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Impact of feed restriction and housing hygiene conditions on specific and inflammatory immune response, the cecal bacterial community and the survival of young rabbits

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Limiting the post-weaning intake of the young rabbit is known to improve its resistance to digestive disorders, whereas a degradation of its housing hygiene is assumed to have a negative impact on its health. This study aims at providing insights into the mechanism of digestive health preservation regarding both host (growth and immune response) and its symbiotic digestive microbiota. A 2 × 2 factorial design from weaning (day 28) to day 64 was set up: ad libitum intake or restricted intake at 70% of ad libitum, and high v. low hygiene of housing (n = 105 per group). At day 36 and day 45, 15 animals/group were subcutaneously immunized with ovalbumin (OVA) to assess their specific immune response. Blood was sampled at 36, 45, 57 and 64 days of age to determine total and anti-OVA immunoglobulin type G (IgG) and haptoglobin levels. The cecal bacterial community was explored (18 per group) by 454 pyrosequencing of genes coding for the 16S ribosomal RNA, whereas cecal pH, NH₃ and volatile fatty acid (VFA) concentrations were measured to characterize fermentative activity. A 30% reduction in feed intake reduced the growth by only 17% (P < 0.001), and improved the feed conversion ratio by 15% (P < 0.001), whereas the degradation of hygiene conditions slightly decreased the feed intake in ad libitum fed rabbits (−3.5%, P < 0.02). As poor hygiene conditions did not affect weight gain, feed conversion was improved from day 42 (P < 0.05). Restricted feeding led to a lower mortality between day 28 and day 40 (P = 0.047), whereas degraded hygiene conditions decreased overall morbidity (7.8% v. 16.6%; P < 0.01). Both a reduced intake and low hygiene conditions of housing affected microbiota composition and especially dominant genera belonging to the Ruminococcaceae family (P < 0.01). Moreover, low hygiene was associated with a higher Ruminococcaceae/Lachnospiraceae ratio (3.7 v. 2.4; P < 0.05). Cecal total VFA and pH were increased (+19%; P < 0.001) and decreased (−0.1 pH unit; P < 0.05), respectively, in feed-restricted rabbits. Neither specific anti-OVA IgG nor haptoglobin was affected by treatments. Total IgG concentrations were the highest in animals raised in poor hygiene conditions after 8 days of restriction, but decreased after 19 days of restriction in high hygiene conditions (−2.15%; P < 0.05). In conclusion, the degradation of hygiene conditions failed to induce a systematic specific and inflammatory response in rabbit, but reduced morbidity instead. Our results suggest that the microbiota composition would be a helpful source of biomarkers of digestive health.

Keywords: rabbit, housing hygiene, feed restriction, specific and inflammatory immune response, cecal microbiota

Implications

Feeding practices such as restricted feed intake are a common strategy to preserve the digestive health of the young rabbit, and to thus reduce medication during critical phases (weaning) or on farms with poor health status or hygiene levels. Feed restriction and housing hygiene have been studied to evaluate how these two factors interact on

rabbit health and performance, and to provide insights into the mechanism of digestive health preservation regarding both host growth and immune response and its symbiotic digestive microbiota adaptation.

Introduction

A high incidence of digestive diseases is observed in young mammals, particularly around the weaning period (feed transition, stress, etc.). The etiology is still poorly understood

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and is often related to distortions and instability in microbiota and the incapacity of the local innate or adaptive immune system to efficiently fight against opportunistic pathogen agents. Preventive administration of antimicrobials is therefore a common practice to control digestive diseases in pig, poultry and rabbit breeding (Chevance and Moulin, 2014), although it favors the emergence and dissemination of antibiotic-resistant bacteria. The transmission of resistance through the horizontal gene transfer (conjugation, transformation or transduction) between human and animal commensal, pathogenic and environmental bacteria represents a public health threat for humans as well as for animals. It is thus essential to find alternatives to reduce antimicrobial use while maintaining animal health.

Feed restriction is one of the most powerful leverages to reduce the incidence of digestive diseases in growing rabbits and particularly epizootic rabbit enteropathy syndrome (for review Gidenne *et al.*, 2012). A 20% intake restriction compared with *ad libitum* led to a 50% reduction of mortality and morbidity (Gidenne *et al.*, 2009). However, the underlying mechanisms supporting this effect are largely unknown. In feed-restricted rabbits, feed intake did not follow the circadian rhythm observed in *ad libitum* animals, and half of the intake occurs within 2 hours (Martignon *et al.*, 2009). This could probably explain the higher stomach acidity in feed-restricted animals compared with the freely fed ones (Gidenne *et al.*, 2009, Martignon *et al.*, 2010a). Neither intestinal morphology (villosity and crypt shape) nor enzymatic secretion is affected in feed-restricted rabbits (Martignon *et al.*, 2010a). In growing pigs, Le Floch *et al.* (2014) subjected growing feed-restricted pigs to poor housing hygiene in order to induce a moderate inflammatory status (Le Floch *et al.*, 2006). They demonstrated that feed restriction did not reduce the systemic inflammatory response caused by poor hygiene, but reduced the occurrence of digestive disorders (Le Floch *et al.*, 2014), irrespective of the hygiene conditions. Such interactions between housing hygiene conditions and feed restriction had never been studied in the growing rabbit, although housing hygiene varies greatly in field conditions and may explain the large variability of animal responses during critical periods such as weaning.

