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PRESLAUGHTER FEED WITHDRAWAL TIME AND ITS EFFECT ON RABBIT BLOOD MEASURES, GASTROINTESTINAL TRACT PARAMETERS AND *LONGISSIMUS LUMBORUM* GLYCOLYTIC POTENTIAL

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Abstract: This study aimed to characterise the physiological response of rabbits to feed withdrawal without stress caused by crating and transport to the slaughterhouse. A total of 72 recently weaned Grimaud rabbits were allocated into 12 cages, each with 6 rabbits (3 females and 3 males, to reflect commercial practices). A preslaughter feed withdrawal time (FWT) was randomly assigned to each of the 12 cages (t=0, 3, 6, 8, 10, 12, 14, 16, 18, 20, 22 or 24 h). Blood lactate and cortisol concentrations were measured at exsanguination. These observations did not indicate an elevated level of stress in the rabbits (P>0.05). The maximum *Longissimus lumborum* glycolytic potential was observed for rabbits that fasted for 3 and 6 h and was relatively stable from 127.78 to 139.04 µmol/g for rabbits with FWT longer than 12 h. As expected, gastrointestinal tract and stomach content weights were lower for rabbits with longer FWT (P<0.0001), while caecum weights did not (P=0.051*).* Rabbits with longer FWT had lower stomach pH and higher caecum pH (both P<0.0001). Metataxonomic 16S analysis revealed that FWT had a significant effect (all P<0.01) on microbiome beta diversity in faeces and caecum. The polymerase chain reaction analysis using specific primers revealed *Enterobacteriaceae* presence in the faeces of male rabbits only at 18 and 22 h. Our results suggest that the caecotrophic behaviour of rabbits allows them to be particularly resistant to hunger despite their small size. However, to limit *Enterobacteriaceae* shedding, the FWT should not exceed 18 h.

Key Words: fasting, glycolytic potential, muscle, preslaughter management, rabbit, stress.

INTRODUCTION

Preslaughter feed withdrawal is commonly used in the livestock industry to reduce the risk of a visceral puncture during evisceration by decreasing the gastrointestinal tract (GIT) weight (Martín-Peláez *et al.*, 2008). The optimal FWT appears to be species dependent. Feed withdrawal reduces economic losses for the producer by decreasing post-transport mortality and saving on feed (Eikelenboom *et al.*, 1991; Petracci *et al.*, 2010). Fasting the animals before slaughter also reduces the amount of non-edible waste at the slaughterhouse (Eikelenboom *et al.*, 1991; Dalle Zotte, 2002). Feed withdrawal can also be applied during the fattening period in rabbit production to reduce the morbidity and mortality related to enteric disease by feed restriction (Tuleda and Lebas, 2006). When feed is restricted, rabbit should have access only once a day to a large amount of feed to limit dominance over feed and to ensure that body weight homogeneity is not affected (Tuleda and Lebas, 2006).

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Feed withdrawal time represents the length of time that an animal is deprived of feed. The feed withdrawal period starts at the moment feed is withdrawn at the farm and ends at the point of slaughter. FWT therefore includes the time at the farm, during transport and during lairage at the slaughterhouse (Verga *et al.*, 2009).

Despite the benefits associated with an adequate FWT, if it is too long, it can lead to poor animal welfare. This is evidenced by the increased aggressiveness observed in pigs, such as longer and more intense fighting (Faucitano *et al.*, 2006). Research suggests that it is best if most of the FWT for pigs occurs in their familiar farm environment to reduce stress. Conducting the majority of the FWT at the slaughterhouse prevented the pigs from properly resting before slaughter due to longer fights and increased the occurrence of dark, firm and dry meat (DFD; Dalla Costa *et al.*, 2016). A prolonged FWT can also reduce live body weight. Although loss of stomach weight occurs in the first few hours of feed withdrawal, longer FWT can cause weight loss through the loss of nutrients and humidity in rabbit corporal tissue, leading to reduced meat quality and carcass yield (Bianchi *et al.*, 2008; Frobose *et al.*, 2014; Cornejo-Espinoza *et al.*, 2016). Longer FWT have also been shown to increase caecum pH to near-neutral levels in pigs, which can alter fermentation and produce favourable conditions for the growth of *Enterobacteriacea* such as *Salmonella* sp. and *Escherichia coli* (Lebas *et al.*, 1997; Nattress and Murray, 2000; Martín-Peláez *et al.*, 2009). This could also increase faecal shedding of those undesirable bacteria, creating a contamination risk for the carcasses and threatening product safety (Nattress and Murray, 2000; Reid *et al.*, 2002; Faucitano *et al.*, 2010). In rabbits, little information is available for the microbiological data in healthy rabbits at slaughter, but Kohler *et al.* (2008) found that *Enterobacteriaceae* were detected on 23.6% of the 500 rabbit carcasses sampled with counts <1.5 Log CFU/cm^2. Furthermore, *Enterobacteriacea* are commonly used as an indicator of faecal contamination in slaughterhouses (Kohler *et al.,* 2008).

Fasting can affect blood glucose levels and muscle energy reserves in animals (Warriss *et al.*, 1998; Leheska *et al.*, 2002). Because carbohydrates are the main source of glucose in the blood of non-ruminant animals, an animal that is fasting must break down liver glycogen reserves to maintain stable blood glucose levels (Leheska *et al.*, 2002; Savenije *et al.*, 2002). When the FWT is too long, liver glycogen reserves are depleted and blood glucose levels decrease (Warriss *et al.*, 1988). In the muscles, glucose and glycogen reserves are used as primary energy sources for muscle contraction during exercise and times of stress. Intense muscular activity before slaughter has been shown to lead to glucose and glycogen depletion (Leheska *et al.*, 2002; Warriss, 2010). However, the effect of fasting on muscle glycogen reserves is variable according to the literature. Some authors reported a decrease in muscle glycogen reserves in pigs (Wittmann *et al.*, 1994; Bertol *et al.*, 2005) and poultry (van Schalkwyk *et al.*, 2005; Wang *et al.*, 2017) following a 24-h FWT. Meanwhile, Bidner *et al.* (2004) found a 36-h FWT to have no effect on muscle glycolytic potential (GP) in pigs. The GP represents all components that can be converted into lactic acid in the muscle after slaughter (Monin and Sellier, 1985). A lower GP has been associated with a higher ultimate pH (pH_u) in *post mortem* skeletal muscle (Hulot and Ouhayoun, 1999; Hamilton *et al.*, 2003). A higher pH_u was also associated with increased FWT in rabbits (Bate-Smith and Bendall, 1949; Kola *et al.*, 1994; Xiong *et al.*, 2008). Meat pH is an important parameter for all livestock species, as it influences meat quality characteristics such as water-holding capacity (WHC), colour and tenderness (Hulot and Ouhayoun, 1999). Meat with a pH already lower than 6 at 45 min *post mortem* (pH_{45min}) can be described as pale, soft and exudative (PSE). When the pH_u is greater than 6, the meat is dark, firm and dry (DFD). To have a good meat quality, ultimate pH in the LL muscle should be lower than 6 at 24 h *post mortem* (Mach *et al.*, 2008; Mazzone *et al.*, 2010). For rabbits, DFD-like meats have been reported in the literature, but PSE-like meats have not (Cavani *et al.*, 2009; Koné *et al.*, 2016; Larivière-Lajoie *et al.*, 2021).

According to the Code of Practice for the Care and Handling of Rabbits, it is recommended that FWT should not exceed 24 h (NFACC, 2018). More recently, the Panel on Animal Health and Welfare (AHAW; EFSA, 2022) concluded that rabbits subjected to FWT longer than 6 h are likely to experience prolonged hunger and that a FWT longer than 12 h will cause the rabbit to lose weight. Although the literature on the impacts of fasting on rabbit meat quality is limited compared to other livestock species, a 12-18 h FWT for rabbits is suggested to achieve acceptable stomach weight without having major effects on meat quality (Kola *et al.*, 1994; Bianchi *et al.*, 2008; Xiong *et al.*, 2008). However, the studies that put forward this recommendation focus mainly on meat quality and not on other parameters. To the best of our knowledge, no study has examined blood and gut digesta parameters or *Longissimus lumborum*

(LL) GP during preslaughter feed withdrawal. Our objective is to study the effect of FWT on the physiological response of rabbits without the stress caused by crating and transport, in order to better understand and optimise preslaughter management practices for rabbit meat production.

MATERIALS AND METHODS

Animal housing and feeding

All experimental procedures involving live rabbits were approved by the Animal Use and Care Committee at Université Laval, which follows the guidelines from the Canadian Council on Animal Care (CCAC, 2009).

