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## **BOTH QUANTITATIVE AND ENERGETIC INTAKE LEVEL AFFECT CAECAL MICROBIOTA COMPOSITION AND ACTIVITY IN THE GROWING RABBIT**

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### **ABSTRACT**

Short-term feed restriction strategies reduce rabbit post-weaning digestive disorders, but little is known about the implications of the caecal ecosystem in these beneficial effects. Our study looks into the consequences of feed (quantitative) and energy intake level on the caecal ecosystem. At weaning 320 rabbits were allotted into four groups: HE100, HE75, LE100 and LE75, differing in dietary digestible energy concentrations (HE = 10.13 vs LE = 9.08 MJ DE/kg, calculated values) and intake levels (100 = *ad libitum* vs 75 = restricted at 75% of *ad libitum*). Caecal content was sampled in ten rabbits per group at 42 and 50 days of age for ammonia and volatile fatty acid concentration measurements and for bacterial composition determination using 16S DNA sequencing. Restricted feed intake increased the acetate proportion (+2.8 units,  $P < 0.001$ ) and decreased that of butyrate (-2.4 units,  $P < 0.001$ ), while high energetic intake (HE diet) mainly decreased the caecal pH (-0.14,  $P < 0.001$ ). Akkermansiaceae were increased with high energetic intake (0.54 vs 0.25%,  $p_{\text{adjust}} < 0.05$ ), while Christensenellaceae were decreased (2.8 vs 5.6%,  $p_{\text{adjust}} < 0.001$ ) and Eubacteriaceae increased with restricted feed intake (10.5 vs 6.9%,  $p_{\text{adjust}} < 0.05$ ). Altogether, our results indicated that quantitative and energetic intake level modified the caecal microbial activity and composition. These modifications deserve further investigation to determine the potential causal link between microbial composition, activity and impacts on the host's health.

**Key words:** rabbit, intake level, microbiota, caecum, energy.

### **INTRODUCTION**

In mammals, weaning generates important changes as the young animal shifts from milk to solid feed, and is often associated with various digestive disorders. In rabbits, these digestive disorders are characterized by an imbalance in the caecal microbiota (Gidenne et al., 2010). Short term feed restriction has been used in French rabbit breeding systems for over 10 years as an efficient method to reduce digestive disorders in the growing rabbit (Gidenne *et al.*, 2012). These beneficial effects might be associated with a change in gut microbial composition and activity. However, the correlations between feed intake level and gut microbiota have been scarcely documented in the rabbit specie. Combes et al. (2017) showed moderate effects of feed restriction on the major caecal bacterial taxa in 63 days old rabbits. However, the microbial community stabilizes between 49 and 63 days of age (Combes et al., 2011), and digestive disorders occur prior to this stabilization, thus indicating that a more marked effect of feed restriction might occur before 49 days of age. Increasing the dietary energy concentration can partially compensate for the reduced growth associated with restricted feed intake, although impacts on digestive health vary according to

general health status of the farm (Knudsen *et al.*, 2014). Impact of dietary energy concentration on the caecal microbiota has not yet been studied in rabbits. Thus, the present study was conducted to evaluate the impact of both quantitative and energetic intake level (dietary digestible energy concentration) on caecal microbial composition and activity in the growing rabbit.

## MATERIALS AND METHODS

### Experimental design, animals, housing and feeds

The experiment was conducted at the INRAE (Castanet-Tolosan, France) breeding unit using hybrid rabbits (*Oryctolagus cuniculus*, INRAE breed). A bifactorial design was used with two levels of feed intake, *ad libitum* feeding vs restricted feeding at 75% of *ad libitum* (namely 100 and 75), and two diets differing in digestible energy (DE) concentration, a “low” energy diet (LE) formulated with 9.08 MJ DE/kg, and a “high” energy diet (HE) formulated with 10.13 MJ DE/kg (detailed formulas and chemical compositions in Knudsen *et al.*, 2014). At weaning (35 days of age) rabbits of both sexes were allotted according to their weaning weight and litter of origin in cages of 5 rabbits (80/group). The animals were then fed freely or restricted according to their experimental treatment and water provided *ad libitum*. 10 healthy animals per treatment were killed at 42 and 50 days of age. Caecal pH was measured and caecal content was collected for further microbiota, VFA (Volatile Fatty Acids) and ammonia (NH<sub>3</sub>) analyses.

### Chemical analyses

The DNA was extracted as previously described (Combes *et al.*, 2011). The DNA samples were 454 sequenced with primers targeting the V3-V4 regions of the 16S rRNA gene. VFA were measured by automated gas chromatography and ammonia concentrations were determined by a colorimetric method.

