-01
			ubiquitin-activating enzyme E1C	**programmed cell death**	**1.68E-03**
			zinc finger protein 403	response to stimulus	9.64E-01
			synaptojanin 2 binding protein	**regulation of endocytosis**	**1.26E-03**
			tax1 (human T-cell leukemia virus type I) binding protein 1	**negative regulation of programmed cell death**	**3.52E-05**
			phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	**negative regulation of programmed cell death**	**3.52E-05**
			RAB5A, member RAS oncogene family	**vesicle-mediated transport**	**2.30E-03**
			sodium channel, voltage-gated, type 6, alpha polypeptide	transport (sodium ion transport)	1.00E+00
			zinc finger protein 265	nucleic acid metabolism	1.00E+00
			15-hydroxyprostaglandin dehydrogenase	**prostanoid metabolism**	**2.02E-02**
			baculoviral IAP repeat-containing 4	**negative regulation of programmed cell death**	**3.52E-05**
			CDC10 (cell division cycle 10, S.cerevisiae, homolog)	cell proliferation	6.96E-01
			zinc-fingers and homeoboxes 1	**negative regulation of transcription**	**1.15E-02**
			iron responsive element binding protein 2	**regulation of protein biosynthesis**	**9.77E-03**
			calcium/calmodulin-dependent protein kinase II beta subunit	cell proliferation	6.96E-01
			kit ligand	**regulation of cell proliferation**	**1.75E-02**
			synaptosomal-associated protein 25	**vesicle-mediated transport**	**2.26E-03**
			carbonic anhydrase 3	response to biotic stimulus	5.55E-01
			heat shock protein 1, alpha	response to biotic stimulus	5.55E-01
			heterogeneous nuclear ribonucleoprotein A3	**mRNA transport**	**2.49E-03**
			bone morphogenetic protein receptor, type 1A	**organogenesis (endoderm development)**	**1.12E-05**
			eukaryotic translation initiation factor 5	**regulation of protein biosynthesis**	**9.77E-03**
			insulin-like growth factor 1	**organogenesis (gliogenesis)**	**1.12E-05**
			YME1-like 1 (S. cerevisiae)	protein metabolism (catabolism)	1.00E+00
			COP9 (constitutive photomorphogenic) homolog, subunit 2	**negative regulation of nucleic acid metabolism**	**1.15E-02**
			kirsten rat sarcoma viral oncogene homologue 2 (active)	signal transduction (Ras protein)	6.49E-01
			septin 7	cell proliferation	6.96E-01
			calpastatin	protein metabolism (catabolism)	1.00E+00
			transcription factor EC	regulation of nucleic acid metabolism	9.89E-01
			Rho-associated coiled-coil forming kinase 2	signal transduction (intracellular signaling cascade)	6.49E-01
			fibroblast growth factor 1	**regulation of cell adhesion**	**2.81E-02**
			N-Acetyltransferase-2 (arylamine N-acetyltransferase)	metabolism	1.00E+00
			utrophin	**somatic muscle development**	**4.90E-03**
			activating transcription factor 2	**catecholamine biosynthesis**	**4.70E-02**
			RAS protein-specific guanine nucleotide-releasing factor 1	signal transduction (small GTPase mediated)	6.49E-01
			protein kinase, AMP-activated, alpha 2 catalytic subunit	**steroid biosynthesis**	**8.21E-03**
			platelet-activating factor acetylhydrolase, alpha subunit 45kDa	**organogenesis (neurogenesis)**	**1.12E-05**

Fig. 3. Modulation of the age-related changes in gene expression by Clamoxyl in proximal small intestine. The subset of genes associated with significant ($P <$ 0.0005) age-dependent expression changes in the control group (Cont; superscript a) is represented in the first column of the heat maps by the fold change between expression levels at d21 and d17. The age-related expression changes for this subset of genes in the antibiotic-treated group (Clam) are given in the second column. The third column represents the interaction of age and treatment computed as the fold change of values in the second vs. the first column. The probe sets found to be significantly ($P < 0.0005$) modulated by the age-treatment interaction were clustered using the Biological Process Gene Ontology (GO) database at 0.05 test rejection level. The genes corresponding to the probe sets found to be associated with a GO term (superscript b) have been expanded in the second heat map, and their title (NetAffx) and deepest node GO biological process are detailed in the accompanying table. *P* values represent the level of significance of the modulation of each identified biological process. Red and green colors indicate up- and downregulation, respectively.

8% in the distal part). Their expression levels and annotation are given in Supplementary Table S1 (available at the *Physiological Genomics* web site).[1] When clustered using the Biological Process GO database, only 25, 38, and 48% of these probe sets in the proximal small intestine, distal small intestine, and colon, respectively, were assigned to a GO node. The expression profiles of the corresponding genes are expanded in the second heat map of Figs. 3–5. Concerning the gut barrier-related functions, Clamoxyl affected significantly the maturation of endocytosis and vesicle-mediated transport in the proximal small intestine, as well as the maturation of the immune function in both distal small intestine and colon.

[1] The Supplemental Material for this article (Supplemental Tables S1 and S2) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00057.2005/DC1**.