This study was performed with 72 recently weaned Grimaud rabbits (35-d old; Laprodéo, Saint-Tite, Quebec, Canada). Animals were kept at a Université Laval animal research facility in conventional commercial cages that allowed for 0.37 m^2 of space per rabbit. Upon arrival, each rabbit was weighed and then allocated according to weight into 12 cages, each with 3 males and 3 females to reflect commercial practices and to allow them to express their natural gregarious behaviour. A cycle of 12 h of light and 12 h in the dark was maintained throughout the experiment. The temperature was set at 20°C and the humidity level at 37%. Animals were fed *ad libitum* with a commercial diet (Supplementary Material 1). Weekly, all rabbits were manually weighed individually to determine body weight (BW), and average daily gain (ADG; g/d) was calculated by weight difference divided by the 7-d period. Daily feed intake (feed given minus refusal per cage; g) was also measured for each week. Average daily feed intake per animal (ADFI; g/d) was calculated by dividing the total feed consumed over a week by the number of rabbits and the number of days (n=7). Feed conversion ratio (FCR) was calculated by dividing ADFI by ADG. Rabbits were fed until they reached an average commercial slaughter live BW of 2318±169 g (55 d old).

Behaviour observations

As requested by the Animal Protection Committee, rabbit behaviour was evaluated using the scan sampling method and was monitored as a critical control point to terminate the experiment if animal welfare was impaired unduly. Before the observations, a period of 10 min with the observer in the room was applied to limit the observer's influence on rabbit behaviour. Ten scans were performed every 1 min during 10 min prior to slaughter, using an observation grid. First, each rabbit's posture was noted. Posture included sitting, lying down or moving. Then, the presence and type of activities such as resting, drinking, grooming, biting the cage, mating, being at the empty feeder, moving, shaking, stamping feet, sneezing and scratching the cage were noted. Resting behaviours included sitting and lying down when rabbit exhibited no other activities. The interactions were noted as either aggressive or non-aggressive. Aggressive behaviours include chasing and triggering escape, leaping, biting another rabbit, bouncing and paw scraping. All behavioural assessments were performed by the same two observers over the 24-h observation period. The observers evaluated the first two groups together to standardise their observations, and then each took a 12-h shift, one following the other.

Slaughter procedures

A preslaughter FWT was randomly assigned to each of the 12 cages (t=0, 3, 6, 8, 10, 12, 14, 16, 18, 20, 22 or 24 h) at the beginning of the feed withdrawal period. Water remained available at all times prior to slaughter. Rabbits were slaughtered in an adjacent room to avoid stress related to transport and lairage at the slaughterhouse. Feed withdrawal started at 7:30 a.m. after the lights were turned on and the feeders were removed. Each animal's jugular vein was excised after proper stunning using a non-penetrative captive bolt pistol (Zephyr E, Bock Industries, Krugersdorp, South Africa).

Blood measures

Blood lactate was measured for all rabbits in duplicate at the time of exsanguination using hand-held lactate analysers (Lactate scout +, EKF Diagnostics, Cardiff, Wales, UK) according to the manufacturer's instructions. Blood samples were collected, maintained on ice and then centrifuged at 3000 $\times g$ for 10 min at 4°C to collect blood serum. Analyses for cortisol, glucose and urea serum content were performed in an external laboratory (Biovet, Ste-Hyacinthe, QC, Canada).

Digesta collection

Full gastrointestinal tracts were removed promptly after slaughter. They were put on ice and then transported to the dissection laboratory in an adjacent building to be weighed and for samples to be taken. Caecum samples for DNA extraction were aseptically harvested, as well as faeces taken directly from the colon, and were immediately frozen at –80°C. Stomach and caecum weights were recorded both when full and emptied of their contents, which were then thoroughly mixed. Stomach and caecum pHs were measured using a portable pH meter (ROSS, Orion Star A221, Thermo Scientific, Beverly, CA, USA) combined with an Orion Kniphe electrode (ThermoFisher, Nepean, ON, Canada) and a temperature compensation probe (928 007 MD, micro probes ATC, Maryland, USA). The dry matter content of the stomach contents was determined using lyophilisation as describe in Larivière-Lajoie *et al.* (2021).

Glycolytic potential

A sample of LL muscle was harvested from each rabbit in the region between the third and fourth left ribs to evaluate muscle GP immediately after slaughter. Within each slaughter time, all samples were transported on dry ice and immediately frozen at –80°C upon arrival at the laboratory until analysis. Glycolytic potential measurements were performed in triplicate using the modified method from Monin and Sellier (1985) as described by Rocha *et al.* (2015). The glycogen was decomposed to glucose and glucose-6-phosphate, and the lactate concentration was evaluated to calculate the GP according to the following formula: 2 ([glycogen]+[glucose]+[glucose-6-phosphate]) +[lactate]. GP is expressed as µmol glucose equivalent/g of muscle.

Molecular characterisation of the gut microbiome

Total genomic DNA was extracted from 250 mg of homogenised faecal and caecum samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA purity and quantity were assessed with a Nanodrop 2000 (Thermo Scientific, Wilmington, USA). DNA (750 ng/rabbit) was pooled to obtain two composite samples per FWT for both the caecum and faeces samples. Each composite sample was composed of DNA from the three female or three male rabbits from the same cage.

For amplification of the 16S RNA gene, equimolar pooling and sequencing were performed at our genomic analysis facility (IBIS, Université Laval, Quebec City, Canada). Briefly, the 16S V3-V4 region was amplified using the sequence-specific regions described in Klindworth *et al.* (2013) using a long oligo polymerase chain reaction (PCR) approach. The PCR reactions were purified using the Axygen PCR cleanup kit (Axygen, Corning, NY, USA). Barcoded amplicons were pooled in equimolar concentrations and sequenced on an Illumina MiSeq (paired-end 300 bp with two index reads).

The quality of the sequenced reads was performed using FastQC (version 0.11.5; Andrew, 2010). An amplicon sequence variant (ASV; Callahan *et al.*, 2017) table was then generated using the DADA2 (Callahan *et al.*, 2016; version 1.20.0) package workflow in R (R Core Team, 2017; version 4.1.1). For the filtration steps, primers were removed by trimming the first 17 nucleotides of the forward and the first 21 of the reverse reads. Sequences that contained ambiguous nucleotides (N) were discarded. The expected error filter was not used, in accordance with Prodan *et al.* (2020). Dereplication, sample inference, chimera identification and merging of the paired-end reads were performed using default parameters, with one exception: samples were pooled at the sample inference step. Taxonomic assignment was conducted using the SILVA rRNA database (release 138.1; Pruesse *et al.*, 2007) with the functions assignTaxonomy and addSpecies. A maximum likelihood phylogenetic tree was then constructed using DECIPHER R package (version 2.20.0; Wright, 2016) and phangorn R package (version 2.8.1; Schliep, 2011). Non-

bacterial ASVs, those without an identified phylum, and those of low prevalence (present in less than 5% of the samples) were filtered. Data were then rarefied to 90% of the minimum sample depth. Filtered and rarefied ASV were searched against the NCBI nr/nt 16S curated database (Bioproject 33175 or 33317; excluding archea; accessed 05-11-2021) in GenBank using BLASTN (version 2.12.0; Altschul *et al.*, 1990). When the queried ASV had more than 97% identity, the species name from the NCBI 16S database was added. The same procedure was performed for genus reassignment at a 90% identity threshold and for Family consensus. The ASV counts were normalised into relative abundances. Bar plots were produced using the phyloseq R package (version 1.36.0; McMurdie and Holmes, 2013). Taxa with an abundance of less than 1% were grouped in the "Other" category. Sequences were deposited in GenBank under accession number SUB12179058.

To improve detection of *Lactobacillaceae* and *Enterobacteriaceae*, specific PCR analyses were performed. The oligonucleotide primers used for PCR amplification were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). For *Lactobacillaceae,* DNA was PCR amplified using the PerfeCTa® SYBR® Green FastMix® Reaction Mixes (Quanta Biosciences, Beverly, MA, USA). Five ng of female or male pooled DNA was used for each specific PCR amplification. For *Enterobacteriaceae,* DNA amplification by PCR was performed using a volume of 25 µL that contained 1 X PCR Buffer with Mg+ (Bioshop Canada Inc., Burlington, Ontario, Canada), 0.2 µM of each primer, 200 µM of each dNTPs, 2.5 U of Taq DNA Polymerase (Bioshop Canada inc.) and 100 ng of pooled sample DNA.