Besides its barrier role, microbiota is involved in immune organ and cell development, diversification of antibodies and mechanisms of oral tolerance. Microbiota stimulates the development of the blood vessel network of the intestinal villi (Stappenbeck *et al.*, 2002) and determines the development of follicles in the intestinal mucosa and in the vermiform appendix (Štěpánková *et al.*, 1980; Hanson and Lanning, 2008). In addition, gut microbiota contributes to innate immunity through stimulation of Paneth cell antimicrobial peptide secretion. Finally, in rabbits, the diversification of the primary repertoire of antibodies, which begins before birth and ends at the age of 10 to 12 postnatal weeks, is dependent on bacterial stimulation (Mage *et al.*, 2006; Hanson and Lanning, 2008). Thus, close interactions between symbiotic microbiota, host intestinal immune and non-immune structures or cells ensure the maintenance of

intestinal homeostasis and prevent the introduction and dissemination of pathogens in the digestive and systemic compartment. Thus, the digestive ecosystem is probably a key target for promoting health in rabbit. For instance, morbid rabbits exhibit altered fermentation profiles (increase in pH and decrease in the concentration of total volatile fatty acid (VFA), and the inversion of the propionate/butyrate ratio) compared with healthy rabbits (Martignon *et al.*, 2010b).

To obtain more insight into the mechanism of feed restriction action in the growing rabbit, this study was undertaken to evaluate the interaction between feed intake level and housing hygiene on growth performance, feed consumption and conversion, specific and inflammatory immune response, selected cecal fermentation parameters, digestive microbiota functioning and composition as well as health in rabbits.

Material and methods

Experimental design and animals

Young rabbits from both sex (*Oryctolagus cuniculus*; New Zealand White × Californian), weaned at 28 days old (day 28), were caged collectively (five rabbits per cage) and divided into four groups (*n* = 105 rabbits/group housed in *n* = 21 cages/group), according to a 2 × 2 factorial design with two levels of feed allowance, *ad libitum* feeding *v.* restricted feeding at 70% of *ad libitum*, and housing that differed in hygiene level, high *v.* low hygiene (Figure 1). Two groups of animals were housed in a room with low hygiene and fed an *ad libitum* diet ('LH-F') or a diet restricted to 70% of the voluntary intake ('LH-R'), and two other groups of animals were housed in a room with a high level of hygiene, and fed an *ad libitum* diet ('HH-F') or a diet restricted to 70% ('HH-R'). The room with a high hygiene level was cleaned before the animals' entry, including the materials: cages, feeders, pipettes and watering ramps. Cleaning with hot water (90°C) and high pressure was conducted, followed by the spraying of antiseptic foam, rinsing, and the spraying of an antiseptic detergent (Virkon; Antec International Ltd, Rotterdam, The Netherlands). In the room with low hygiene

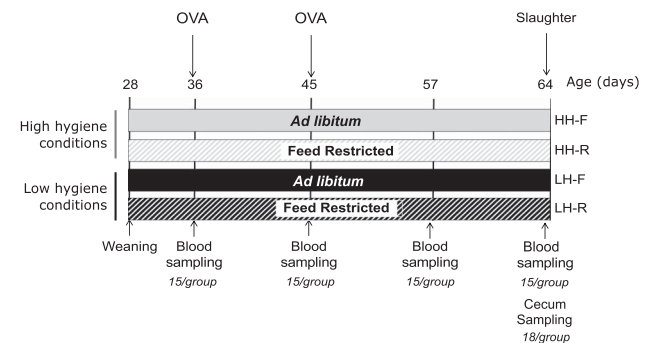


Figure 1 Schematic representation of the experimental design. The experimental groups are HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F); OVA = ovalbumin.

conditions, previous animal manure was not eliminated (only hairs were cleaned before the start of the protocol) and the room was not disinfected before the animals' entry. Similarly, no room cleaning or removal of excreta took place during the experiment. The lighting schedule alternated between 14 h of night with 10 h of light from 0100 h to 1100 h, and feed-restricted animals were fed daily in a single distribution between 0900 h and 0930 h in order to synchronize the ingestion profile of these animals and rabbits fed *ad libitum*. The amounts of feed distributed to the feed-restricted groups were calculated on the basis of a theoretical *ad libitum* ingestion curve, and readjusted according to the real ingestion of the rabbits fed *ad libitum*. Water was provided *ad libitum*. The diet was manufactured at the PECTOUL experimental unit, (INRA, Castanet-Tolosan, France), and was formulated to cover the nutritional requirements of the growing rabbits (ingredients: dehydrated alfalfa: 33.0%, barley: 14.5%, wheat: 15.0%, soybean meal: 12.0%, beet pulp: 12.0%, sunflower meal: 11.0%, minerals and premix: 2.5%). The measured chemical composition was dry matter (DM): 90.0%, CP: 20.4% DM, ether extract: 1.5% DM, starch: 18.8% DM, NDF: 33.5% DM, ADF: 20.12% DM, ADL: 6.35% DM. Animals were weighed individually once a week and feed consumption was measured per cage twice a week. Morbidity and mortality were checked daily: a control of clinical signs of digestive disorders such as diarrhea, cecal impaction, suspicion of epizootic rabbit enteropathy or other pathophysiological/pathological disorders (respiratory problems, injuries, etc.) was carried out. A health risk index was then calculated as the sum of the morbid and the dead for the initial number of animals for a given period (Gidenne *et al.*, 2009). At day 36, 15 rabbits/group were subcutaneously immunized with an injection of 600 µg ovalbumin (OVA) (Sigma-Aldrich, Lyon, France) in incomplete Freund's adjuvant (Sigma-Aldrich) and challenged 8 days later with 300 µg OVA in incomplete Freund's adjuvant. Animals were handled according to the guidelines for animals used in experiments, in agreement with French legislation (Anonymous, 2009).