### Bioinformatics and statistical analyses

Bioinformatics treatment of the sequencing was performed using the FROGS pipeline (Escudie *et al.*, 2018). Reads were clustered into Operational Taxonomic Units (OTU) and OTUs with abundances above 0.005% of total sequences and present in at least 6 samples were kept. Taxonomic assignment was performed using the SILVA 132 database.

Three animals were removed from all analyses due to physiological signs of illness. Statistical analyses were performed using R (version 3.5.1), an open source statistical software. Fermentative parameters, diversity and taxonomic relative abundances were analyzed with a linear model with the feed intake level, the diet, the age and the interactions between those three factors as fixed effects. For the microbial community an FDR correction was applied to the p-values. Beta diversity was represented using a Bray Curtis dissimilarity measure and an nMDS ordination method.

## RESULTS AND DISCUSSION

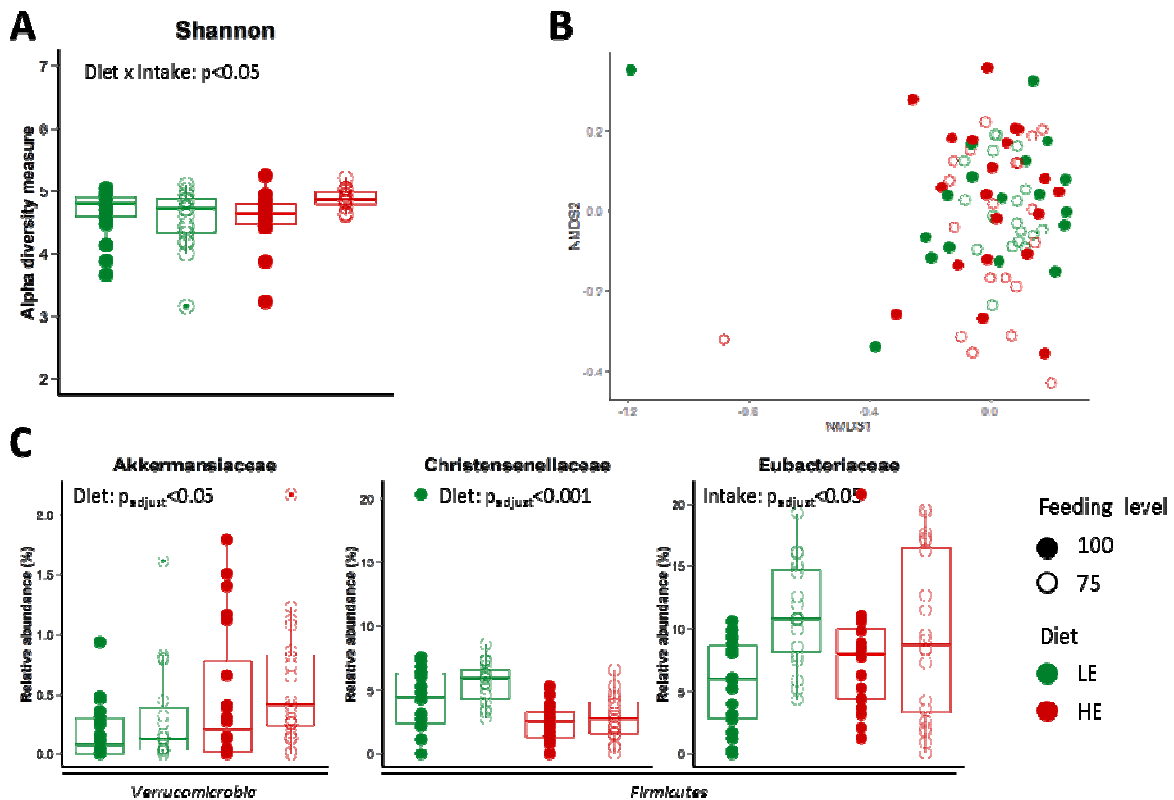
High energetic intake (HE vs LE diet) was associated with a decreased pH regardless of the age (-0.14, P<0.001, Table 1) possibly due to the higher proportion of digestible fibers in that diet (Trocino *et al.*, 2013). Restricted feed intake decreased the VFA level with the HE diet while it increased it with the LE diet regardless of age (IxD p<0.05). Concerning the fermentative profile, restricted feed intake increased acetate proportions (+2.8%, P<0.001) while decreasing those of butyrate (-2.4%, P<0.001), this effect being stronger with the HE diet (IxD, p<0.05). Similar switches occur with an increased soluble fiber fraction of the diet (Trocino *et al.*, 2013) associated with a decrease in digestive disorders (Gidenne, 2003). Thus, the beneficial effects of feed restriction upon health might be associated with modifications in the caecal fermentative parameters. Restricted feed intake also decreased propionate proportions with the LE diet while increasing them with the HE diet (IxD, p<0.001). Finally, ammonia levels increased with restricted feed intake at 42 days of age (AxI, p<0.001), but not at 50 days of age. This could be