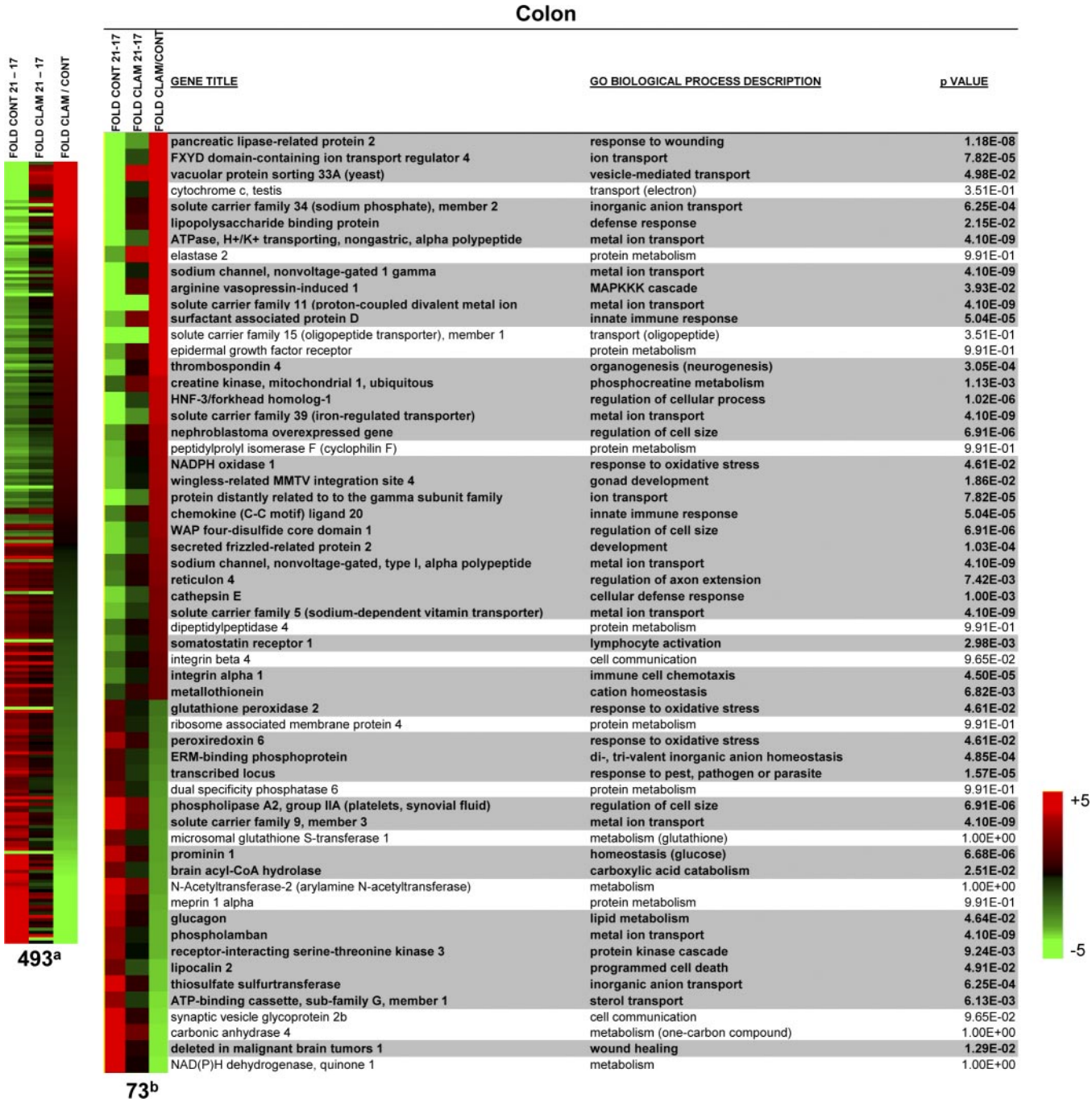
FOLD CONT 21-17	FOLD CLAM 21-17	FOLD CLAM/CONT	GENE TITLE	GO BIOLOGICAL PROCESS DESCRIPTION	p VALUE
			calpain 3	macromolecule metabolism	9.98E-01
			phosphate regulating gene with homologies to endopeptidases	bone remodeling	3.96E-02
			unc-13 homolog C (C. elegans)	regulation of neurotransmitter levels	2.06E-03
			phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	B-cell activation	2.66E-02
			transcription factor EC	transcription, DNA-dependent	9.38E-01
			E2F transcription factor 2	coagulation	8.58E-05
			transforming growth factor, beta 1	innate immune response	3.40E-08
			V-ral simian leukemia viral oncogene homolog A (ras related)	cell communication	2.71E-02
			argininosuccinate lyase	arginine metabolism	4.84E-03
			ATP-binding cassette, sub-family C (CFTR/MRP), member 3	bile acid transport	1.18E-03
			solute carrier family 2, (facilitated glucose transporter)	hexose transport	1.79E-02
			cathepsin L	macromolecule metabolism	9.98E-01
			transcription factor 2	transcription from Pol II promoter	2.94E-02
			mevalonate (diphospho) decarboxylase	sterol biosynthesis	5.60E-04
			neuraminidase 1	macromolecule metabolism	9.98E-01
			fatty acid binding protein 3	fatty acid oxidation	3.20E-04
			insulin-like growth factor 2	morphogenesis	3.10E-06
			3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	ketone body metabolism	1.47E-04
			serine (or cysteine) proteinase inhibitor, clade B, member 5	immune response	5.71E-04
			arginosuccinate synthetase	arginine metabolism	4.84E-03
			Prostaglandin E receptor 3 (subtype EP3)	innate immune response	3.40E-08
			solute carrier family 3, member 1	carboxylic acid transport	4.00E-02
			tumor protein p53	transcription from Pol II promoter	2.94E-02
			BMP and activin membrane-bound inhibitor	serine/threonine kinase signaling pathway	8.19E-04
			insulin-like growth factor binding protein 5	regulation of cell size	4.73E-06
			chemokine (C-C motif) ligand 5	innate immune response	3.40E-08
			attractin	innate immune response	3.40E-08
			Growth arrest specific 7	regulation of cell size	4.73E-06
			nuclear factor I/X	regulation of transcription	9.41E-01
			elastase 1, pancreatic	macromolecule metabolism	9.98E-01
			dipeptidylpeptidase 7	macromolecule metabolism	9.98E-01
			N-acylsphingosine amidohydrolase 2	cell growth and/or maintenance	9.99E-01
			ER transmembrane protein Dri 42	gastrulation (sensu Vertebrata)	3.38E-03
			Disabled homolog 2, mitogen-responsive phosphoprotein	negative regulation of cell size	2.10E-02
			cyclin-dependent kinase inhibitor 1C, p57	development	3.83E-05
			ectonucleoside triphosphate diphosphohydrolase 2	purine nucleoside diphosphate catabolism	1.18E-03
			lectin, galactose binding, soluble 3	cell communication	2.71E-02
			ribosome associated membrane protein 4	macromolecule metabolism	9.98E-01
			cold shock domain protein A	transcription from Pol II promoter	2.94E-02
			enoyl-Coenzyme A, hydratase	fatty acid oxidation	3.20E-04
			sodium-dependent neutral amino acid transporter ASCT2	extracellular transport	1.72E-02
			fatty acid binding protein 6, ileal (gastrotropin)	carboxylic acid metabolism	4.28E-01
			phosphoglucomutase 1	di-, tri-valent inorganic cation homeostasis	1.45E-02
			fatty acid binding protein 4, adipocyte	transport	1.00E+00
			clusterin	male gamete generation	6.59E-03
			chondroitin sulfate proteoglycan 2	cell communication	2.71E-02
			tachykinin 1	innate immune response	3.40E-08
			stearoyl-Coenzyme A desaturase 1	fatty acid metabolism	7.13E-02
			chloride channel 2	anion transport	2.26E-01
			carbonic anhydrase 3	response to stress	2.15E-04
			synaptic vesicle glycoprotein 2b	regulation of neurotransmitter levels	2.06E-03
			neurogenic differentiation 3	organogenesis (neurogenensis)	1.42E-03
			kininogen 1	innate immune response	3.40E-08
			triadin	di-, tri-valent inorganic cation homeostasis	1.45E-02
			pancreatic lipase-related protein 2	immune response	5.71E-04

Fig. 4. Modulation of the age-related changes in gene expression by Clamoxyl in distal small intestine. For details, see legend to Fig. 3.