Sampling procedure and analyses of cecal physicochemical parameters

Four blood samples were taken from the 15 immunized rabbits at days 36, 45, 57 and 64. Blood was collected in heparin tubes. At 64 days of age, 18 rabbits/group were sacrificed: animals were first anesthetized using an intra-muscular injection of ketamine (Imalgene™, Merial, Lyon, France; 20 to 25 mg/kg live mass), then euthanized with an intra-cardiac injection of embutramide (T61™; Intervet International GmbH, Unterschleissheim, Germany; 0.3 ml/kg live mass). The full cecum was then quickly removed and weighed, and cecal contents were sampled immediately. Lymphoid organs (spleen and vermiform appendix) were removed and weighed to measure their development. The pH was then measured in the cecum using a glass pH 'Unitrode' electrode (Pt1000/B/2/3MKCl; Metrohm™, Villebon Courtaboeuf, France). The DM of cecal content was determined by heating a 10 g sample at 103°C for 24 h. Two samples of 1 g of

fresh cecal content were diluted in storage solutions: one in HgCl₂ (2 ml, 2% w/v) and one in H₂SO₄ (3 ml, 2% w/v), for further analysis of VFA and NH₃, respectively. VFA was measured by automated gas chromatography (Chrompack CP 9000 gas chromatograph; Chrompack B.V., Middleburg, The Netherlands) and NH₃ concentration was determined by a colorimetric method using a Continuous Flow Analyzer (SAN11; Skalar, Norcross, GA, USA).

Measurements of ovalbumin-specific and total immunoglobulin type G and haptoglobin

The total concentration of the immunoglobulin type G (IgG) was measured by ELISA as already described (Accensi *et al.*, 2006). Briefly, goat anti-rabbit IgG (Fc fragment specific) was used to capture antibodies (Bethyl; Interchim, Montluçon, France). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG was used to detect antibodies in conjunction with TMB substrate (3,3',5,5'-tetramethyl benzidine; Thermo Fisher Scientific, Courtaboeuf, France). The enzyme-substrate reaction was stopped by the addition of H₂SO₄. Absorbance was read at 450 nm using an ELISA plate reader (Spectra Thermo, Tecan France, Lyon, France) and Biolise 2.0 data management software. Plasma samples were quantified by reference to standard curves constructed with known amounts of rabbit IgG. Titers of specific antibody anti-OVA were also measured by ELISA (Meissonnier *et al.*, 2008). Briefly, the anti-OVA antibodies were detected with peroxidase-labeled anti-rabbit IgG (Bethyl). Anti-OVA IgG was also measured by ELISA. Briefly, ELISA plates were coated with OVA diluted in carbonate buffer (4 mg/ml 0.1 M NaHCO₃, pH 9.6). Diluted plasma samples were then added to the plates and the anti-OVA antibodies were detected with HRP-labeled anti-rabbit IgG (Bethyl). Absorbance was read at 450 nm as previously indicated. Plasma samples were quantified by reference to standard curves constructed with rabbit hyperimmune serum. The plasma concentration of haptoglobin, an acute-phase protein, was measured on samples from 45 days to 64 days of age using a colorimetric method (Phase Haptoglobin assay T801; Tridelta Development Limited, Maynooth, Ireland).

Cecal content sample DNA extraction

Total DNA was extracted from 0.2 g of cecal content sample, combining a mechanical lysis with a FastPrep instrument (MP Biomedicals, Illkirch, France) and the QIAamp DNA stool minikit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The quality and quantity of DNA extracts were checked using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The V3 to V4 hypervariable regions of 16S ribosomal RNA genes (460 bp on *Escherichia coli*) of d64 samples were amplified with bacterial forward 343F (TACGGRAGGCAGCAG Liu *et al.*, 2007) and reverse 784R (TACCAGGTATCTAATCCT Andersson *et al.*, 2008) primers. Each primer had a barcode sequence of 11 nucleotides on 5', which was unique for each sample. PCRs were performed in a total volume of 100 µl containing 1× PCR buffer, 200 µM of

dNTP (deoxyribonucleotide), 0.5 U Isis™ DNA polymerase (MP Biomedicals, Illkirch Graphenstaden, France), 0.5 µM of forward and reverse primers, and 1 µl of DNA (1 to 5 ng/µl). The amplification program consisted of an initial denaturation step at 94°C for 2 min; 32 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, and a final extension step at 72°C for 7 min. PCR products were purified with the MiniElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Size and quality of the barcoded purified amplicon from individual samples were checked on 2% (w/v) agarose gels using a NanoDrop ND-1000 spectrophotometer. The purified PCR products were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Saint Aubin, France) on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) and then mixed in equimolar amounts to a final DNA concentration of 1000 ng/µl for each library. The pooled amplicons were pyrosequenced using a 454 FLX Titanium sequencer (454 Life Sciences, Roche Applied Science, Branford, CT, USA) at the GeT (Genomic and Transcriptomic) platform in Toulouse, France.

Sequence analysis

A total of 606 296 16S ribosomal DNA amplicon sequences were sorted based on their respective barcodes, representing the 72 collected cecal content samples. Using FROGS (Escudie *et al.*, 2015) sequences were filtered by removing sequences that did not match both proximal PCR primer sequences (no mismatch allowed), with a short sequencing length (<400 nucleotides), with at least one ambiguous base or with a long homopolymer (greater than eight). Chimeric DNA sequences were detected using VSEARCH and removed. After trimming barcodes and adaptor sequences, the average read length was 430 ± 7 nucleotides. Sequences were deposited in Sequence Read Archive: accession number SRP035383. Reads were clustered into Operational Taxonomic Units (OTUs) using SWARM (Mahé *et al.*, 2014). OTU taxonomic assignment was performed using the BLAST algorithm against the SILVA SSU Ref NR 119 database (Pruesse *et al.*, 2007).

Statistical analyses

All statistical analyses were carried out using R software, version 3.0.2 (R Development Core Team, 2008). Categorical data (morbidity, mortality and health risk index) were analyzed using a χ^2 -test. Non-repeated numerical data (growth performance, fermentative parameters and bacterial relative abundances) were subjected to ANOVA using a two-factor model – housing hygiene and intake level – and their interaction. To stabilize variance, a square root transformation was applied to bacterial community taxonomic data. Repeated numerical data (specific and total IgG and haptoglobin) were analyzed by a linear mixed model including age, and a two-factor model – housing hygiene and intake level – with their interactions as fixed effects and the rabbits as a random effect. Differences were considered significant at $P < 0.05$. The Tukey HSD test was used to perform mean comparisons. A sparse Partial Least Square Discriminant Analysis was performed in order to identify the most discriminative OTUs

in relation to treatments using the mixOmics package of R software (Lê Cao *et al.*, 2011). Three components were retained to discriminate the four groups. The number of OTU to be considered on each of the three components was assessed through the calculation of the lowest average error rate classification using a cross-validation procedure.

