COPROPHAGY IN RABBIT UPREGULATES IMMUNE SYSTEM GENE EXPRESSION IN ILEUM

Cauquil L.[1]*, Beaumont M.[1], Schmaltz-Panneau B.[2], Liaubet L.[1], Lippi Y.[3], Gress L.[1], Bluy L.[1], Duranthon V.[2], Combes S.[1]

[1]GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France
[2]BDR, INRAE, ENVa, Université Paris Saclay, Jouy en Josas, France
[3]Toxalim, Université de Toulouse, INRAE, ENVT, INP-Purpan, Toulouse, France;
*Corresponding author: laurent.cauquil@inrae.fr

ABSTRACT

Amplification of coprophagous behavior in young rabbits strongly reduces mortality and stimulates the maturation of the microbiota. We hypothesized that this positive effect of coprophagy is immune-mediated at the intestinal level. We thus compared the ileum transcriptome of rabbits for which coprophagic behavior was enhanced to those where this behavior was prevented. Young rabbits were allocated to three groups: in NF (No access to Feces) group ingestion of mother's hard feces was prevented, while in two further groups FF (access to Feces from Foreign does without antibiotic treatment) and FFab (access to Feces from Foreign does supplemented with antibiotics, in drinking water with tetracycline [50 mg/kg BW] and tiamulin [10 mg/kg BW]), kits had access in the nest to feces excreted by foreign females receiving either no antibiotic or tiamulin and tetracycline. Ileum mucosa was sampled in 35 and 49 days old rabbits (n=9-10 rabbits per group per age) and transcriptome analysis was carried on using an Agilent G3 Rabbit 60K microarray. As expected, a total of 209 genes were differentially expressed (DE) according to age ($P<0.05$) but none according to treatment. However a significant interaction between age and treatment was observed ($P<0.05$). Between 35 and 49 days of age, FF group exhibited 350 DE genes while the NF and FFab groups showed only 10 and 9 DE genes respectively ($P<0.05$). Upregulated genes coded for antimicrobial peptides, mucine production, cytokines and chemokines, pattern recognition receptors and proteins involved in immunoglobulin A secretion or antiviral responses. To gain mechanistic insight into the FF group DE annotated genes list, pathway enrichment analysis was carried out. Gene ontology analysis revealed that the 231 annotated upregulated DE genes (Ensembl gene annotation for rabbit) in FF according to age were significantly implicated in 28 biological process all related to immune system. All together, these results suggested that the beneficial effect of coprophagous behavior on rabbit survival might be mediated through an immune activation in the ileum. Interestingly, the effect of coprophagy on intestinal immune gene expression was not observed when kits ingested feces from antibiotics medicated does, probably because key immune-stimulating bacteria were missing.

Key words: suckling rabbits, transcriptome, microarray, ileum, coprophagous behavior

INTRODUCTION

Several studies highlighted that within 2 weeks after giving birth, lactating doe left hard feces in the nest, which are partly ingested by the young rabbits (Hudson and Distel, 1982, Kovács *et al.*, 2006, Combes *et al.*, 2014). These studies agree on an excretion rate of 2-3 hard feces/day during the first six days after kindling, with however a high variability of the emission (5% of females excrete no droppings while 20% have an excretion higher than 6 faeces/day). At the same time, the ingestion of feces by the suckling rabbits starts as early as day 2-3 (<1 feces per litter of 10), peaks around day 10 (1-2 feces/day/litter) and continues until day 20. This behavior would constitute a vector for vertical

transmission of the microbiota from the mother to her progeny, and might compensate for the short contact time of the mother with its offspring. It might allow a targeted and early microbial colonization of the digestive tract from the first days of neonatal life. Indeed, deprivation of coprophagy delays the implantation dynamics of the microbiota and leads to an increase mortality. Interestingly, providing feces from foreign female increased by 3 fold the coprophagous behavior which improves the health of kits (strong reduction of mortality) and speed up the implantation of the microbiota (Combes *et al.*, 2014). Despite a similar doe feces ingestion by pups, the beneficial effect of coprophagy was no longer observed when feces came from antibiotic medicated females. We hypothesized that the positive effect of coprophagic behavior on health is immune-mediated at the level of ileum. The objective of this study was to compare the effect on ileum transcriptome of impairment of feces ingestion, and ingestion of foreign feces from does with and without antibiotic medication.

MATERIALS AND METHODS

Animals and experimental design

All animal housing and handling procedures (experimental unit, INRAE, Castanet-Tolosan, France) complied with the guidelines for animal research of the French Ministry of Agriculture and were described in Combes et al (2014). Briefly, the does were kept in wire cages containing a nest box for kits and a controlled nursing was performed once a day. The rabbits, both mothers and growing rabbits, were fed ad libitum a pelleted feed manufactured at the PECTOUL experimental unit, INRAE (Castanet-Tolosan, France) and had free access to fresh water. The diet contained neither coccidiostatic nor antibiotics. At birth (day-old [d] 0), litters were adjusted to 8-10 kits with no cross-fostering. In all the groups, the maternal feces excreted by the mothers in the nest boxes were counted immediately after the suckling. In the NF group (No access to Feces); the maternal hard feces were removed immediately after counting. In the FF group and FFab group, the maternal feces were removed after counting and replaced with foreign feces. Weaning occurred at age 35 d, but offspring had access to doe's solid feed before weaning as soon as they were able to leave the nest. Ileum mucosa was sampled in 35 and 49 d rabbits (n=9-10 rabbits per group per age).