Clamoxyl Differentially Affected Gene Expression All Along the Gut

The GeneArray analysis was also performed to determine how the antibiotic treatment affected the expression of genes along the intestine at the suckling-weaning interface. The complete list of the significantly modulated probe sets is provided in Supplementary Table S2. Figure 6 summarizes the quantitative effect of the antibiotic treatment on the different intestinal parts. Only a restrained number of probe sets, nine at d17 and seven at d21, were significantly affected in the three regions (Supplementary Table S2*A*). The amount of probe sets showing a common behavior in two intestinal parts ranged from 17 to 48 at d17 and from 22 to 43 at d21 (Supplementary Table S2, *B–D*). At d17, the number of probe sets significantly affected by the treatment was higher in the proximal small intestine (894) than in the distal small intestine (420) or in the large intestine (358). Conversely, at d21, Clamoxyl induced more changes in the large (509) and distal small intestine (377) than in the proximal small intestine (137).

Clamoxyl downregulated the expression of genes related to antigen presentation. Various genes related to antigen presentation, such as MHC class II and nonclassical class Ib, were downregulated with Clamoxyl treatment in the different parts of the intestine (Table 1). In particular, the expression of MHC



FOLD CONT 21-17	FOLD CLAM 21-17	FOLD CLAM/CONT	GENE TITLE	GO BIOLOGICAL PROCESS DESCRIPTION	p VALUE
			pancreatic lipase-related protein 2	response to wounding	1.18E-08
			FXYD domain-containing ion transport regulator 4	ion transport	7.82E-05
			vacuolar protein sorting 33A (yeast)	vesicle-mediated transport	4.98E-02
			cytochrome c, testis	transport (electron)	3.51E-01
			solute carrier family 34 (sodium phosphate), member 2	inorganic anion transport	6.25E-04
			lipopolysaccharide binding protein	defense response	2.15E-02
			ATPase, H+/K+ transporting, nongastric, alpha polypeptide	metal ion transport	4.10E-09
			elastase 2	protein metabolism	9.91E-01
			sodium channel, nonvoltage-gated 1 gamma	metal ion transport	4.10E-09
			arginine vasopressin-induced 1	MAPKKK cascade	3.93E-02
			solute carrier family 11 (proton-coupled divalent metal ion	metal ion transport	4.10E-09
			surfactant associated protein D	innate immune response	5.04E-05
			solute carrier family 15 (oligopeptide transporter), member 1	transport (oligopeptide)	3.51E-01
			epidermal growth factor receptor	protein metabolism	9.91E-01
			thrombospondin 4	organogenesis (neurogenesis)	3.05E-04
			creatine kinase, mitochondrial 1, ubiquitous	phosphocreatine metabolism	1.13E-03
			HNF-3/forkhead homolog-1	regulation of cellular process	1.02E-06
			solute carrier family 39 (iron-regulated transporter)	metal ion transport	4.10E-09
			nephroblastoma overexpressed gene	regulation of cell size	6.91E-06
			peptidylprolyl isomerase F (cyclophilin F)	protein metabolism	9.91E-01
			NADPH oxidase 1	response to oxidative stress	4.61E-02
			wingless-related MMTV integration site 4	gonad development	1.86E-02
			protein distantly related to to the gamma subunit family	ion transport	7.82E-05
			chemokine (C-C motif) ligand 20	innate immune response	5.04E-05
			WAP four-disulfide core domain 1	regulation of cell size	6.91E-06
			secreted frizzled-related protein 2	development	1.03E-04
			sodium channel, nonvoltage-gated, type I, alpha polypeptide	metal ion transport	4.10E-09
			reticulon 4	regulation of axon extension	7.42E-03
			cathepsin E	cellular defense response	1.00E-03
			solute carrier family 5 (sodium-dependent vitamin transporter)	metal ion transport	4.10E-09
			dipeptidylpeptidase 4	protein metabolism	9.91E-01
			somatostatin receptor 1	lymphocyte activation	2.98E-03
			integrin beta 4	cell communication	9.65E-02
			integrin alpha 1	immune cell chemotaxis	4.50E-05
			metallothionein	cation homeostasis	6.82E-03
			glutathione peroxidase 2	response to oxidative stress	4.61E-02
			ribosome associated membrane protein 4	protein metabolism	9.91E-01
			peroxiredoxin 6	response to oxidative stress	4.61E-02
			ERM-binding phosphoprotein	di-, tri-valent inorganic anion homeostasis	4.85E-04
			transcribed locus	response to pest, pathogen or parasite	1.57E-05
			dual specificity phosphatase 6	protein metabolism	9.91E-01
			phospholipase A2, group IIA (platelets, synovial fluid)	regulation of cell size	6.91E-06
			solute carrier family 9, member 3	metal ion transport	4.10E-09
			microsomal glutathione S-transferase 1	metabolism (glutathione)	1.00E+00
			prominin 1	homeostasis (glucose)	6.68E-06
			brain acyl-CoA hydrolase	carboxylic acid catabolism	2.51E-02
			N-Acetyltransferase-2 (arylamine N-acetyltransferase)	metabolism	1.00E+00
			meprin 1 alpha	protein metabolism	9.91E-01
			glucagon	lipid metabolism	4.64E-02
			phospholamban	metal ion transport	4.10E-09
			receptor-interacting serine-threonine kinase 3	protein kinase cascade	9.24E-03
			lipocalin 2	programmed cell death	4.91E-02
			thiosulfate sulfurtransferase	inorganic anion transport	6.25E-04
			ATP-binding cassette, sub-family G, member 1	sterol transport	6.13E-03
			synaptic vesicle glycoprotein 2b	cell communication	9.65E-02
			carbonic anhydrase 4	metabolism (one-carbon compound)	1.00E+00
			deleted in malignant brain tumors 1	wound healing	1.29E-02
			NAD(P)H dehydrogenase, quinone 1	metabolism	1.00E+00

Fig. 5. Modulation of the age-related changes in gene expression by Clamoxyl in colon. For details, see legend to Fig. 3.

class II RT1Bb chain was downregulated by nine-, four-, and threefold in the proximal and distal small intestine and in colon, respectively. In addition, the expression of CD1d, a gene associated with the presentation of lipid-containing antigens, was significantly downregulated in the proximal small intestine at d21.