For *Lactobacillaceae*-specific 16S gene amplification, the following cycling conditions were applied: initial denaturation at 95°C for 10 min, 50 cycles of denaturation at 95°C for 10 s, annealing at 54°C for 10 s, extension at 72°C for 20 s, and another extension at 72°C for 2 min (Forward primer: AGC AGT AGG GAA TCT TCC A; Reverse Primer: CAC CGC TAC ACA TGG AG; Walter *et al.*, 2001; Heilig *et al.*, 2002). For *Enterobacteriaceae,* two specific primers were used: PS1 and PS6. PS1 is a primer with a larger detection spectrum that can detect all the bacteria formerly known as *Enterobacteriaceae* (known as *Enterobacteriales* since 2016; Adeolu *et al.*, 2016), while PS6 is a more specific primer that targets the correct and up-to-date *Enterobacteriaceae* taxonomic group (Resendiz-Nava *et al.*, 2022). The following cycling conditions were applied for both primers: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and another extension at 72°C for 5 min (PS1 forward primer: 5'-GGGGATAACYACTGGAAACGGTRGC-3'; reverse primer: ACT 5'-GCATGGCTGCATCAGGSTTKC-3'; Kurina *et al.*, 2020 and PS6 forward primer: 5'-GACCTCGCGAGAGCA-3'; reverse primer: 5'-CCTACTTCTTTTGCAACCCA-3'; Hansen *et al.*, 2012). PCR amplifications were conducted in a Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany). PCR reaction yield was verified using agarose gel electrophoresis. A 5-µL volume of PCR product was mixed with 4 µL of DNA-free water and 1 µL of 10X BlueJuice gel loading buffer (Thermo Fisher Scientific, Waltham, MA, USA). The PCR product was migrated on an agarose gel (1% agarose; Agarose A, Biobasic, Markham, On, Canada; 99% 1X TEA buffer; Tris Acetate-EDTA buffer, Milipore Sigma, Oakville, On, Canada; and 1X GelRed® nucleic acid stain; Biotium, Fremont, CA, USA) for 45 min at 240 V. Gel illumination was performed at 254 nm using a BioDoc-It 220 Imaging System (Ultra-Violet Products Ltd., Cambridge, UK). The PCR amplifications were carried out in a Lightcycler480 (Roche Diagnostic, Laval, Quebec, Canada). The positive PCR amplicons were sequenced to confirm the specific amplification product using Sanger sequencing at the *Plateforme de séquençage et de génotypage des génomes* of the *Centre hospitalier universitaire* (Université Laval, Quebec City, Canada) and aligned using NCBI blast (version 2.10.0).

Statistical analysis

A random experimental design was used in which the experimental unit was the rabbit, since the animals were all raised and fasted under the same conditions and because the final data was obtained at slaughter on an individual basis on the carcass as they formed a stable group, were of the same age, weight and fed the same diet during the growing period. For blood and gut digesta parameters as well as GP, data were checked for normality and homogeneity of variances. When variance normality and homogeneity could not be assumed, data were transformed to achieve normality and/or homogeneity of variances. Values for blood lactate and cortisol concentrations were log-transformed (log_{10}) to normalise the data prior to analysis. Then, the data were assessed with an analysis of variance (ANOVA) using the R software GLM procedure (R Core Team, 2017; version 4.1.1) to determine the effects of FWT on blood parameters, gut digesta parameters and GP. In this model, FWT was considered to be a fixed effect. For blood lactate and cortisol concentrations, data were also analysed using the same method as previously described. The effect

of slaughter order on blood lactate was also measured to evaluate stress variation amongst individuals, as rabbits are known to exhibit gregarious behaviour (Chu *et al.,* 2004). Results were considered significantly different when a *P*-value was lower than 0.05 and a tendency when *P*-value was lower than 0.10. If significant, ($P<0.05$), differences between experimental groups were compared using the Emmeans R package (version 1.7.0) and adjusted with a Tukey test. When transformed for statistical analysis, data were then back-transformed to their original scale for graphical representation and interpretation. A confidence interval of 95% was then used as the dispersion parameter instead of standard error, since this allows for a better understanding of the dispersion in the original scale when data are back-transformed (Lee, 2020). Pearson's correlation coefficients were calculated to measure the correlation with physiological variables using the R package corrplot (version 0.92). The 16S rRNA gene sequences were categorised based on sample type (faeces or caecum), sex (female or male) and FWT (0 to 24 h). Alpha diversity (within a sample) was calculated with phyloseq (version 1.30.0). The species richness was evaluated with the Observed and Chao1 indexes and both richness and evenness were evaluated with the Shannon and Simpson indexes. To evaluate the differences between each FWT, a Tukey HSD test was performed between all the samples using the Emmeans R package (version 1.7.0). A *P*-value lower than 0.05 was considered significant for the tests.

A clustering analysis based on principal component analysis (PCA) was performed using the FactoMineR and Factoextra R packages (version 2.4 and 1.0.7; Lê *et al.*, 2008; Kassambara and Mundt, 2020). A dendrogram was then fitted onto the generated bar plots using the R package ggdendro (version 0.1.23; de Vries and Ripley, 2022).

Beta diversity (between samples) was calculated using the unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) and the Bray-Curtis dissimilarities (Bray and Curtis, 1957) with the phyloseq R package (version 1.36.0). Principal coordinate analysis (PCoA) was used to visualise the distances using ampvis2 (version 2.7.10). Correlation with physiological variables (blood lactate, cortisol, glucose and urea concentrations, GIT weight, stomach content weight and caecum weight, stomach and caecum pH and stomach DM) was added to the graphs as red arrows, using the envfit function of the vegan R package (Oksanen *et al.*, 2020; version 2.5-7). The arrows were scaled to 25% to properly display the data. Permutational multivariate analysis of variance (PERMANOVA) was performed using the Adonis function of the vegan R package with 9999 permutations to analyse the unweighted and weighted UniFrac distances and the Bray-Curtis dissimilarities for sex and FWT. Permutational analysis of multivariate dispersions (PERMDISP) was used to test the homogeneity of the dispersion for each metadata category using the betadisper function of the vegan R package. Differences were considered significant when a *P*-value was lower than 0.05 and a tendency when *P*-value was lower than 0.10.

The linear discriminant analysis effect size (LEfSe) method was performed on non-normalised data (raw ASV counts) using microbiomeMarker R package (version 1.6.0; Cao *et al.*, 2022). Phyla with higher relative abundances in the three FWT groups were identified by LEfSe. The size effect of each of these phyla was calculated using linear discriminant analysis (LDA; Segata *et al.*, 2011). A LDA score (Log10) of 2.0 was used as the cut-off for identifying biomarkers.

RESULTS

Growth performance

From the beginning to the end of the trial (23 d), rabbits weighed on average 1202±66 g (minimum: 1003 g, maximum: 1302 g) and 2318±169 g (minimum: 1675 g, maximum: 2670 g), respectively. For the first and last week of the trial, the ADG went from 57.8±4.2 to 45.4±3.6 g, the ADFI from 125.5±9.4 g to 162.3±12.5 g and the FCR from 2.18±0.14 to 3.59±0.30, respectively.

Behaviour analysis

The animals remained calm during the observation periods prior to being slaughtered and hardly moved at all (≤6.1%). They mostly either remained sitting or lying down (*i.e.*, resting), with very few rabbits moving (Figure 1A). Furthermore, interactions between animals were limited (<7.6%) and no aggressive interactions were observed (Figure 1B).

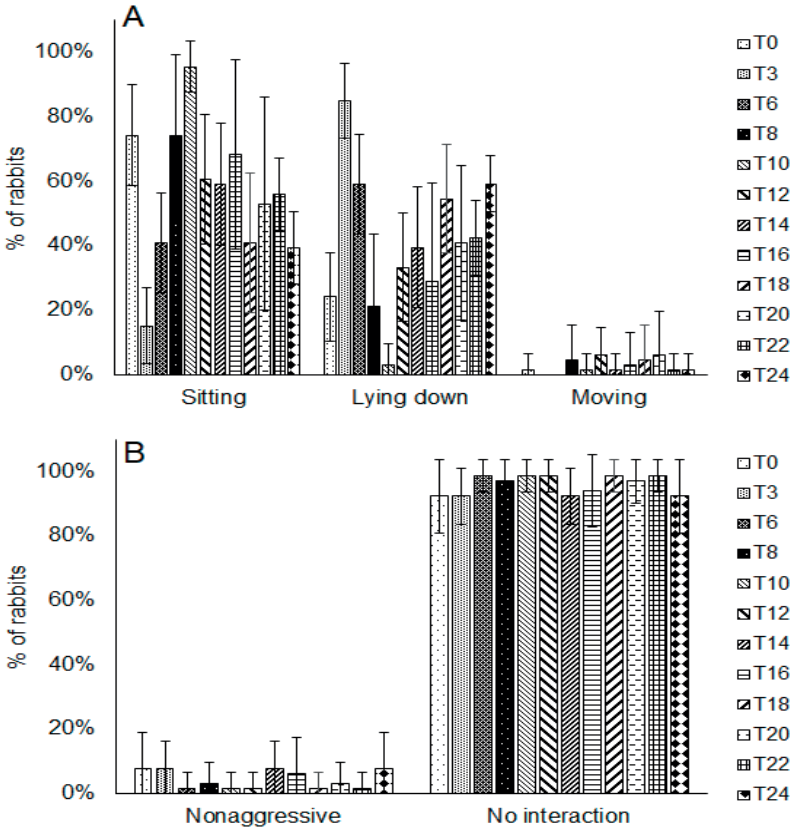

Figure 1: Rabbit posture (A) and type of interaction (B) during observations, prior to slaughter (mean±standard deviation). ▫ T0; ▩ T3; ▩ T6; ■ T8; ▧ T10; ◪ T12; ▨ T14; ▤ T16; ◩ T18; ▫ T20; ▦ T22; ◧ T24. No aggressive interactions were observed.