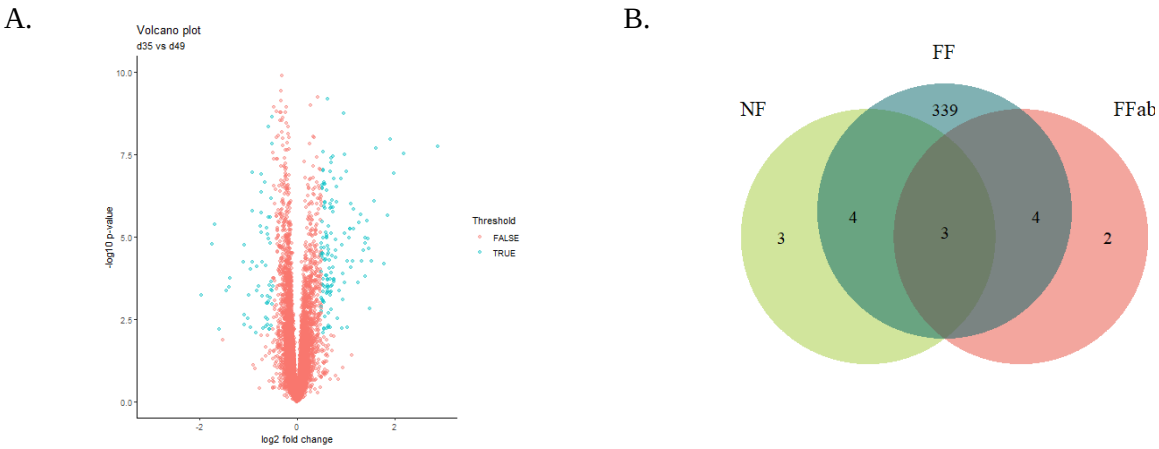

Figure 1 A. Volcano plot showing DE of probes according to age (35 vs 49 days). Blue points indicate DE genes considered as significant (P.adj< 0.05 and logFC > |0.5|
B. Venn diagram of DE genes between 35 and 49 days of age in NF group, where ingestion of hard feces was prevented, in FF and FFab groups where kits had access in the nest to feces excreted by foreign females receiving either no antibiotic or medicated with tiamulin and tetracycline

Transcriptomic Analyses

Total RNA was isolated from each of the 56 ileum samples. Briefly, samples were grounded to a fine powder in a liquid-nitrogen cooled grinder with stainless steel beads and processed for total RNA isolation using Trizol (Invitrogen, France) and the Nucleospin RNA II kit (Macherey-Nagel, France)

according to the manufacturer's instructions. The microarray Agilent SurePrint G3 Rabbit GE 8x60K (Design 042421) used in this experiment consisted in 61657 spots, which correspond to 42089 probes targeting 29026 unique gene. The raw data of the microarray is available on NCBI (GEO platform) with the accession number GSE104838. After quality control and a quantile normalization step, 33284 spots were kept for further analysis and log2 transformed. Differential expression analysis was assessed using the R/Bioconductor software package limma (Linear Models for Microarray Data). All fold-changes associated with these analyses are represented in log2 scale (logFC) and we show only data with a P-value (adjusted with BH) <0.05 and a logFC > |0.5|. Finally, the significant DE genes were analysed with g:profiler (Liis Kolberg and Uku Raudvere, 2019) to obtain the top biological functions.

Table 1: Number of unique genes found up- or downregulated (logFC > |0.5| and adj. P< 0.05) between 35 and 49 days in NF group, ingestion of hard feces was prevented, in FF and FFab groups where kits had access in the nest to feces excreted by foreign females receiving either no antibiotic or tiamulin and tetracycline, respectively

	d35vs49_NF	d35vs49_FF	d35vs49_FFab
Probes down-regulated	3	119	1
Probes up-regulated	7	231	8
Total DE probes	10	350	9

Table 2: Top-twenty upregulated DE annotated genes between 35 and 49 days in FF group where kits had access in the nest to feces excreted by foreign females not medicated with antibiotics

Gene symbol*	logFC	Adj. Pval	Gene symbol	logFC	Adj. P value
IDO1	3.96	0.000	**CYP1A1**	2.10	0.003
REG3G	3.59	0.042	**IRF7**	1.98	0.002
DDX60	3.17	0.000	**LYG2**	1.96	0.021
HERC5	2.85	0.006	ITLN2	1.92	0.008
USP18	2.40	0.005	**ISG15**	1.81	0.002
UBD	2.39	0.001	**XAF1**	1.81	0.001
FABP4	2.25	0.002	AQP4	1.77	0.001
CCL5	2.24	0.001	**GBP1**	1.74	0.032
GZMH	2.17	0.005	**OASL**	1.72	0.028
RSAD2	2.16	0.000	**IFI44**	1.70	0.008