Results

Feed intake, growth and health status of growing rabbits

Throughout the whole experiment, the intake level of the feed-restricted group was limited to 70% of the control group fed *ad libitum*, 87 v. 123 g/day for both hygiene conditions (Table 1). No significant interaction between intake level and hygiene was observed. The average daily weight gain was reduced by 16% for the feed-restricted rabbits, whereas the intake was reduced by 30%. At the end of the experiment, these animals weighed 12% less than the control ones. After a 14-day period of adaptation, feed restriction led to a significant improvement in feed efficiency for the second period (from day 42 to day 63; –30%), resulting in an improved feed conversion of 15% throughout the experimental period. Low hygiene decreased the intake for the rabbits fed *ad libitum* (–4.1%, $P < 0.02$ between day 15 and day 36; and –3.5%, $P < 0.02$ for the entire experimental period). As hygiene conditions did not affect weight gain, feed conversion was thus slightly improved under low hygiene conditions from day 42 (Table 1). Overall mortality and morbidity reached 10.9% and 11.9%, respectively, which results in a health risk index of 22.8% (Supplementary Table S1). Mortality was not statistically different between the different groups and no significant difference was detected for the incidence of digestive disorders among the groups. However, the study of the mortality kinetics (Figure 2) shows a positive early effect on the mortality rate of restricted feed intake ($P < 0.05$ between day 28 and day 40). Surprisingly, poor hygiene conditions decreased morbidity between day 28 and day 42 ($P < 0.05$), and for the entire experimental period ($P < 0.01$). Finally, the health index risk was not affected by treatments over the entire experimental period.

Cecal physicochemical and fermentation parameters

Housing hygiene did not impact the relative weight of the cecum or the appendix, or any of the environmental parameters of the cecum. The relative weight of the cecum was greater (–1 point; $P < 0.001$; Table 2), and that of the vermiform appendix was lower in feed-restricted rabbits (–0.3 point; $P < 0.001$; Table 2). Cecal content pH was lower for the animals under a restricted diet (5.51 v. 5.60; $P < 0.05$; Table 2). The total amount of VFA was also higher for feed-restricted animals (121 v. 102 mmol/l; $P < 0.001$), but the molar proportion of acetate, butyrate and propionate were similar between feed-restricted and *ad libitum* fed animals.

Cecal bacterial community: diversity and composition

The richness and diversity index of the bacterial community remained stable regardless of the hygiene of housing or diet.

Table 1 Effect of housing hygiene and intake level (IL) on growth and feed consumption in young rabbits

	Groups				RMSE	P-levels		
	HH-F	HH-R	LH-F	LH-R		Hygiene	IL	Hygiene × IL
Feed consumption (g/day)								
28 to 42 days of age	88.1	58.8 ^u	87.1	58.6 ^u	4.5*	0.310*	nc	
42 to 63 days of age	149.6	108.5 ^u	143.4	103.3 ^u	6.6*	0.016*	nc	
28 to 63 days of age	125.3	88.6 ^u	120.9	85.4 ^u	4.8*	0.017*	nc	
Live weight (g)								
28 days of age	650	649	651	647	84	0.96	0.76	0.86
42 days of age	1363 ^a	1127 ^b	1337 ^a	1118 ^b	152	0.26	<0.001	0.61
63 days of age	2410 ^a	2115 ^b	2377 ^a	2105 ^b	211	0.33	<0.001	0.62
Weight gain (g/day)								
28 to 42 days of age	51.0 ^a	34.2 ^b	49.2 ^a	33.4 ^b	7.4	0.086	<0.001	0.47
42 to 63 days of age	49.6 ^a	47.0 ^b	49.0 ^a	46.7 ^b	6.4	0.46	<0.001	0.83
28 to 63 days of age	50.3 ^a	41.9 ^b	49.4 ^a	41.5 ^b	4.9	0.21	<0.001	0.62
Feed conversion								
28 to 42 days of age	1.75	1.73	1.78	1.76	0.09	0.13	0.30	0.88
42 to 63 days of age	3.00 ^a	2.31 ^b	2.93 ^a	2.21 ^b	0.12	<0.01	<0.001	0.77
28 to 63 days of age	2.48 ^a	2.11 ^b	2.45 ^a	2.05 ^b	0.08	0.030	<0.001	0.62

HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F); RMSE, root mean square error; nc = not calculable.

^{a,b}Means with a common superscript did not differ at $P = 0.05$ level according to a monofactorial variance analysis (group effect).

*RMSE and P -levels for the comparison of LH-F and HH-F groups only.

^uThe intake level for LH-R and HH-R groups is fixed, signifying that means have a null variance and were not compared.

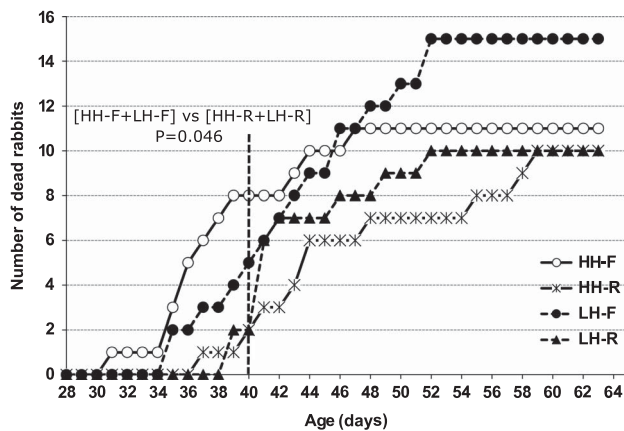


Figure 2 Effect of housing hygiene and of intake level on the post-weaning mortality kinetics in growing rabbits. HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F).