Clamoxyl downregulated the expression of genes coding for anti-microbial products. Some genes associated with Paneth cell secretion products were differentially regulated with the Clamoxyl treatment, especially in proximal small intestine at d17 (Table 2). Therefore, the gene expression of several anti-microbial peptides such as defensins, lysozyme, and phospholipase A2 was downregulated with the antibiotic treatment. In addition, the gene expression of matrilysin, a metalloproteinase known to hydrolyze defensin precusors into their mature and active forms, was more than sixfold lower in the proximal small intestine of Clam than in that of Cont at d17. The changes in matrilysin gene expression were confirmed by real-time PCR, which showed a significant downregulation of this gene with Clamoxyl (1.7-fold, $P < 0.05$).

Clamoxyl upregulated the expression of mast cell proteases in the distal small intestine. Numerous proteases secreted by mast cells were upregulated by the treatment in the distal part of the small intestine (Table 3). Changes affected the pups at

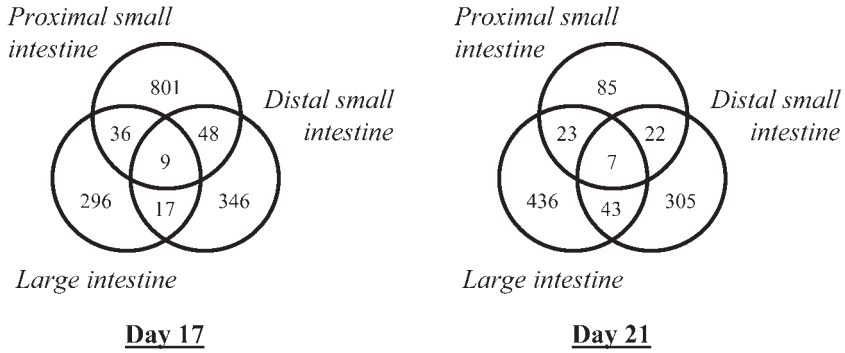


Fig. 6. Quantitative effect of the Clamoxyl treatment on the different intestinal segments at d17 and d21. Only the probes that were differentially regulated ($P < 0.001$) with the treatment are shown. Each circle represents the number of probe sets differentially regulated in one intestinal segment, and the intersections represent the number of probe sets showing similar changes in the corresponding intestinal segments.

both d17 and d21. Real-time PCR analysis of mast cell protease 4 confirmed the upregulation of this gene in the distal small intestine of Clam rats at d21 (1.2-fold, $P < 0.05$).

DISCUSSION

The goal of the described study was to determine the impact of a commercial antibiotic intervention early in life on the maturation of the genes involved in the intestinal barrier function. To this aim, we developed an antibiotic-treated suckling rat model. Amoxicillin, the active component of Clamoxyl, is a broad-spectrum antibiotic frequently used in the treatment of infant infectious diseases and usually administered by oral route. The dose of amoxicillin (100 $mg \cdot kg^{-1} \cdot day^{-1}$) given to the rat pups was in the range of that currently employed in neonatal therapy (50–100 $mg \cdot kg^{-1} \cdot day^{-1}$) (43). Pharmacokinetics of amoxicillin show close similarity in rats and humans (57), and the dose employed in our study was shown to significantly decrease fecal Enterococci counts in mice (26). The animals treated with Clamoxyl were compared with a control group in which the Clamoxyl solution was replaced by saline. We deliberately decided to use amoxicillin in its commercial form to be closer to the clinical use of the antibiotic. Therefore, the potential contribution of the commercial vehicle (including sodium benzoate, aroma, vanillin, bergamot oil, antioxidant E320, aspartame) on the effects observed and discussed here cannot be evaluated.

The effect of Clamoxyl administration on the intestinal microbiota balance was monitored by assessing the luminal contents of total aerobic and anaerobic living bacteria, as well as the concentration of *Lactobacillus, Enterobacteriacae*, and *Enterococcus,* major genera in the young rat (16, 42). The intervention affected the balance of the microbiota, especially in colon, where all the bacterial populations studied were significantly reduced in the Clamoxyl group. In particular, *Lactobacillus*, which is one of the most abundant genus in rodents (16), was almost totally depleted. These results are in accordance with the reported specificity of amoxicillin, which acts mainly against gram-positive bacteria (*Lactobacillus* and *Enterococcus*) as well as against some *Enterobacteriacae* (i.e., *Escherichia coli, Salmonella, Shigella*). In contrast, in the small intestine, the total bacterial counts were not affected, suggesting that the reduction in the Lactobacilli density was

Table 1. *Gene expression of antigen presentation molecules*

Probe Set	Gene Title	Day 17 Clam	Day 17 Cont	Day 17 *P* value	Day 21 Clam	Day 21 Cont	Day 21 *P* value
		Proximal small intestine					
1367679_at	CD74 antigen	1,675	2,336	$4 \cdot 10^{-5}$*	1,808	1,675	$4 \cdot 10^{-1}$
1370822_at	RT1 class II, locus Ba	419	809	$2 \cdot 10^{-10}$*	592	568	$7 \cdot 10^{-1}$
1370383_s_at	RT1 class II, locus Db1	385	648	$2 \cdot 10^{-6}$*	600	523	$2 \cdot 10^{-1}$
1370382_at	RT1 class II, locus Bb	15	131	$P < 1 \cdot 10^{-16}$*	42	78	$4 \cdot 10^{-3}$
1388236_x_at	RT1-CE11 class I	2,876	2,504	$1 \cdot 10^{-1}$	898	1,428	$2 \cdot 10^{-8}$*
1368593_at	CD1d1 antigen	657	760	$2 \cdot 10^{-1}$	189	276	$4 \cdot 10^{-4}$*
		Distal small intestine					
1370382_at	RT1 class II, locus Bb	11	46	$2 \cdot 10^{-15}$*	75	91	$2 \cdot 10^{-1}$
1370383_s_at	RT1 class II, locus Db1	315	474	$2 \cdot 10^{-5}$*	491	501	$7 \cdot 10^{-1}$
1370822_at	RT1 class II, locus Ba	352	584	$2 \cdot 10^{-8}$*	588	564	$6 \cdot 10^{-1}$
1388202_at	RT1 class Ib, locus Aw2	28	124	$P < 1 \cdot 10^{-16}$*	119	97	$4 \cdot 10^{-1}$
1388236_x_at	RT1-CE11 class I	3,875	2,592	$1 \cdot 10^{-6}$*	1,370	1,269	$9 \cdot 10^{-1}$
1388694_at	MHC class I RT1·O type 149	58	37	$1 \cdot 10^{-2}$	26	63	$2 \cdot 10^{-4}$*
		Large intestine					
1371033_at	RT1 class II, locus Bb	168	478	$1 \cdot 10^{-16}$*	292	605	$8 \cdot 10^{-11}$*
1388212_a_at	MHC class Ib RT1·S3	288	172	$2 \cdot 10^{-4}$*	179	167	$6 \cdot 10^{-1}$
1388202_at	RT1 class Ib, locus Aw2	36	37	$9 \cdot 10^{-1}$	31	63	$9 \cdot 10^{-4}$*
1367595_s_at	β-2 microglobulin	4,300	4,182	$8 \cdot 10^{-1}$	3,666	4,771	$6 \cdot 10^{-5}$*
1388536_at	HLA-B associated transcript 2	278	407	$1 \cdot 10^{-3}$*	280	254	$4 \cdot 10^{-1}$

Probe set, Affymatrix probe set no.; Gene title, NetAffx annotations; Clam and Cont, mean average difference intensity (ADI) data for the Clamoxyl (Clam) and control (Cont) groups; *P* value, level of significance. *Significant differences between Clam and Cont groups ($P < 0.001$).