explained by the altered intake behavior of feed restricted animals (Martignon, 2010) as they consume their daily feed portion over a very short period of time leading to a greater digestive nitrogen flow over that time period. This might lead to an overload of protein in the caecum, as the pancreatic enzymatic activity is not fully mature before 42 days of age (Scapinello *et al.*, 1999), thus leading to higher ammonia levels.

**Table 1:** Fermentative microbial activity at 42 and 50 days of age according to energetic (HE vs LE) and quantitative intake level (75 vs 100%)

	42 days of age				50 days of age				SEM	p-values					
	LE100	LE75	HE100	HE75	LE100	LE75	HE100	HE75		Age	I	D	AxI	AxD	IxD
pH	5.44	5.45	5.33	5.42	5.47	5.4	5.15	5.29	0.02	*	***				
VFA (mmol/l)	77.7	84.0	89.8	76.6	88.4	96.2	99.0	91.6	1.9	***					*
Acetate (%)	82.3	84.1	80.2	85.5	81.2	83.6	80.7	82.2	0.4		***				
Prop. (%)	4.4	3.6	4.4	4.4	5.3	3.8	3.7	4.4	0.1					**	***
Butyrate (%)	12.8	11.8	14.9	9.6	13.1	12.2	15.2	13.0	0.4		***				*
Prop./But.	0.35	0.31	0.31	0.50	0.49	0.32	0.25	0.35	0.02					*	**
NH3 (mmol/l)	8.0	13.8	6.4	14.7	4.2	3.7	5.0	6.8	0.6	***	***		***		

\* p<0.05, \*\* p<0.01, \*\*\* p<0.001; Prop.: Propionate, I: Intake level (100 vs 75), D: Diet (HE vs LE), A: Age.



**Figure 1:** Modulation of the microbial community according to energetic (HE vs LE) and quantitative intake level (75 vs 100%)

A) Alpha diversity; B) nMDS representation of the beta diversity (Bray Curtis, stress= 0.19) according to axes 1 and 2; C) significantly modulated families.

The alpha diversity (Shannon index, Figure 1A) of the caecal microbiota was differentially affected by feed intake level according to the dietary energy level (IxI: p<0.05), even though no significant differences were obtained when comparing the groups. Beta diversity was unaffected by feeding level,

diet (Figure 1B) and age (data not shown). After filtering, when taxonomic assignment was performed, 5 bacterial phyla, 12 families and 42 genera were detected. Only a minor phylum, Verrucomicrobia, was increased with high energetic intake (0.54 vs 0.25%,  $p_{\text{adjust}} < 0.05$ , data not shown). 3 families were modulated by either diet or feeding level. Akkermansiaceae, being the sole representative member of the Verrucomicrobia, was increased with high energetic intake (Figure 1C) while Christensenellaceae (Firmicutes) were decreased (2.8 vs 5.6%,  $p_{\text{adjust}} < 0.001$ ). Eubacteriaceae (Firmicutes) increased with restricted feed intake (10.5 vs 6.9%,  $p_{\text{adjust}} < 0.05$ ). These families' proportion drastically increases as of 43 days of age (Read *et al.*, 2019), and presents distinct metabolic specificities that might be associated with the beneficial health effects of feed restriction. At genus level only a minor genus of the Ruminococcaeae family (UCG-010) was increased with high energetic intake at 50 days of age (DxA  $p_{\text{adjust}} < 0.05$ , 0.41% vs 0.05%,  $p < 0.05$ ). Thus, quantitative and energetic intake level had notable effects on the caecal biotope and on a couple of bacterial taxa.

## CONCLUSIONS

Altogether, our results indicated that quantitative and energetic intake level modified the caecal microbial activity, while no major changes occurred on the microbial composition. Three bacterial families – Akkermansiaceae, Christensenellaceae and Eubacteriaceae – were however strongly affected, thus deserving further investigation to determine the potential causal effects between microbial composition, activity and impacts on the host's health.

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