The average number of OTUs per sample was 308 ± 92 with a coverage of $95.5 \pm 2.5\%$. Chao1 index and ACE (abundance-based coverage estimators), richness estimators, represented 415 and 416, respectively, whereas the Shannon index accounted for 4.3. Using a BLAST algorithm with a minimum coverage and identity of 95%, five different phyla were found across all samples (Supplementary Figure S1). Among them, *Firmicutes* represented 83% of the total sequenced DNA. Among the other low abundant phyla, *Bacteroides* represented 5.8% of the total sequences, followed by *Proteobacteria* (0.58%) and *Actinobacteria* (0.37%). Within the *Firmicutes* phylum, the families of

Ruminococcaceae and *Lachnospiraceae* were dominant (50% and 22% of whole sequences, respectively), followed by *Christensenellaceae* (7.6%). The major families of the *Bacteroidetes* phylum are *Rikenellaceae* and *Bacteroidaceae*, representing 2.4% and 1.9%, respectively, of the sequences. Using a BLAST algorithm with a minimum coverage and identity of 95% and 97%, respectively, 76 genera could be assigned, representing 76.4% of the sequences. Among these genera, 14.2% of the sequences belonged to the genus *Ruminococcaceae* NK4A214 group, followed by the *Lachnospiraceae* NK4A136 group (9.0%) and the *Ruminococcaceae* V9D2013 group (7.4%).

Effects of treatments on the bacterial community

Low hygiene is responsible for a decrease in bacteria belonging to the *Lachnospiraceae* family, the second most important bacterial family that inhabits the cecal microbiota (19.8% v. 23.9% of total sequences; $P = 0.043$), with the *Ruminococcaceae* family being the most abundant (50.2%). Thus, a significant increase in the *Ruminococcaceae*/*Lachnospiraceae* ratio for rabbits raised in poor hygiene conditions (3.7 v. 2.4; $P < 0.05$) was observed. At the genus level, an increased abundance of bacteria belonging to the *Ruminococcaceae* UCG-008 group was observed (0.7% v. 0.4% of total sequences; $P = 0.008$, Table 3) in low hygiene rabbits. Restricted feed intake slightly increased the abundance of the *Proteobacteria* phylum (0.64 v. 0.51; $P = 0.031$, Table 3), the *Desulfovibrionaceae* family (0.59 v. 0.64; $P = 0.007$, Table 3), the *Tyzzellerella* genus (0.74 v. 0.29; $P < 0.001$, Table 3) and the *Christensenellaceae* R-7 group (5.7% v. 4.0%; $P = 0.017$, Table 3), whereas two

Table 2 Organ weight and cecal fermentation parameters according to housing hygiene and intake level (IL) in young rabbits.

	Groups				RMSE	P-values		
	HH-F	HH-R	LH-F	LH-R		Hygiene	IL	Hygiene × IL
Cecum weight/live weight* (%)	6.6 ^b	7.8 ^a	7.2 ^{ab}	8.0 ^a	0.96	0.09	<0.001	0.44
Appendix weight/live weight (%)	0.31 ^{ab}	0.30 ^{ab}	0.33 ^a	0.28 ^b	0.041	0.88	<0.001	0.07
Spleen weight/live weight (%)	0.072	0.074	0.082	0.071	0.014	0.28	0.19	0.06
Dry matter level (%)	21.7	21.3	21.5	21.2	1.4	0.54	0.30	0.87
pH	5.64 ^a	5.49 ^b	5.55 ^{ab}	5.54 ^{ab}	0.14	0.58	0.014	0.06
Total VFA (mmol/l)	105.8 ^{bc}	118.7 ^{ab}	97.6 ^c	124.3 ^a	0.14	0.77	<0.001	0.10
Acetate (%)	80.5	80.6	79.0	79.9	3.0	0.11	0.46	0.60
Butyrate (%)	14.7	14.3	15.7	15.4	2.9	0.14	0.60	0.94
Propionate (%)	4.4	4.7	4.9	4.4	1.0	0.64	0.64	0.10

HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F); VFA = volatile fatty acid.

^{a,b,c}Means with a common superscript did not differ at $P = 0.05$ level according to a monofactorial variance analysis (group effect).

*Full cecum weight (organ + content).

Table 3 Core genus according to housing hygiene and intake level (IL) in young rabbit cecum (abundance in percent of the total number of sequences)