Rabbits were mostly resting prior to slaughter (≥62.1% of rabbits resting), except for rabbits after 10 h of FWT (36.4%; Table 1). No rabbits were observed stretching, stamping their feet or eating their caecotrophs (Table 1).

Blood parameters and muscle glycolytic potential

Blood lactate concentrations were not affected by the FWT (P=0.18). However, the maximum mean was observed at 0 h. Concentrations went from 5.01 to 1.72 mmol/L within the first 6 h of feed withdrawal and then remained relatively stable at <2.98 mmol/L (Figure 2A). The slaughter order had no effect on blood lactate concentrations (data not shown). Blood glucose concentrations were lower (P<0.0001) and blood urea concentrations were higher (P<0.0001) for animals with longer FWT (Figure 2B). The concentrations of LL G6P were not significantly affected by the FWT (Figure 2C). The LL muscle lactate concentrations were lower for rabbits that fasted for 0 h (37.49±0.57 µmol/g) and, for the other groups, it remained relatively stable between 52.65±0.80 and 62.60±1.10 µmol/g (Figure 2D). A maximum LL GP of 151.55±5.77 µmol/g was reached for rabbits that fasted for 3 and 6 h of FWT (P=0.001) and decreased to 126.30±5.82 µmol/g for rabbits with a 10 h of FWT (Figure 2E). Levels remained relatively stable between 127.78 and 139.04 µmol/g for the longer FWT. No significant difference was found between fasting groups for cortisol levels (P=0.34) and were, on average, 30.10±17.93 nmol/L (data not shown). The first rabbits slaughtered in each group had blood cortisol concentrations that were lower than in the fifth and sixth rabbits slaughtered (P=0.01; Figure 2F).

Table 1: Percentage (%) of rabbits displaying different types activities during the observation period prior to slaughter.[1]

Variables	T0	T3	T6	T8	T10	T12	T14	T16	T18	T20	T22	T24
Type of activity, %												
Resting	90.9±8.7	95.5±7.8	78.8±15.1	65.2±15.7	36.4±26.7	63.6±14.6	65.2±18.9	51.5±18.9	77.3±21.4	62.1±24.8	65.2±17.4	83.3±12.9
Drinking	1.5±5.0	NO[2]	NO	18.2±17.4	18.2±9.0	7.6±11.5	10.6±8.4	9.1±13.7	4.5±10.8	7.6±11.5	6.1±8.4	NO
Grooming	4.5±7.8	3.0±6.7	10.6±13.5	7.6±11.5	31.8±17.4	16.7±10.5	18.2±13.9	33.3±14.9	10.6±11.2	19.7±16.4	15.2±11.7	4.5±7.8
Biting their cage	1.5±5.0	NO	NO	1.5±5.0	6.1±8.4	7.6±8.7	4.5±7.8	1.5±5.0	4.5±7.8	1.5±5.0	9.1±11.5	9.1±8.7
Mating	NO	NO	NO	NO	NO	NO	NO	1.5±5.0	NO	NO	NO	NO
At the empty feeder	NO	1.5±5.0	1.5±5.0	4.5±7.8	4.5±7.8	NO	NO	1.5±5.0	NO	1.5±5.0	NO	NO
Moving	1.5±5.0	NO	NO	1.5±5.0	1.5±5.0	4.5±7.8	NO	1.5±5.0	3.0±10.1	6.1±13.5	1.5±5.0	1.5±5.0
Shaking	NO	NO	NO	NO	NO	NO	NO	NO	NO	1.5±5.0	NO	NO
Stamping their feet	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Caecotrophy	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Sneezing	NO	NO	NO	1.5±5.0	NO	NO	1.5±5.0	NO	NO	NO	NO	1.5±5.0
Scratching the cage	NO	NO	9.1±8.7	NO	NO	NO	NO	NO	NO	NO	3.0±6.7	NO

[1]Mean of the 11 observations per cage±standard deviation. Number of rabbits observed by scan sampling per cage=6, for a total of 72 rabbits.

[2]NO: not observed.

Gastrointestinal tract measurements

The weights of full gastrointestinal tracts (Figure 3A) and stomach content (Figure 3B) were significantly lower for rabbit submitted to longer FWT (P<0.0001), while caecum weight remained relatively stable until 20 h FWT (P=0.051; Figure 3B). Stomach pH was significantly lower for rabbits after 6 h compared to 0 h (P<0.0001) and ranged from 2.20±0.15 at 0 h to 1.22±0.05 after 24 h FWT (Figure 3C). As for caecum pH, it was only significantly higher (P<0.0001) at 14, 18, 22 and 24 h when compared to rabbits that had not fasted (0 h FWT; Figure 3D). Caecum pH fluctuated marginally over the 24-h feed withdrawal but gained 0.5 unit overall; it started at 6.13±0.04 for rabbits with a 0 h FWT and reached 6.63±0.07 for the 24 h FWT group. Stomach content dry matter (DM) was lower after a 10 h FWT compared to 0, 3 and 6 h (P<0.0001) and remained stable at around 11% thereafter (Figure 3E). Stomach content DM therefore follows a similar pattern as LL GP; Figure 2E). Stomach content weight was highly correlated with stomach pH and stomach DM (r=0.63 and 0.70, respectively; P<0.0001; data not shown).

Caecum and faecal microbial genomic analysis

For the alpha and beta diversity analysis, three FWT groups were created. Group 1 includes FWT between 0 and 6 h, Group 2 between 8 and 14 h and Group 3 between 16 and 24 h (Figures 4 and 5). No significant differences were observed for alpha diversity between FWT groups for caecum using Observed, Chao1, Shannon and Simpson indexes (Figure 4A). Faeces from rabbits with longer FWT (Group 3) had lower Observed, Shannon and Simpsons indexes compared to shorter FWT (Groups 1 and 2; all P<0.01), but for Chao1 index, Group 3 was only different from shorter FWT (Group 1; P=0.03; Figure 4B). This indicates that there were fewer total species in the faeces from rabbits with longer FWT and that they were less evenly distributed than samples from rabbits with short (Group 1) and moderate FWT. Beta diversity is presented on principal coordinate analysis (PCoA) plots based on a Bray-Curtis distance matrix (Figures 5A and 5B) and Unweighted (Figures 5C and 5D) and Weighted UniFrac distances (Figures 5E and 5F) for both caecum and faeces. In these figures, dots that are further apart from one another suggest that the microbial community compositions of the samples