Table 2. *Gene expression of anti-microbial products*

Probe Set	Gene Title	Day 17 Clam	Day 17 Cont	Day 17 P value	Day 21 Clam	Day 21 Cont	Day 21 P value
		Proximal small intestine					
1388238_at	defensin 5 precursor	1,552	2,353	$1{\cdot}10^{-6}$*	2,798	3,148	$1{\cdot}10^{-1}$
1387943_at	α-defensin 5	7	8	$8{\cdot}10^{-1}$	9	8	$9{\cdot}10^{-1}$
1370154_at	lysozyme	1,783	2,684	$9{\cdot}10^{-7}$*	3,083	2,665	$8{\cdot}10^{-2}$
1368128_at	phospholipase A2, group IIA	274	685	$1{\cdot}10^{-14}$*	820	1,053	$3{\cdot}10^{-2}$
1368766_at	matrix metalloproteinase 7	11	77	$1{\cdot}10^{-16}$*	64	86	$2{\cdot}10^{-1}$
1369660_at	β-defensin 1	20	11	$2{\cdot}10^{-1}$	16	17	$9{\cdot}10^{-1}$
1387960_at	β-defensin 3	15	32	$1{\cdot}10^{-1}$	26	14	$2{\cdot}10^{-1}$
1370470_at	defensin NP-4 precursor	14	18	$6{\cdot}10^{-1}$	3	18	$6{\cdot}10^{-3}$
1370791_at	defensin NP-3 precursor	3	17	$4{\cdot}10^{-6}$*	86	195	$2{\cdot}10^{-2}$
		Distal small intestine					
1388238_at	defensin 5 precursor	3,666	4,300	$2{\cdot}10^{-2}$	4,390	4,608	$9{\cdot}10^{-1}$
1387943_at	α-defensin 5	4	5	$8{\cdot}10^{-1}$	5	7	$6{\cdot}10^{-1}$
1370154_at	lysozyme	2,809	3,304	$1{\cdot}10^{-2}$	3,822	3,819	$2{\cdot}10^{-1}$
1368128_at	phospholipase A2, group IIA	1,448	2,241	$3{\cdot}10^{-8}$*	2,592	3,040	$4{\cdot}10^{-1}$
1368766_at	matrix metalloproteinase 7	237	276	$1{\cdot}10^{-1}$	274	324	$1{\cdot}10^{-1}$
1369660_at	β-defensin 1	12	11	$9{\cdot}10^{-1}$	29	13	$6{\cdot}10^{-2}$
1387960_at	β-defensin 3	22	15	$3{\cdot}10^{-1}$	22	18	$5{\cdot}10^{-1}$
1370470_at	defensin NP-4 precursor	4	8	$2{\cdot}10^{-1}$	5	12	$4{\cdot}10^{-3}$
1370791_at	defensin NP-3 precursor	105	187	$5{\cdot}10^{-9}$*	393	750	$6{\cdot}10^{-2}$
		Large intestine					
1388238_at	defensin 5 precursor	284	265	$6{\cdot}10^{-1}$	158	271	$8{\cdot}10^{-7}$*
1387943_at	α-defensin 5	5	4	$7{\cdot}10^{-1}$	5	5	1
1370154_at	lysozyme	873	787	$3{\cdot}10^{-1}$	685	644	$5{\cdot}10^{-1}$
1368128_at	phospholipase A2, group IIA	223	709	$P < 1{\cdot}10^{-16}$*	388	2,385	$P < 1{\cdot}10^{-16}$*
1368766_at	matrix metalloproteinase 7	6	5	$7{\cdot}10^{-1}$	4	8	$2{\cdot}10^{-1}$
1369660_at	β-defensin 1	13	8	$4{\cdot}10^{-1}$	11	21	$2{\cdot}10^{-1}$
1387960_at	β-defensin 3	20	21	$9{\cdot}10^{-1}$	6	25	$2{\cdot}10^{-3}$
1370470_at	defensin NP-4 precursor	25	7	$2{\cdot}10^{-2}$	7	8	$8{\cdot}10^{-1}$
1370791_at	defensin NP-3 precursor	2	4	$7{\cdot}10^{-2}$	8	17	$1{\cdot}10^{-1}$

*Significant differences between Clam and Cont groups ($P < 0.001$). For more details, see legend to Table 1.

compensated by the overgrowth of other nonanalyzed populations. In line with this, Mentula et al. (39) already reported a significant increase, specific to the small intestine, in the number of coliforms in dogs treated with ampicillin, an antibiotic that, like amoxicillin, belongs to the amino-penicillin family. The different response of the microbiota in small intestine and colon may be linked to differences in the bacterial diversity (16) as well as in the ecological conditions (34) of both intestinal parts. For instance, the transit time is several times slower in the colon than in the small intestine (27), likely leading to larger periods of contact of the colonic populations with the antibiotic and, therefore, to increased efficacy of the amoxicillin in this intestinal part.

The impact of Clamoxyl on the intestinal transcriptome was analyzed with the GeneArray technology. The GeneArray analysis was performed within the interface between the suckling and weaning periods. Drastic changes in the intestinal function have been reported during this period, especially in the small intestine (13, 25, 31). Accordingly, our data show that important changes occur at the transcriptional level between *days 17* and *21*. Quantitatively, the small intestine appeared to be more affected than the colon, with ~10 and 3% of probe sets, respectively, undergoing significant age-related modulation (Figs. 3–5).