* in bold genes encoding for protein involved in immune system regulation; IDO1: Indoleamine 2,3-dioxygenase 1; REG3G: Regenerating islet-derived protein 3-gamma; DDX60: DEXD/H box helicase; HERC5: HERC family of ubiquitin ligase; USP18: Ubiquitin Specific Peptidase 18; UBD: Ubiquitin D; FABP4: Fatty Acid Binding Protein 4; CCL5: Chemokine (C-C motif) ligand 5; GZMH Granzyme H; RASD2: RASD Family Member 2; CYAP1A1: Cytochrome P450 Family 1 Subfamily A Member 1; IRF7: Interferon regulatory factor 7; LYG2 lyzozyme G2; ITLN: Intelectin 1; ISG15: Interferon-stimulated gene 15; XAF1: XIAP Associated Factor 1; AQP4: Aquaporin 4; GBP1: Guanylate Binding Protein 1; OASL: 2'-5'-Oligoadenylate Synthetase Like; IFI44: Interferon-induced protein 44.

RESULTS AND DISCUSSION

We compared the ileum transcriptome of rabbits for which coprophagic behavior was enhanced to those where this behavior was prevented using an Agilent G3 Rabbit 60K microarray. As expected, transcription level was affected by age with a total of 250 genes differentially expressed (DE) (P<0.05, Figure 1A) but no genes were found DE between the three groups of rabbits either with enhanced (FF and FFab groups) or prevented coprophagous behavior. However, the number of DE genes according to age differed between treatments. When kits ingested feces from foreign females with antibiotics medication (tiamuline and tetracyclin, FFab group) the number of DE genes was very low (9 DE genes) and was equivalent to the number of DE gene of kits with no feces ingestion (10 DE genes, NF group). Between 35 and 49 days of age, a total of 209 genes (Table 1) had a differential expression in ileum mucosa from kits with access to feces excreted by foreign females with no antibiotics medication (FF group). Almost two third of the DE genes of FF group were upregulated. Eighteen out of the 20 most upregulated DE genes were involved in immunity (Table 2). Upregulated genes coded for antimicrobial peptides (REG3G, LYG2, PLA2G16), mucine production (MUC3), cytokines and chemokines (CCL4, CCL5, CCL13, CXCL9, CXCL10, CXCR6, IL15, IL17B, IL18, IFNG), pattern recognition receptors (TLR3, CLEC9A, IRF7) and proteins involved in immunoglobulin A secretion

(PIGR, TNFS13, TNFRSF17) or antiviral responses (ISG15, DDX60; DDX58; ISG15, HERC5, MX1, GZMH).

To gain mechanistic insight into the FF group annotated upregulated genes list, pathway enrichment analysis was carried out. The GO functional enrichment analysis was performed on the 231 annotated upregulated DE genes (Ensembl gene annotation for rabbit) in FF according to age. The top significant GO annotations indicated that the enriched biological processes were all related to immune response.

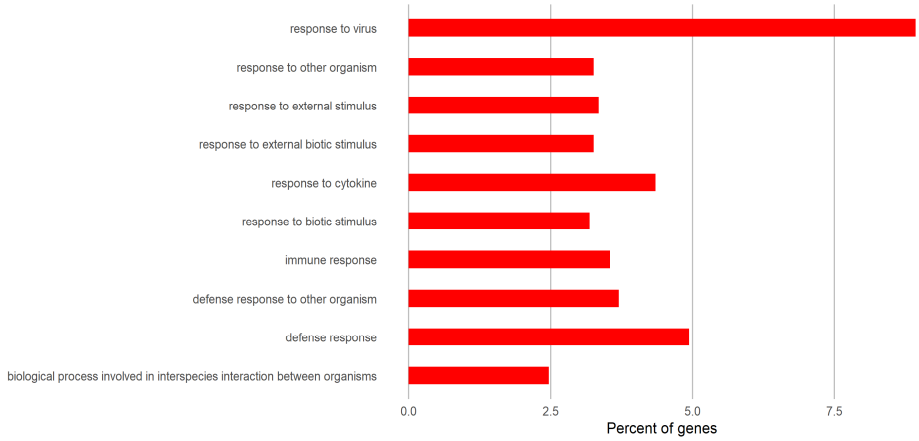

Figure 1: GO analysis of annotated upregulated differentially expressed genes in FF group where kits had access in the nest to feces excreted by foreign females not medicated with antibiotics. Bars represent the 10 first Biological Process (p adj<0.025) and y-axis shows the percentage of enriched genes in each category

CONCLUSIONS

All together, these results suggested that beneficial effect of coprophagous behavior previously observed on rabbit survival might be mediated through an activation of immune responses in the ileum. Although the quantity of ingested feces was equivalent, the coprophagous behavior effect on ileum gene expression was not observed when kits ingested feces from medicated does. Antibiotic medication might have eradicated from doe feces key bacteria implicated in education of immune system. These results highlight the importance of coprophagy for microbiota implantation and immune maturation in the gut of young rabbits.

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