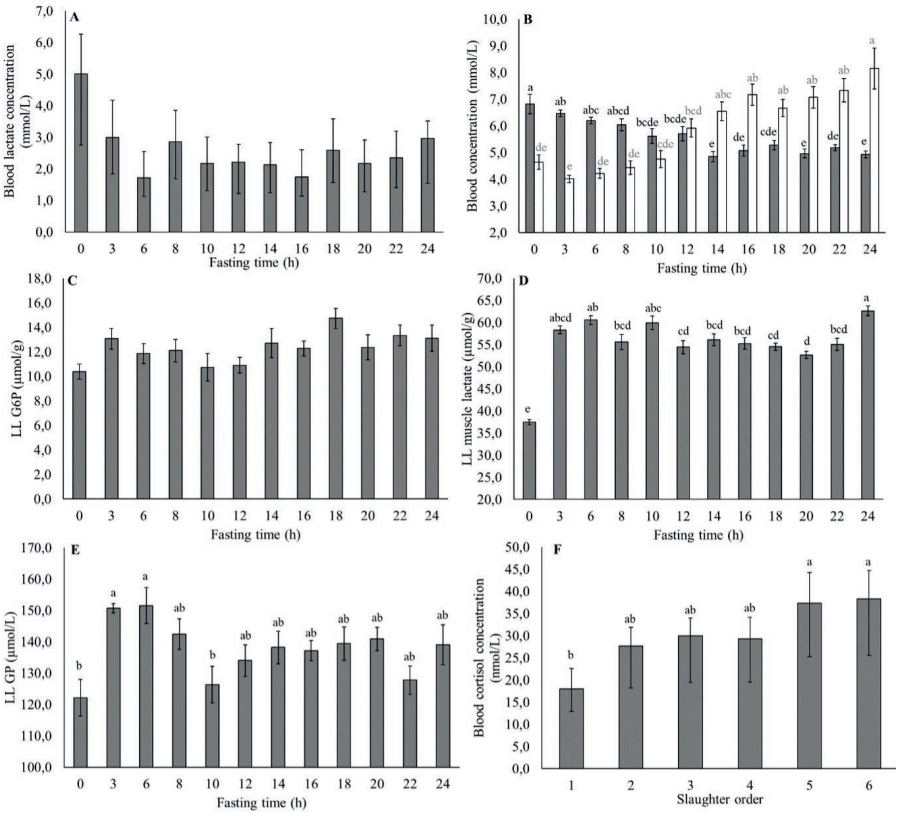

Figure 2: Effect of rabbit feed withdrawal time on (A) blood lactate, (B) glucose (■) and urea (□) concentrations, (C) *Longissimus lumborum* (LL) muscle lactate, (D) Glucose-6-phosphate concentrations (G6P), and (E) GP, as well as the effect of (F) slaughter order on blood cortisol concentrations. Values shown in A represent the mean of six rabbits (three males, three females) with a 95% confidence interval. Each value in B-E represents the mean of six rabbits (three males, three females) with standard error. Each value in F represents the mean value of 12 rabbits, with one rabbit per feed withdrawal time for each slaughter order; bars indicate the 95% confidence intervals. [abcde]Means with different letters indicate a significant difference at $P<0.05$. NS: not significant.

are evolving differently. These PCoA results reveal changes in the microbial community structures between the three groups of increasing FWT (all $P<0.002$). Similar results were found when a PLS-DA analysis was performed for the caecum and faeces (Supplementary material 3). The diversity pattern is, however, less evident in Figure 5F for the weighted UniFrac beta diversity. For both caecum and faeces, the FWT groups had the highest R^2 values for Bray-Curtis, Weighted and Unweighted UniFrac methods (0.15 and 0.14, 0.25 and 0.21, and 0.11 and 0.12, respectively). This indicates that compared to sex, the FWT led to more microbiota variation between samples. The vectors shown overlapping the PCoA analyses based on the Bray-Curtis distance matrix indicate that blood urea concentrations and caecum pH increased with longer FWT. However, blood glucose concentrations, stomach pH, stomach DM, stomach content weight and GIT weight increased with shorter FWT.

Samples from the caecum and faeces exhibited similar relative abundances (%) of phyla, with the predominant phyla being *Firmicutes* (57.2-79.4% in caecum and 64.4-82.5% in faeces), *Bacteroidota* (14.3-33.4% in caecum and 6.2-25.0% in faeces) and *Verrucomicrobiota* (2.8-10.2% in caecum and 2.8-14.8% in faeces). In caecum samples, the phylum with the fourth highest abundance was *Cyanobacteria* (0.6-1.4%), followed by *Desulfobacterota* (0.4-

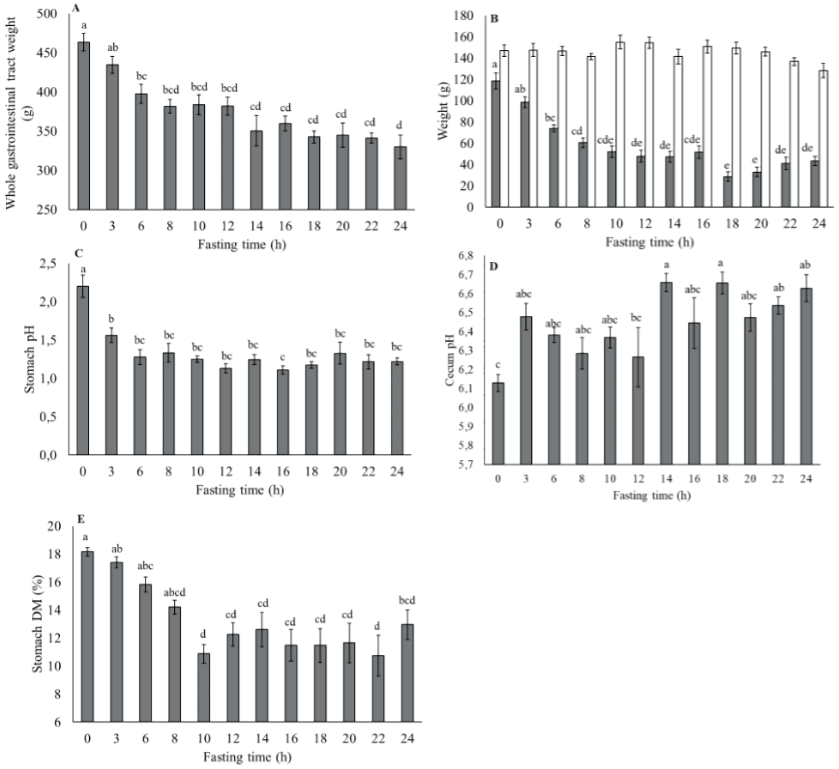

Figure 3: Effect of feed withdrawal on (A) the whole gastrointestinal tract, (B) stomach (■) and caecum (□) weights, (C) stomach pH, (D) caecum pH and (E) dry matter in the stomach content (DM) of rabbits slaughtered on site without transport. Each value represents the mean of six rabbits (three males, three females) with the standard error. [abcde]Means with different letters indicate a significant difference at $P<0.05$.

1.6%), *Campylobacterota* (0.3-1.0%), *Proteobacteria* (0.4-0.9%), *Actinobacteriota* (0.1-2.2%) and *Patescibacteria* (0.0-0.1%; Figure 6). In faeces samples, the phylum with fourth highest relative abundance was *Actinobacteriota* (0.2-4.8%), followed by *Desulfobacterota* (0.4-1.1%), *Proteobacteria* (0.2-1.0%), *Cyanobacteria* (0.0-0.6%), *Campilobacterota* (0.0-0.3%), *Patescibacteria* (0.0-0.3%) and *Chloroflexi* (0.0-0.2%; Figure 6). Nevertheless, linear discriminant analysis effect size (LEfSe) results indicate that the *Firmicutes* discriminated Group 1 from 3 in the caecum and Group 2 and 3 in the faeces, whereas *Verrucomicrobiota*, *Desulfobacterota Campilobacterota* and *Proteobacteria* characterised Group 3 (Figure 7).

Detection of Lactobacillaceae, Enterobacterales and Enterobacteriaceae by PCR analysis

Using specific primers, *Lactobacillaceae* were found at 3, 6, 12, 20 and 24 h of FWT in the caecum of females and at 0, 6, 8, 12, 16 and 18 h for males (Table 2). No *Lactobacillaceae* were detected at 10 and 14 h of FWT in the caecum of either male or female rabbits. *Enterobacterales* were detected for every FWT, but no *Enterobacteriaceae* were found in either female or male caecum (Table 2).

In faeces, *Lactobacillaceae* were detected for every FWT except at 10, 12 and 14 h for female rabbits (Table 2). In male rabbits, *Lactobacillaceae* were found at 6, 8, 12 and 18 h of FWT. *Enterobacterales* were detected for every FWT in the faeces of both female and male rabbits. *Enterobacteriaceae* were found in male faeces after 18 and 22 h (Table 2).

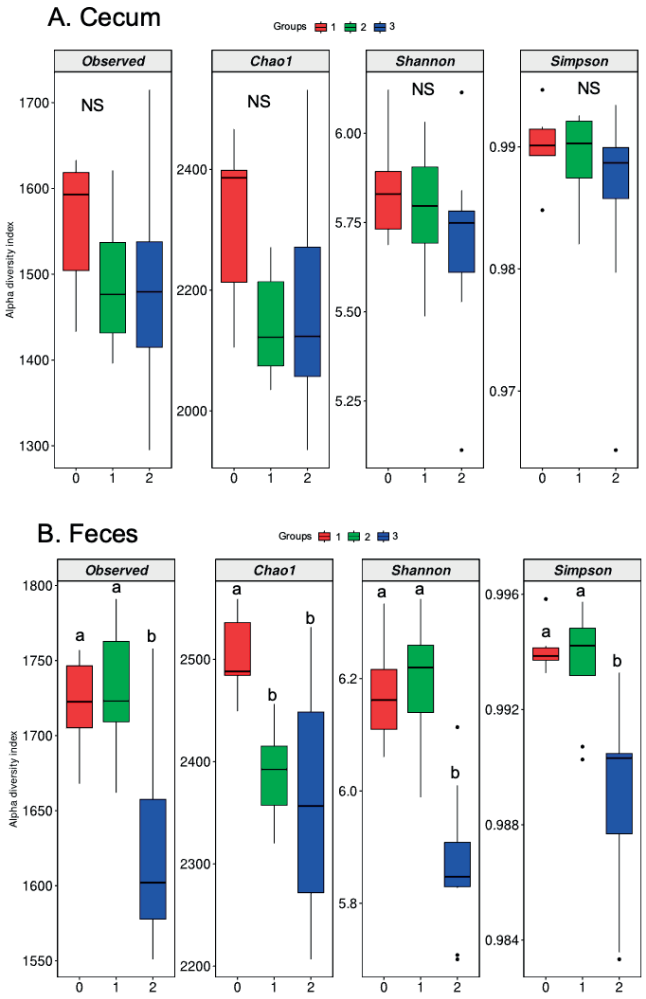

Figure 4: Alpha diversity indexes for richness (Observed, Chao1) and both richness and evenness (Shannon, Simpson) for caecum (A) and faeces (B) according to FWT from rabbits slaughtered onsite. Group 1=0-6 h, Group 2=8-14 h and Group 3=16-24 h of FWT. [ab]Means with different letters indicate a significant difference at $P<0.05$.