	Groups				SEM	P-values		
	LH-F	LH-R	HH-F	HH-R		Hygiene	IL	Hygiene × IL
<i>Ruminococcaceae</i> NK4A214 group	13.75	16.01	13.42	13.77	0.93	0.736	0.765	0.504
<i>Ruminococcaceae</i> V9D2013 group	8.77	10.10	7.67	6.94	0.67	0.221	0.631	0.591
<i>Ruminococcaceae</i> UCG-014 group	7.28 ^a	3.93 ^b	5.21 ^{ab}	3.58 ^b	0.42	0.179	0.001	0.261
<i>Lachnospiraceae</i> NK4A136 group	6.24	9.12	8.94	11.64	1.46	0.067	0.126	0.387
<i>Ruminiclostridium</i>	5.33 ^{ab}	3.44 ^b	3.74 ^b	5.97 ^a	0.32	0.707	0.736	<0.001
<i>Marvinbryantia</i>	3.99	1.66	2.00	2.73	0.374	0.339	0.707	0.043
<i>Christensenellaceae</i> R-7 group	3.71	5.43	4.46	5.89	0.363	0.334	0.017	0.684
<i>Ruminococcaceae</i> UCG-013 group	2.55	2.35	1.80	2.15	0.187	0.278	0.391	0.579
<i>Bacteroides</i>	2.54	1.36	2.17	1.74	0.302	0.790	0.438	0.308
<i>Ruminococcus</i>	2.37	2.05	2.75	2.44	0.224	0.315	0.755	0.745
<i>Ruminococcaceae</i> UCG-005 group	1.90	1.34	2.00	1.25	0.218	0.902	0.214	0.814
<i>Subdoligranulum</i>	1.38	3.29	1.84	1.76	0.301	0.665	0.068	0.101
<i>Fusicatenibacter</i>	1.18	0.843	1.34	1.16	0.155	0.065	0.758	0.909
<i>Ruminococcaceae</i> UCG-011 group	1.05 ^{ab}	0.504 ^b	1.56 ^a	0.681 ^b	0.109	0.327	<0.001	0.527
<i>Roseburia</i>	0.803	0.805	0.483	0.935	0.094	0.949	0.058	0.773
<i>Blautia</i>	0.785	1.270	0.966	1.01	0.075	0.987	0.186	0.153
<i>Lachnoclostridium</i>	0.709	0.192	0.331	0.462	0.086	0.953	0.407	0.160
<i>Eubacterium ruminantium</i> group	0.66	0.629	0.268	0.209	0.074	0.058	0.731	0.633
<i>Ruminococcaceae</i> UCG-008 group	0.647 ^{ab}	0.770 ^a	0.348 ^b	0.441 ^{ab}	0.054	0.008	0.096	0.845
<i>Ruminococcaceae</i> UCG-010 group	0.632	0.471	0.643	0.404	0.057	0.893	0.604	0.422
<i>Rikenellaceae</i> RC9 gut group	0.541	1.59	0.31	0.545	0.311	0.365	0.472	0.421
<i>Rikenellaceae</i> dgA-11 gut group	0.472	0.357	0.587	1.07	0.108	0.084	0.324	0.148
<i>Tyzerella</i>	0.284 ^{bc}	0.617 ^{ab}	0.289 ^c	0.870 ^a	0.069	0.504	<0.001	0.051

HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F).

^{a,b,c}Means with a common superscript did not differ at the $P = 0.05$ level according to a monofactorial variance analysis (group effect).

Ruminococcaceae groups were reduced by half (the UCG-011 group: 1.3% v. 0.6%; $P < 0.001$; and the UCG-014 group: 6.2% v. 3.7%; $P < 0.01$, Table 3).

Among the 20 first most abundant OTUs, 12 were previously found in rabbits (Ley *et al.*, 2008, Monteils *et al.*, 2008), confirming the high specificity of the bacterial species hosting rabbit cecum. Discriminant analysis was performed to highlight the most effective among the 1160 OTUs to discriminate

between treatments (Figure 3). The first component leads to the separation of LH-F rabbits from the three other groups and was correlated with two OTUs (OTU_32 and OTU_168 affiliated with the *Ruminococcaceae* V9D2013 group and the *Ruminococcus* genus, respectively). The second component separated HH-F rabbits from the three other groups with a high level of two OTUs (OTU_66, OTU_320 *Ruminococcaceae* UCG-011 and *Ruminococcus*, respectively). Finally, the third