Interestingly, a significant proportion of the genes undergoing maturational changes (10–30% depending on the intestinal part) showed modulation by the Clamoxyl treatment so that their normal pattern of maturation was either accelerated or slowed down. The importance of the luminal environment, in particular of the intestinal microbiota, on the process of gut

Table 3. *Gene expression of mast cell proteases in distal small intestine*

Probe Set	Gene Title	Localization	Day 17 Clam	Day 17 Cont	Day 17 P value	Day 21 Clam	Day 21 Cont	Day 21 P value
1369572_at	mast cell protease 1	15p13	156	109	$3{\cdot}10^{-3}$	137	135	$1{\cdot}10^{-2}$
1388231_at	mast cell protease 4	15p13	48	47	$9{\cdot}10^{-1}$	78	64	$7{\cdot}10^{-6}$*
1387334_at	mast cell protease 6	10q12	43	43	$9{\cdot}10^{-1}$	61	92	$3{\cdot}10^{-6}$*
1369586_at	mast cell protease 8	15p13	5	1	$3{\cdot}10^{-4}$*	1	2	$2{\cdot}10^{-1}$
1387220_at	mast cell protease 9	15p13	59	61	$9{\cdot}10^{-1}$	87	42	$2{\cdot}10^{-12}$*
1371164_at	mast cell protease 10	15p13	18	7	$4{\cdot}10^{-4}$*	19	38	$9{\cdot}10^{-3}$

Localization, chromosomal localization. *Significant differences between Clam and Cont groups ($P < 0.001$). For more details, see legend to Table 1.

postnatal development has been recently reviewed by Hooper (28). This group has also shown, by comparing germ-free and monocolonized mice, that commensal bacteria modulate the expression of genes involved in postnatal maturation (29). In our study, the modulatory effect of Clamoxyl was proportionally stronger in the colon, with 34% of the age-related genes affected, than in the small intestine, with only 17 and 8% of genes affected in proximal and distal small intestine, respectively. In the small intestine, probe sets modulated by the treatment clustered evenly into different functional probe sets, including endocytosis and vesicule-mediated transport in the proximal small intestine and immune response in the distal part (Figs. 3–5 and Supplementary Table S1). Moreover, in colon, most of the probe sets, in which maturation was up- or downregulated by the antibiotic treatment, were related to ion transport processes (Fig. 5 and Supplementary Table S1). The relevance of these results is limited by the fact that only 32% of the probe sets contained in the chip were associated with a biological process with the databases currently available. Nevertheless, these data give interesting hints on the modulation by the antibiotic treatment of biological functions critical in this period of life.

Interestingly, the probe sets that were altered by the treatment all along the intestinal tract belong to the MHC, a family of genes that are involved in antigen presentation. The main function of MHC molecules is to bind antigenic peptide fragments derived, for example, from pathogens and to display them on the cell surface for recognition by immune T cells (48). Both RT1 class Ib and class II genes were globally downregulated by the antibiotic treatment. These results are in agreement with the data reported by Matsumoto et al. (38), who showed that the intestinal epithelium of germ-free mice does not express MHC molecules. This paper also demonstrated that colonization of the germ-free mouse gut induced expression of MHC class II molecules in epithelial cells (38). Adequate antigen presentation by MHC class II is required for the tolerization to luminal antigens, as shown in MHC class II-deficient mice, which lack the ability to acquire oral tolerance (14). The role of RT1 class Ib molecules is less clear. A large number of MHC class Ib genes have been described (23) and are distinguished from the "classical" class Ia by their limited polymorphism (52). The specific patterns of expression of RT1 Ib suggest specialized roles in antigen presentation. For example, in mice, the class Ib locus M molecules can present peptides with NH_2-formylated amino termini from infectious organisms, such as *Listeria monocytogenes* (52). However, other MHC class Ib loci (HLA-A) have functions unrelated to the immune system such as iron metabolism and uptake in the intestinal tract (10). Unfortunately, the specificity of the MHC class Ib locus Aw2, downregulated with Clamoxyl, is unknown.

Taken together, our results suggest that early antibiotic treatment may affect the acquisition of tolerance to luminal antigens by altering the mechanisms of antigen presentation. It is important to note that the presence of intestinal microbiota is required for the maintenance of oral tolerance, as evidenced in germ-free rodent models (55). In this regard, some population-based studies have found a positive association between the early administration of antibiotics and the risk of later development of atopy (15, 21, 58, 60) or Crohn's disease (9), both allergy and Crohn's disease being characterized by a hyperresponsiveness of the immune system to harmless antigenic components (i.e., protein and commensal microbiota).

Paneth cells are key players in the mucosal handling of luminal bacteria, acting specifically at the level of the small intestinal crypts (44). Indeed, they are able to synthesize and secrete compounds such as defensins, lysozyme, and type IIA phospholipase A2, with anti-microbial properties (24, 46, 59). The gene expression of several antimicrobial peptides, defensins 3 and 5, lysozyme, and phospholipase A2, as well as matrilysin was down regulated in the Clamoxyl group. Interestingly, the expression of defensins 3 and 5 synthesized predominantly by Paneth cells or neutrophils (5) was specifically reduced by the antibiotic treatment, whereas the defensins predominantly found in epithelial cells (5), β-defensins, were not significantly affected. Contrary to our results, Putsep et al. (45) did not find any differences in the qualitative profile of prodefensin peptides present in germ-free or conventional mice. They concluded that the intestinal microbiota did not affect the production of defensins by the Paneth cells. However, this paper did not present quantitative data, and, therefore, an effect of the microbiota on the actual amount of synthesized defensins cannot be ruled out. Moreover, Ayabe et al. (4) showed a dose-dependent secretion of defensins when the crypts from germ-free mice were isolated and exposed to bacteria or bacterial antigens, indicating that, indeed, the presence of bacteria stimulates the secretion of these molecules. As indicated, the matrilysin gene was significantly downregulated in the Clamoxyl-treated group. In line with these results, Lopez-Boado et al. (33) failed to detect matrilysin protein in germ-free mice. Matrilysin is a protease expressed in Paneth cells that plays a crucial role in mucosal defense by activating the prodefensin molecules (59). Therefore, a reduction in the activity of this protease may compromise the anti-pathogenic activity of the defensins (59). We also observed reduced expression of the phospholipase A2 gene after antibiotic intervention. This enzyme has been shown to inactivate both gram-positive (46) and gram-negative bacteria (24). Accordingly, Qu et al. (47) showed that in vivo intraluminal perfusion with lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, increased phospholipase A2 and lysozyme activity in the luminal perfusates of the LPS-exposed rats. The changes observed in the level of expression of Paneth cell products were specific to postnatal *day 17*, suggesting higher susceptibility before weaning to the alterations occurring at the luminal level. Although these data should be confirmed by appropriate functional assays, they strongly suggest that an antibiotic treatment may reduce the innate ability of the host to deal with pathogens in the still-immature gut. The results appear to be related to an alteration of the secretory ability of the cell rather than to a change in the number of cells, as the immunohistochemical analysis of the intestinal tissues did not reveal any effect in the number of Paneth cells (data not shown).