DISCUSSION

Rabbit behaviour

Rabbit behaviour was observed through scan sampling to detect any welfare issues before slaughter, according to the Code of Practice for the Care and Handling of Rabbits (NFACC, 2018). No aggressive or concerning behaviour was observed, and rabbits were mostly sitting or lying down. The results suggest that the FWT did not impose excessive levels of stress on the animals that would have required stopping the experiment before the end. However, to access rabbit behaviour properly upon feed withdrawal, further research with more replicates is needed.

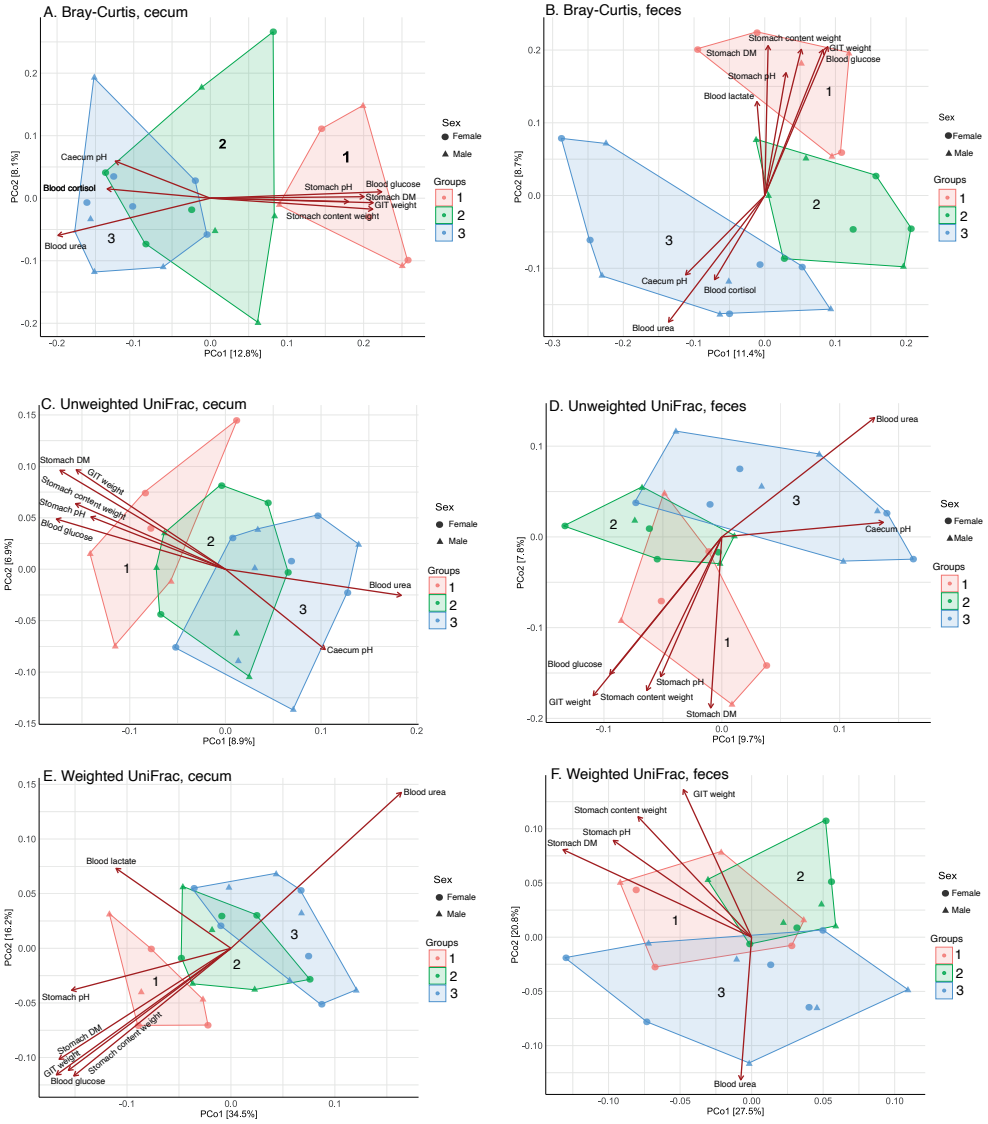

Figure 5: Principal coordinate analysis (PCoA) illustrating the beta diversity based on the sequencing of the 16S gene amplicons (A, B) using Bray-Curtis, (C, D) Unweighted UniFrac and (E, F) Weighted UniFrac methods according to sample type (caecum or faeces) and FWT from rabbits slaughtered onsite. Group 1=0-6 h, Group 2=8-14 h and Group 3=16-24 h of FWT. Correlation vectors (in red) of the top 10 contributive physiological parameters for the sample distribution are also represented. Arrows are displayed at a scale of 25%. Stomach DM: Stomach dry matter. GIT weight: Gastrointestinal tract weight.

Blood parameters and muscular GP

Blood lactate concentration is used as an indication of short-term stress levels before slaughter (Rocha *et al.*, 2015; Trocino *et al.*, 2018). Physical exertion can cause a maximum blood lactate concentration to be reached in 4 min before returning to basal levels after 2 h (Warriss *et al.*, 1994; Edwards *et al.*, 2010; Rocha *et al.*, 2015). High blood

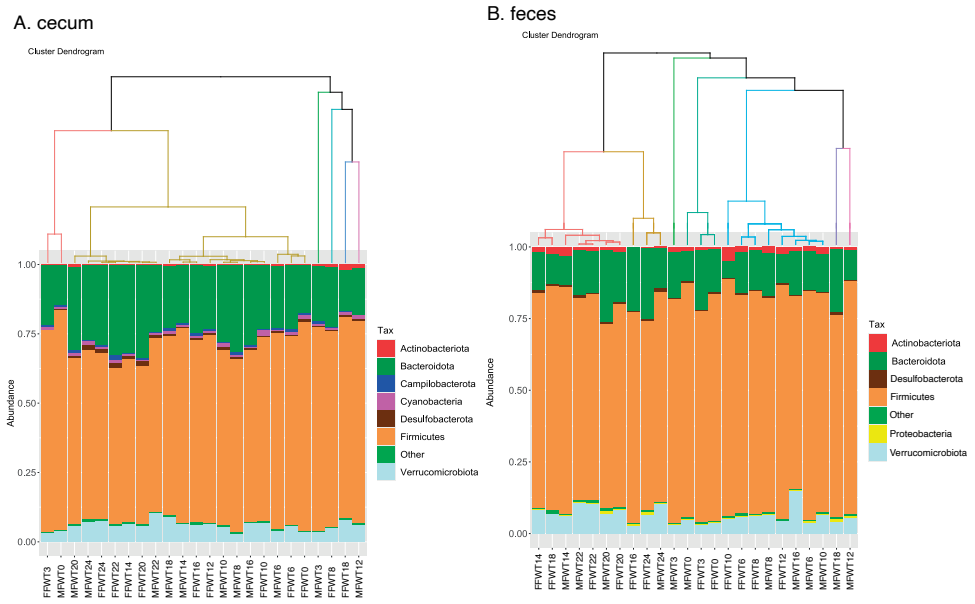

Figure 6: Cluster dendrogram of the identified phyla with the top seven highest relative abundances (%) for (A) caecum and (B) faeces samples for each feed withdrawal time. F: female, M: male, FWT: feed withdrawal time.

lactate levels are associated with preslaughter stress in rabbits (Nakyinsige *et al.*, 2013; Fazio *et al.*, 2015; Trocino *et al.*, 2018). In our study, blood lactate was not influenced by the applied FWT (Figure 2A). Blood lactate remained mostly below the range reported in Langlois *et al.* (2014) for healthy rabbits (6.9±2.7 mmol/L), with a maximum value of 8.5 mmol/L. Since the rabbits were not subjected to crating, transport or preslaughter lairage and always had access to water, this suggests that feed withdrawal alone is not particularly stressful for rabbits. Furthermore, rabbits prefer to eat during dark periods (Lebas and Gidenne, 2006), which could have limited the stress caused by removing the feed after opening the lights at the beginning of feed withdrawal. This seems to be the case especially when feed withdrawal occurs in a familiar environment, as reported with pigs (Dalla Costa *et al.*, 2016). Similarly, Nijdam *et al.* (2005) found that a 13-h feed withdrawal period had no impact on blood lactate concentrations in broilers. In pigs, no impact was found on blood lactate levels for FWT of up to 36 h (Bertol *et al.*, 2005; Frobose *et al.*, 2014; Acevedo-Giraldo *et al.*, 2020).