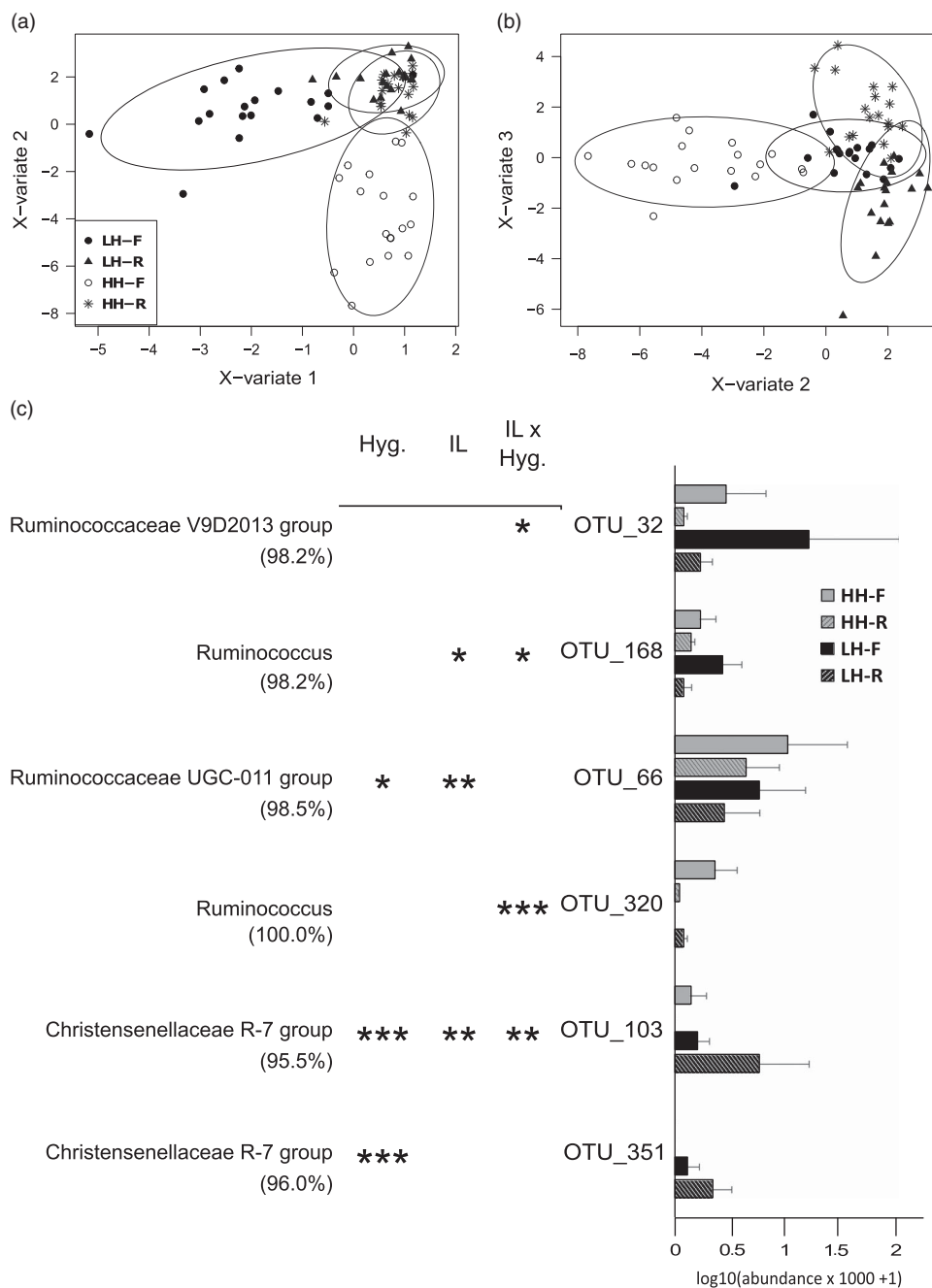


Figure 3 Rabbit cecal bacterial community sample representation from sparse partial least square discriminant analysis (a) using the first two latent variables, (b) using the second and the third latent variables. (c) Relative abundance of the six most discriminant operational taxonomic units (OTUs) among the 1160 OTUs. In brackets the percentage of identity blast hits; HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for effect of housing hygien (Hyg.), intake level (IL) and their interaction.

component barely separated LH-R from HH-R with the highest abundance for the LH-R group in OTU_103 and OTU_351, both of which belong to the *Christensenellaceae* R-7 group.

Specific and inflammatory immune response

Total plasma IgG concentrations evolved with age ($P < 0.001$) to reach their maximum level at day 57, and significantly decreased thereafter ($P < 0.05$) (Figure 4). Repeated analyses revealed a significant interaction between

the level of feed intake and the age ($P = 0.006$). At 36 days of age, total IgG were the highest in LH-R ($P < 0.05$). At day 57, feed restriction induced a reduction of the total IgG concentration in high hygiene conditions (-2.15% ; $P < 0.05$). As expected, specific plasma anti-OVA IgG concentrations were not detected before immunization (day 36). Before the second OVA immunization (day 45), anti-OVA IgG could only be measured in five out of 60 rabbits. The anti-OVA IgG concentrations increased between day 57 and

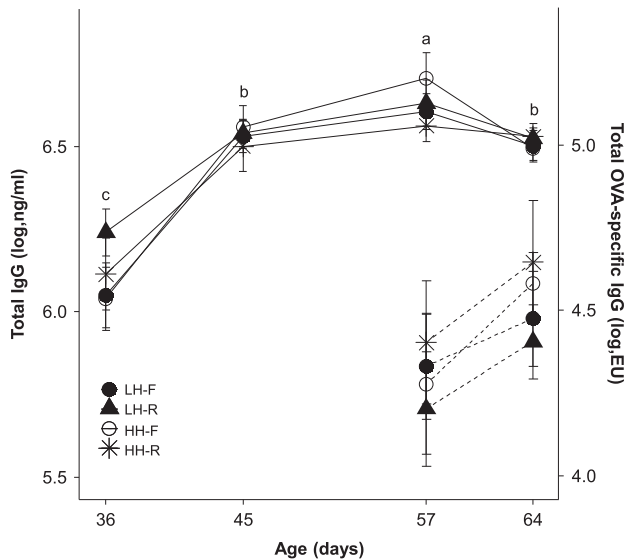


Figure 4 Effect of housing hygiene and intake level on the plasma anti-ovalbumin immunoglobulin type G (IgG) and the total plasma IgG concentrations from 36 to 64 days of age in growing rabbits. HH-F = high hygiene conditions and free intake; HH-R = high hygiene conditions and restricted intake (70% from F); LH-F = low hygiene conditions and free intake; LH-R = low hygiene conditions and restricted intake (70% from F). ^{a,b,c}Means with a common superscript did not differ at the $P=0.05$ level according to a monofactorial variance analysis (age effect).

day 64 ($P < 0.01$). Haptoglobin concentrations were significantly lower in 45-day-old rabbits than at the other ages (0.29 ± 0.17 v. 0.42 ± 0.18 mg/ml; $P < 0.05$). Neither hygiene conditions nor intake level affected total or anti-OVA IgG concentrations and haptoglobin.

Discussion

Preserving health is a major concern in livestock production. From an economic point of view, mortality and morbidity represent major economic losses. Moreover, animal health and its management directly impact the social acceptability of livestock production. During the last decades, antimicrobial substances have been massively used as a tool for health maintenance. Livestock production has recently had to address the immediate necessity of limiting the use of antimicrobial substances due to the increase in the occurrence of antimicrobial resistance. Some feeding practices such as limiting feed ingestion are known to reduce health disturbances in rabbits (Gidenne *et al.*, 2012). Conversely, poor housing hygiene conditions are suspected to be a risk factor for health (Madec *et al.*, 1998). This study was undertaken to evaluate the interaction of feed restriction and hygiene conditions on growth performance, feed consumption and conversion, specific and inflammatory immune response, selected cecal fermentation parameters, digestive microbiota functioning and composition as well as health in rabbits.

As expected, the beneficial effect of post-weaning feed intake limitation on feed efficiency in rabbits is confirmed in this study. Indeed, a reduction of 30% of the intake led only

to a 16% reduction in daily weight gain (Gidenne *et al.*, 2012; Knudsen *et al.*, 2014). However, the resulting 15% of improvement of the feed conversion ratio observed in our study was higher than previously recorded with similar limited intake levels (Gidenne *et al.*, 2009; Martignon *et al.*, 2010a). This difference might be primarily related to differences in the feed composition. Knudsen *et al.* (2014) reported that an increase of 1 MJ of digestible energy of the feed induced an improvement of nutrient digestibility (organic matter, energy and ADF) in feed-restricted rabbits.