Finally, our GeneArray analysis revealed increased gene expression of a number of mast cell proteases in the distal part of the small intestine of Clamoxyl-treated rats. Interestingly, these protease genes are located on the same chromosome (15p12 or p13). In contrast, mast cell protease 6, which is not located on chromosome 15, was downregulated by the antibiotic treatment. These data strongly suggest that the upregulated genes were all modulated by the same transcription factor. The

observed upregulation of mast cell protease expression is in agreement with the data reported by Enerback et al. (20), who showed that administration of the antibiotic Polymyxin to rats induced an increase of the mucosal mast cell number. Mast cells are considered effector components of the immune system, playing an important role in host defense against pathogens (35, 36). However, they also play a central role in the onset and maintenance of chronic intestinal inflammation (61) and in the clinical manifestations of gastrointestinal allergic disorders (8). Although we did not observe any signs of macro- or microscopic inflammation in the intestine of the Clamoxyl-treated animals (data not shown), an increase in the mast cell number and/or activity may reflect a subinflammatory status that, as in other conditions (53), can predispose the animal to an exacerbated inflammatory reaction in the case of insult.

In summary, the Clamoxyl treatment altered the normal colonization pattern of the gut microbiota and interfered with the normal maturation profile of 10–30% of genes in the different intestinal segments. In particular, the antibiotic treatment resulted in the downregulation of genes related to innate host defense and to antigen presentation, and in the upregulation of genes related to mast cell activity. The design of our study did not allow us to determine whether the observed effects were attributable to the microbiota changes or to the Clamoxyl chemical composition (either the antibiotic itself or the vehicle). However, our results suggest that the early administration of a commercial antibiotic preparation widely employed in pediatric settings interferes with the maturation of various parameters of the mucosal barrier. These changes occur at the suckling-weaning transition, a critical period in which the gut and the subjacent immune tissue deal with an array of novel food antigens and bacteria. It would be interesting to evaluate whether these changes are associated with an increased risk of contracting infection or developing inflammatory or atopic diseases later in life.

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REFERENCES

1. **Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402, 1997.
2. **Andremont A.** Consequences of antibiotic therapy to the intestinal ecosystem. *Ann Fr Anesth Reanim* 19: 395–402, 2000.
3. **Axelsson I, Jakobsson I, Lindberg T, Polberger S, Benediktsson B, and Raiha N.** Macromolecular absorption in preterm and term infants. *Acta Paediatr Scand* 78: 532–537, 1989.
4. **Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, and Ouellette AJ.** Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. *Nat Immun* 1: 113–118, 2000.
5. **Bevins CL, Martin-Porter E, and Ganz T.** Defensins and innate host defence of the gastrointestinal tract. *Gut* 45: 911–915, 1999.
6. **Biol-N'garagba MC and Louisot P.** Regulation of the intestinal glycoprotein glycosylation during postnatal development: role of hormonal and nutritional factors. *Biochimie* 85: 331–352, 2003.
7. **Bonnemaison E, Lanotte P, Cantagrel S, Thionois S, Quentin R, Chamboux C, and Laugier J.** Comparison of fecal flora following administration of two antibiotic protocols for suspected maternofetal infection. *Biol Neonate* 84: 304–310, 2003.
8. **Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, and Rothenberg ME.** Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 112: 1666–1677, 2003.
9. **Card T, Logan RF, Rodrigues LC, and Wheeler JG.** Antibiotic use and the development of Crohn's disease. *Gut* 53: 246–250, 2004.
10. **Cardoso CS and de Sousa M.** HFE, the MHC and hemochromatosis: paradigm for an extended function for MHC class I. *Tissue Antigens* 61: 263–275, 2003.
11. **Cebra JJ.** Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 69: 1046S–1051S, 1999.
12. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
13. **Davies PH and Messer M.** Intestinal cathepsin B and D activities of suckling rats. *Biol Neonate* 45: 197–202, 1984.
14. **Desvignes C, Bour H, Nicolas JF, and Kaiserlian D.** Lack of oral tolerance but oral priming for contact sensitivity to dinitrofluorobenzene in major histocompatibility complex class II-deficient mice and in CD4+ T cell-depleted mice. *Eur J Immunol* 26: 1756–1761, 1996.
15. **Droste JH, Wieringa MH, Weyler JJ, Nelen VJ, Vermeire PA, and Van Bever HP.** Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? *Clin Exp Allergy* 30: 1547–1553, 2000.
16. **Ducluzeau R and Raibaud P.** Bacterial interactions within the digestive tract. *Rev Sci Tech Off Int Epiz* 8: 313–332, 1989.
17. **Durbin R, Eddy S, Krogh A, and Itchisor G.** *Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids*. Cambridge, MA: Cambridge Univ. Press, 1998.
18. **Eastham EJ, Lichauco T, Grady MI, and Walker WA.** Antigenicity of infant formulas: role of immature intestine on protein permeability. *J Pediatr* 93: 561–564, 1978.
19. **Eisen MB, Spellman PT, Brown PO, and Botstein D.** Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95: 14863–14868, 1998.
20. **Enerback L, Lowhagen G, Lowhagen O, and Wingren U.** The effect of polymyxin B and some mast-cell constituents on mucosal mast cells in the duodenum of the rat. *Cell Tissue Res* 214: 239–246, 1981.
21. **Farooqi IS and Hopkin JM.** Early childhood infection and atopic disorder. *Thorax* 53: 927–932, 1998.
22. **Gardner ML.** Gastrointestinal absorption of intact proteins. *Annu Rev Nutr* 8: 329–350, 1988.
23. **Gunther E and Walter L.** The major histocompatibility complex of the rat (*Rattus norvegicus*). *Immunogenetics* 53: 520–542, 2001.
24. **Harwig SS, Tan L, Qu XD, Cho Y, Eisenhauer PB, and Lehrer RI.** Bactericidal properties of murine intestinal phospholipase A2. *J Clin Invest* 95: 603–610, 1995.
25. **Henning SJ and Kretchmer N.** Development of intestinal function in mammals. *Enzyme* 15: 3–23, 1973.
26. **Hofstra W, Welling GW, and Van der Waaij D.** A comparative study of the effect of oral treatment with augmentin, amoxycillin and bacampicillin on the faecal flora in mice. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 269: 78–85, 1988.
27. **Holzapfel WH, Haberer P, Snel J, Schillinger U, and Huis in't Veld JH.** Overview of gut flora and probiotics. *Int J Food Microbiol* 41: 85–101, 1998.
28. **Hooper LV.** Bacterial contributions to mammalian gut development. *Trends Microbiol* 12: 129–134, 2004.
29. **Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, and Gordon JI.** Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291: 881–884, 2001.
30. **Husebye E, Hellstrom PM, and Midtvedt T.** Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. *Dig Dis Sci* 39: 946–956, 1994.
31. **Iiboshi Y, Nezu R, Khan J, Chen K, Cui L, Yoshida H, Wasa M, Fukuzawa M, Kamata S, Takagi Y, and Okada A.** Developmental changes in distribution of the mucous gel layer and intestinal permeability in rat small intestine. *JPEN J Parenter Enteral Nutr* 20: 406–411, 1996.
32. **Kandori H, Hirayama K, Takeda M, and Doi K.** Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. *Exp Anim* 45: 155–160, 1996.