Blood cortisol concentrations were also measured and used as an indicator of long-term psychological stress prior to slaughter (Warriss *et al.*, 1994; Choe, 2018). Cortisol is released into the blood at a slower rate than lactate, reaching peak levels within 15-20 min of a stressful event (Warriss *et al.*, 1994; Lay *et al.*, 1998). For pigs, cortisol levels in blood return to basal levels 4 h after the stress is alleviated (Dalin *et al.*, 1993; Warriss *et al.*, 1994; Lay *et al.*, 1998). In our study, blood cortisol concentrations were not affected by the applied FWT ($P>0.05$). Nijdam *et al.* (2005) found that a 13-h FWT had no effect on blood cortisol concentrations in broilers. Conversely, Acevedo-Giraldo *et al.* (2020) found that a 24-hFWT increased blood cortisol levels in pigs. Karaca *et al.* (2016) observed no effect for a 12-h FWT but found higher blood cortisol levels when lambs fasted for 24 and 48 h compared to 0 h (27.45 and 27.67 *vs.* 11.04 nmol/L). In our study, since rabbits experienced only feed withdrawal and did not experience crating, transport and preslaughter lairage, the results suggest that rabbits were not particularly stressed by the FWT. This result could also suggest that the period without food during a feed restriction is not particularly stressful for rabbits (Tuleda and

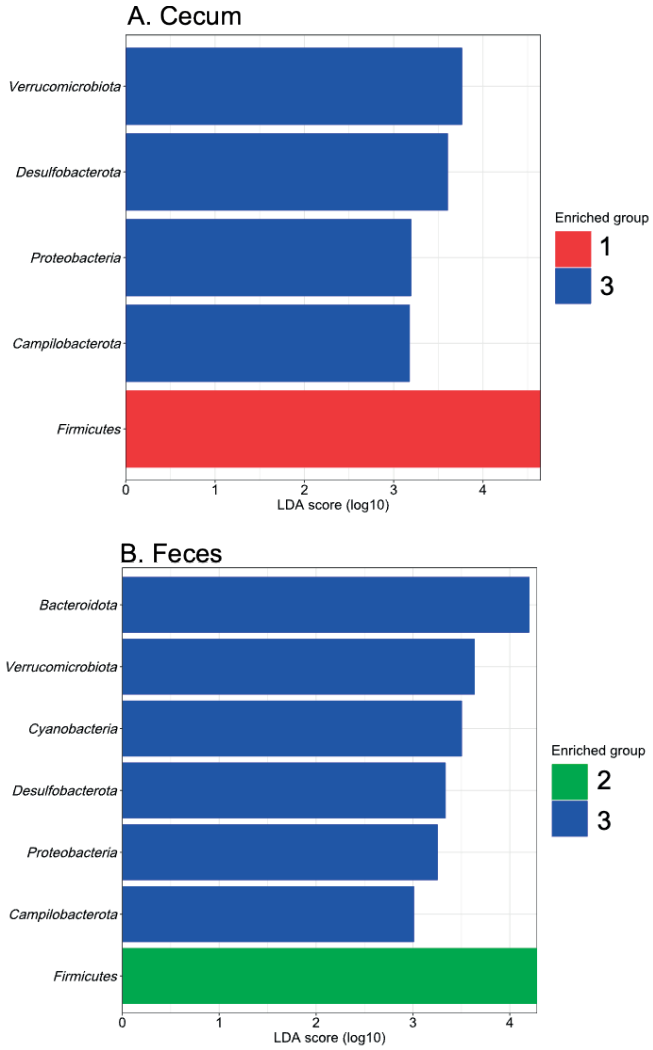

Figure 7: Differentially abundant phyla across FWT groups as assessed using LDA with effect size (LEfSe) measurements for Group 1=0-6 h, Group 2=8-14 h and Group 3=16-24 h of FWT. Only those genera with a LDA score (log10) of >2.0 are displayed.

Lebas, 2006). However, blood cortisol is also influenced by the circadian rhythm, reaching peak levels in the afternoon and the lowest levels at 6 AM (Szeto *et al.*, 2004). Since our experiment was conducted over a 24-h period, blood cortisol results may have been influenced by the circadian rhythm.

Interestingly, the first rabbits slaughtered in each group had blood cortisol concentrations that were lower than in the fifth and sixth rabbits slaughtered ($P<0.05$; Figure 2F). Some studies have indicated that stress pheromones found in the blood, urine and faecal matter of stressed animals can be detected by other animals nearby, which could induce stress before slaughter (Terlouw *et al.*, 1998; Buil *et al.*, 2010). As all the rabbits that shared a cage were in the same room at the time of slaughter in our study, the remaining rabbits may have detected stress pheromones being released as rabbits were slaughtered, as indicated by the increasing blood cortisol concentrations in ascending

Table 2: Effect of feed withdrawal time on the presence or absence of *Lactobacillaceae, Enterobacteriales* and *Enterobacteriaceae* in caecum content and feces[1] of rabbits slaughtered onsite (without transport).

Feed withdrawal time (h)	0	3	6	8	10	12	14	16	18	20	22	24
Caecum female												
Lactobacillaceae		+	+			+				+		+
Enterobacterales	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacteriaceae												
Caecum male												
Lactobacillaceae	+		+	+		+		+	+			
Enterobacterales	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacteriaceae												
Faeces female												
Lactobacillaceae	+	+	+	+				+	+	+	+	+
Enterobacterales	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacteriaceae												
Faeces male												
Lactobacillaceae			+	+		+			+			
Enterobacterales	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacteriaceae									+		+	

[1] Presence or absence was determined by PCR analysis.

slaughter order. The last rabbits in each group experienced a longer period before being handled by humans to be slaughtered, which may have contributed to the increased blood cortisol levels (Rushen *et al.*, 1999). Moreover, rabbits are social animals that can develop physiological symptoms from stress when not in the presence of other rabbits (Held *et al.*, 1995; Chu *et al.*, 2004). Thus, as the rabbits were slaughtered, the remaining rabbits in the group may have experienced further stress due to the dwindling numbers, leading to increased blood cortisol levels.

As feed withdrawal starts, the liver breaks down glycogen to balance out the decrease in blood glucose levels. These glycogen reserves prevent large changes in glucose concentrations. When the reserves are depleted, blood glucose levels will begin to drop (Savenije *et al.*, 2002; Karaca *et al.*, 2016). The results from our study indicate a decrease in blood glucose with longer FWT (Figure 2B). This is consistent with the study by Hauguel *et al.* (1988), which reported a 15% decrease in blood glucose levels from 5.77±0.27 to 4.88±0.21 mmol/L after a 96-h FWT for rabbits. Conversely, Brecchia *et al.* (2006) found no differences in blood glucose concentrations when rabbits fasted for 24 or 48 h. However, this could be due to a more consistent flow of nutrients from the ingestion of caecotrophs (Kozma *et al.*, 1974). Although significant differences were observed in our study, blood glucose values were within the normal reported range for healthy rabbits (4.16-8.60 mmol/L; Melillo, 2007). This could indicate that the applied FWT were not long enough to lower blood glucose concentrations to a point where the rabbits' metabolisms could not adjust.

When rabbits fast and glucose and glycogen reserves are depleted, amino acids are catabolised resulting in increased urea production that is then released into the bloodstream (Marín-García *et al.*, 2020). In our study, urea concentrations in the bloodstream were significantly higher after a 14-h FWT and remained high thereafter, while blood glucose concentrations were significantly higher for 0-8 h of FWT (Figure 2B). Blood urea concentrations ranged between 4.02±0.14 and 8.15±0.77 mmol/L during the experiment. This is consistent with results from Marín-García *et al.* (2020), who reported higher plasma urea after rabbits fasted for 18 h, compared to 8 h (6.50±0.05 *vs.* 6.10±0.08 mmol/L). However, caecotrophs are a source of amino acids and could have caused the increased urea levels observed from 14 h onward, after they were ingested (Hamlin, 2011; Marín-García *et al.*, 2020). Overall, the values for blood urea concentrations were below or in the lower range of those observed by Melillo (2007) for healthy rabbits (7.14-16.07 mmol/L).