Strategies based on intake limitation have proven to be effective in reducing morbidity and mortality in rabbits (Gidenne *et al.*, 2009). This feeding practice is efficient to reduce the incidence of digestive disorders, particularly those related to epizootic rabbit enteropathy (Gidenne *et al.*, 2012). Despite the limited number of animals, a positive effect of intake limitation could be seen in this study in the 12 days following weaning. During this period, feed-restricted rabbits also had the highest level of plasma total IgG, but this difference was only significant in low hygiene conditions. However, after a 29-day period of intake limitation in high hygiene conditions, feed restriction induced a reduction of the total IgG concentration. As previously discussed by Knudsen *et al.* (2015), the depletion in total IgG in feed-restricted rabbits is probably the result of the reduction of available energy for the immune system, which is a costly process, or a reduction in necessary available nutrients for the synthesis of these proteins. Accordingly, fecal IgA concentrations were correlated with digestible energy intake (Knudsen *et al.*, 2015). However, contrary to humoral immune response (immunoglobulins), the inflammatory response (haptoglobin) was not affected by intake limitation. This last observation is in agreement with a recent study that did not reveal any effect of the feeding level on the inflammatory response to an lipopolysaccharide (LPS) injection on the mean rectal temperature change or on the TNF- α (Tumor Necrosis Factor) concentration in rabbits fed a restricted diet for 1 week (Knudsen *et al.*, 2016). Conversely, a reduction of the LPS inflammatory response was observed in feed-restricted mice (Matsuzaki *et al.*, 2001) and rats (MacDonald *et al.*, 2011).

It is now accepted that gut intestinal microbiota is involved in health preservation. Indeed, this symbiotic partner has evolved with its host and is largely involved in gut associated lymphoid tissue trophic development and stimulation. More specifically in rabbits, it has been shown that microbiota determines the development of follicles in the intestinal mucosa and in the vermiform appendix (Štěpánková *et al.*, 1980; Hanson and Lanning, 2008). In addition, the diversification of the primary repertoire of antibodies in rabbits is dependent on bacterial stimulation (Mage *et al.*, 2006; Hanson and Lanning, 2008). In the present study, we explored microbiota composition and functioning changes according to hygiene conditions and feed intake level. Cecal ecosystem functioning as observed through fermentative parameters was not affected by hygiene conditions or feed intake level. The higher total VFA concentration and the associated lower pH

observed in feed-restricted compared with *ad libitum* rabbits is probably the result of the difference in ingestion behavior. Feed-restricted rabbits did not follow the circadian rhythm observed in rabbits fed *ad libitum* and ate half of the intake within 2 h (Martignon *et al.*, 2009; Gidenne *et al.*, 2012). We demonstrate here for the first time that microbiota composition was modified in feed-restricted young rabbits and that this mainly concerns the genera belonging to the *Ruminococcaceae* family. In steer colon, OTUs classified within *Ruminococcaceae*, *Lachnospiraceae* and *Clostridiaceae* families have been associated with feed intake levels (Myer *et al.*, 2015). Using fingerprint techniques, Abecia *et al.* (2007) showed that the structure of the cecal microbiota of lactating rabbit was influenced by the level of intake.

In the present study, low housing hygiene conditions had no negative impact on health and growth. We instead observed a positive effect on morbidity. Conversely in pig, low hygiene conditions decreased growth performance and induced a systematic inflammatory response (Le Floc'h *et al.*, 2014). This discrepancy might be related to a species-specific response to hygiene degradation or to the extent and duration of hygiene degradation. In our conditions, a poor hygiene environment had a positive action on microbial colonization, as described in a study conducted on pig that compared indoor, outdoor and isolator-reared piglets (Mulder *et al.*, 2009). Low hygiene conditions were associated with an increase in the *Ruminococcaceae*/*Lachnospiraceae* ratio that could best be explained by a lower abundance of *Lachnospiraceae*. *Lachnospiraceae* and *Ruminococcaceae* are two of the most abundant families found in rabbit gut (Huybens *et al.*, 2013; Bauerl *et al.*, 2014; Combes *et al.*, 2014) and in other mammalian gut environments (Biddle *et al.*, 2013). Both families have been associated with gut health (Berry and Reinisch, 2013; Biddle *et al.*, 2013). In a study comparing healthy rabbits and rabbits suffering from epizootic rabbit enteropathy, the former group was linked to two uncharacterized genera of the *Ruminococcaceae* family and one genus of *Lachnospiraceae* family (Bauerl *et al.*, 2014). Moreover, in rabbit, stimulation of coprophagia behavior in the nest sped up microbiota maturation, reduced the mortality rate and, a reduction of *Lachnospiraceae* abundance was also observed (Combes *et al.*, 2014). We confirmed the uniqueness of the rabbit cecal microbiota composition (Ley *et al.*, 2008; Monteils *et al.*, 2008; Bauerl *et al.*, 2014), and suggested some putative OTUs whose abundances were correlated with treatments. Unfortunately, the microbial species that inhabit the rabbit cecal ecosystem are largely unknown. Further studies are needed to confirm that the taxonomic units highlighted in this study (e.g. bacterial families, genera and OTUs) could be relevant microbial biomarkers of intestinal health in rabbit. If this hypothesis is confirmed, the definition of microbial biomarkers could be harnessed to define new rearing strategies to preserve health. Moreover, metagenomic studies on single bacterial cell (e.g. the exhaustive exploration of whole bacteria genomes) would be helpful to obtain insights into species living in the rabbit gastrointestinal tract and to understand the functional link between the presence of a set of microbe species and its effect on the host.

Conclusion

We confirmed that feed restriction in growing rabbits is a relevant strategy to preserve health and to improve feed efficiency. Contrary to what was expected, the degradation of hygiene conditions failed to induce a systematic specific and inflammatory response in rabbit, but reduced morbidity instead. However, both a reduced intake and low housing hygiene conditions after weaning affected microbiota composition and particularly genera and OTUs belonging to the dominant *Ruminococcaceae* family. Together, these results suggest that the microbiota composition would be a helpful source of biomarkers of health. However, further studies are needed to obtain a greater insight into the microbiota composition signature of health and diseases.

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Supplementary material

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