33. **Lopez-Boado YS, Wilson CL, Hooper LV, Gordon JI, Hultgren SJ, and Parks WC.** Bacterial exposure induces and activates matrilysin in mucosal epithelial cells. *J Cell Biol* 148: 1305–1315, 2000.
34. **Mackie RI, Sghir A, and Gaskins HR.** Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* 69: 1035S–1045S, 1999.
35. **Malaviya R, Ikeda T, Ross E, and Abraham SN.** Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. *Nature* 381: 77–80, 1996.
36. **Malaviya R, Twesten NJ, Ross EA, Abraham SN, and Pfeifer JD.** Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol* 156: 1490–1496, 1996.
37. **Mansourian R, Mutch DM, Antille N, Aubert J, Fogel P, Le Goff JM, Moulin J, Petrov A, Rytz A, Voegel JJ, and Roberts MA.** The global error assessment (GEA) model for the selection of differentially expressed genes in microarray data. *Bioinformatics* 20: 2726–2737, 2004.
38. **Matsumoto S, Setoyama H, and Umesaki Y.** Differential induction of major histocompatibility complex molecules on mouse intestine by bacterial colonization. *Gastroenterology* 103: 1777–1782, 1992.
39. **Mentula S, Harmoinen J, Koski P, Westermarck E, Rautio M, Huovinen P, and Kononen E.** Inhibition of ampicillin-induced emergence of resistance in intestinal coliforms by targeted recombinant β-lactamase. *Int J Antimicrob Agents* 24: 555–561, 2004.
40. **Mullink JW and Morsink FH.** Presence of IgA-producing cells in rats and mice in relation to microbial status. *Lab Anim* 18: 252–257, 1984.
41. **Mutch DM, Simmering R, Donnicola D, Fotopoulos G, Holzwarth JA, Williamson G, and Corthesy-Theulaz I.** Impact of commensal microbiota on murine gastrointestinal tract gene ontologies. *Physiol Genomics* 19: 22–31, 2004.
42. **Nakayama M, Yajima M, Hatano S, Yajima T, and Kuwata T.** Intestinal adherent bacteria and bacterial translocation in breast-fed and formula-fed rats in relation to susceptibility to infection. *Pediatr Res* 54: 364–371, 2003.
43. **Otters HB, van der Wouden JC, Schellevis FG, Suijlekom-Smit LW, and Koes BW.** Trends in prescribing antibiotics for children in Dutch general practice. *J Antimicrob Chemother* 53: 361–366, 2004.
44. **Porter EM, Bevins CL, Ghosh D, and Ganz T.** The multifaceted Paneth cell. *Cell Mol Life Sci* 59: 156–170, 2002.
45. **Putsep K, Axelsson LG, Boman A, Midtvedt T, Normark S, Boman HG, and Andersson M.** Germ-free and colonized mice generate the same products from enteric prodefensins. *J Biol Chem* 275: 40478–40482, 2000.
46. **Qu XD and Lehrer RI.** Secretory phospholipase A2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. *Infect Immun* 66: 2791–2797, 1998.
47. **Qu XD, Lloyd KC, Walsh JH, and Lehrer RI.** Secretion of type II phospholipase A2 and cryptdin by rat small intestinal Paneth cells. *Infect Immun* 64: 5161–5165, 1996.
48. **Rajagopal D, Bal V, George A, and Rath S.** Diversity and overlap in the mechanisms of processing protein antigens for presentation to T cells. *Indian J Med Res* 120: 75–85, 2004.
49. **Resi D, Milandri M, and Moro ML.** Antibiotic prescriptions in children. *J Antimicrob Chemother* 52: 282–286, 2003.
50. **Rothberg RM.** Immunoglobulin and specific antibody synthesis during the first weeks of life of premature infants. *J Pediatr* 75: 391–399, 1969.
51. **Schindler C, Krappweis J, Morgenstern I, and Kirch W.** Prescriptions of systemic antibiotics for children in Germany aged between 0 and 6 years. *Pharmacoepidemiol Drug Saf* 12: 113–120, 2003.
52. **Shawar SM, Vyas JM, Rodgers JR, and Rich RR.** Antigen presentation by major histocompatibility complex class I-B molecules. *Annu Rev Immunol* 12: 839–880, 1994.
53. **Soderholm JD, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, and Perdue MH.** Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 123: 1099–1108, 2002.
54. **Stepankova R, Kocna P, Sterzl J, and Dvorak B.** Expression of MHC class II antigens and enzymatic activity in enterocytes of germ-free and conventional rats: dependence on nutritional factors during suckling. *Adv Exp Med Biol* 371A: 501–505, 1995.
55. **Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, and Koga Y.** The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 159: 1739–1745, 1997.
56. **Szentkuti L, Riedesel H, Enss ML, Gaertner K, and Von Engelhardt W.** Pre-epithelial mucus layer in the colon of conventional and germ-free rats. *Histochem J* 22: 491–497, 1990.
57. **Torres-Molina F, Peris-Ribera JE, Garcia-Carbonell MC, Aristorena JC, and Pla-Delfina JM.** Nonlinearities in amoxycillin pharmacokinetics. II. Absorption studies in the rat. *Biopharm Drug Dispos* 13: 39–53, 1992.
58. **Wickens K, Pearce N, Crane J, and Beasley R.** Antibiotic use in early childhood and the development of asthma. *Clin Exp Allergy* 29: 766–771, 1999.
59. **Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, and Parks WC.** Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286: 113–117, 1999.
60. **Wjst M, Hoelscher B, Frye C, Wichmann HE, Dold S, and Heinrich J.** Early antibiotic treatment and later asthma. *Eur J Med Res* 6: 263–271, 2001.
61. **Xu X, Weksler-Zangen S, Pikarsky A, Pappo O, Wengrower D, Bischoff SC, Pines M, Rivkind A, Goldin E, and Levi-Schaffer F.** Mast cells involvement in the inflammation and fibrosis development of the TNBS-induced rat model of colitis. *Scand J Gastroenterol* 37: 330–337, 2002.