Along with the other mechanisms used by rabbits to maintain their metabolism homeostasis, the impact of caecotrophy on muscle reserves is confirmed by the stabilisation of the LL GP at between 127.78 and 139.04 µmol/g after a 10-h FWT in our study (Figure 2E). According to Lebas *et al.* (1997), caecotrophy begins at 8-12 h after a meal, which coincides with the GP concentrations we observed. During this period, rabbits will eat their caecotrophs, which are a source of energy and protein. This appears to replenish their energy reserves (Cheeke, 1987). Rabbits may be particularly resistant to hunger despite their small size for this reason, supporting previous claims of this nature (Lebas *et al.*, 1997).

To our knowledge, our study is the first to report the effects of FWT on rabbit LL GP. Although Żelechowska and Przybylski (2015) studied LL GP values (reporting levels between 98.8 and 117.8 µmol/g), they only investigated rabbits that were not fasted before slaughter. Muscle GP can be used as an indicator of the muscle's capacity to support glycolysis *post mortem*. At the time of slaughter, it has also been known to influence meat pH_u. In pigs, Faucitano *et al.* (2020) did not find any effects from a 32-h FWT on pig LL GP and pH_u. Faucitano *et al.* (2006) suggested that when FWT is conducted alone, without transport or lairage, muscle glycogen does not deplete enough to affect pork meat quality. These results are consistent with a study by Dalla Costa *et al.* (2016) that examined pigs in lairage and concluded that although recovering from the stress of loading, transport and unloading is important, feed withdrawal should not be extended at the slaughterhouse. Instead, it is best to have livestock fast in a familiar environment. However, in our study, rabbits always had access to water before slaughter and feed was withdrawn when lights were opened which likely reduced stress. Further research is needed, for rabbits, to confirm this hypothesis under commercial conditions where animals are crated, transported and rested before slaughter.

Gastrointestinal tract measurements

As expected, total GIT weight decreased over time (Figure 3A). The GIT tract weights were expressed as a percentage of the slaughter weights and ranged from 19.8 to 14.4% after 0 and 24 h, respectively (data not shown). These results are consistent with Masoero *et al.* (1992), who found that GIT weights were lower when rabbits fasted for 24 h compared to no fasting (349 *vs.* 417 g). Similarly, Bianchi *et al.* (2008) observed a decrease in GIT weights (21.2, 19.8 and 18.6%) after 3, 9 and 15 h of FWT, respectively.

Stomach content weight also decreased with longer FWT (Figure 3B). Coppings *et al.* (1989) reported a reduction in stomach weight in rabbits from 73.2 to 35.7 g after a 12-h FWT, remaining relatively stable thereafter.

A trend of lower caecum weights with longer FWT (Figure 3B) was observed in our study. Coppings *et al.* (1989) and Carmichael *et al.* (1945) reported that a 12 and 24-h FWT, respectively, had a limited impact on rabbit caecum weight. However, when they compared rabbits that fasted with a muzzle for 48 h to prevent caecotrophy, they observed lower caecum dry content weights. This could indicate that the ingestion of caecotrophs contributes to the dry matter content and weight of caecum during the feed withdrawal period. More studies are needed to confirm this hypothesis.

Stomach pH decreased with longer FWT up to 14 h and remained relatively stable thereafter (Figure 3C). Vernay *et al.* (1975) also observed a reduction after rabbits fasted for 48 h (2.22±0.17 *vs.* 1.36±0.13). The drop in pH for FWT under 14 h could be caused by the removal of the buffering effect of the feed, as seen with the decreases in stomach weight and DM in our study. The mixing of gastric content and increased fluidity (Lang *et al.*, 1998; Friendship *et al.*, 2000) also suggest that when no further feed is available, rabbit stomach pH should gradually drop as the stomach continues to secrete acid (Carabaño *et al.*, 2010). However, Rees Davies and Rees Davies (2003) found that caecotrophs release buffering substances in the stomach after feed ingestion, which stabilises stomach pH and prevents further pH reduction.

In our study, stomach content DM was higher at the beginning of the experiment and decreased to its lowest levels after a 10-h FWT (10.88±0.68%; Figure 3E). These results contradict those of Carmichael *et al.* (1945), which showed that a 24-h FWT had limited impacts on rabbit stomach DM compared to non-fasted rabbits. Those authors suggested that the limited impact of FWT on stomach DM was due to faeces in the digestive tract as a result of caecotrophy. In our study, stomach DM remained relatively stable after 12 h of FWT, which coincides with the time rabbits usually eat their caecotrophs after initial feed ingestion (Lebas *et al.*, 1997).

Caecum pH increased from 6.13±0.04 at 0 h to 6.63±0.07 at 24 h of FWT, which is an increase of 0.5 pH units between the shortest and longest FWT (Figure 3D). This variation suggests a certain change, over time, in the microbial activity in the caecum toward the end of the experiment (Martín-Peláez *et al*., 2008). Vernay *et al*. (1975) observed an increase from 6.2±0.2 to 6.9±0.1 after a FWT of 48 h for rabbits. Similarly, Piattoni *et al*. (1997) reported a caecum pH of 6.7±0.1 after 16 h of FWT compared to 6.2±0.2 for non-fasted rabbits. It has been suggested that caecum pH increases with longer FWT due to a decrease in the availability of fermentable substrate. This leads to reduced production of short-chain fatty acids (Martín-Peláez *et al*., 2008) and changes in the microbiota profile. It is important to remember that as caecum pH approaches near-neutral levels, a more favourable environment is produced for the growth of undesirable bacteria such as *Salmonella* sp. and *E. coli* (Lebas *et al*., 1997; Martín-Peláez *et al*., 2009; Eicher *et al*., 2017).

The absence of difference for alpha diversity between FWT for the caecum with a concomitant significant difference with the beta diversity suggests that the bacteria varied mainly in their proportion from one another (Figure 4). In faeces, alpha diversity analyses revealed that longer FWT had a significant effect on microbial richness and evenness. As for microbiome beta diversity analyses using Bray-Curtis, Weighted and Unweighted UniFrac methods, it revealed that FWT had a significant effect on microbiome beta diversity. Thompson *et al*. (2008) used PCR analysis and found that there was a reduction in microbial species and diversity in the small intestine of poultry as FWT increased from 0 to 24 h. This variation was attributed to either an increase in gut pH, which is known to alter microbial growth, or the absence of food, which causes a severe energy crisis for microorganisms (Kohl *et al*., 2014). However, in our study, stomach pH decreased as FWT increased, suggesting that because rabbits exhibit caecotrophy, their response to FWT is most likely different than for monogastric animals (such as poultry and swine).

The predominant phyla detected in the caecum and faeces are relatively similar to the findings from Combes *et al*. (2017) and Velasco-Galilea *et al*. (2018). The predominant phyla were *Firmicutes*, *Bacteroidota*, *Proteobacteria* and *Actinobacteria*. There were some differences in the relative abundances between studies, but *Firmicutes* was the most abundant phylum in all of them, including ours. No *Tenericutes* were detected in the top 10 phyla for relative abundance in our study, unlike in Velasco-Galilea *et al*. (2018), where it was the second most abundant. In our study, *Bacteroidota* was the second most abundant phylum in both caecum and faeces, which is similar to the results found by Combes *et al*. (2017) in caecum. However, Velasco-Galilea *et al*. (2018) found the *Bacteroidota* to be in third rank in both caecum and faeces. For *Proteobacteria,* Combes *et al*. (2017) found them in third rank in the caecum and Velasco-Galilea *et al*. (2018) found them in fifth and sixth rank in faeces and caecum, respectively. In our study, they were the sixth and seventh most abundance phyla in faeces and caecum, respectively.

Using PCR analysis and specific primers, we only detected *Enterobacteriaceae* in the faeces of males and for 18 and 22 h of FWT (Table 2). This suggests greater shedding risk of these bacteria when male rabbits are subject to longer FWT compared to females. Both sexes are usually raised together for rabbit meat production and the FWT is applied to all animals at the same time. Hence, to limit the shedding of these bacteria and contamination of the other animals in the cage, FWT should not be longer than 18 h according to our results. Contrary to what has been observed in other species such as pigs, cattle and poultry (Schierack *et al*., 2007; Shang *et al*., 2018; Pang *et al*., 2020), the presence of *Enterobacteriaceae* was limited in our study, as confirmed by the lack of identification using 16S amplicon analysis. The lack of identification of *Enterobacteriaceae* from the sequencing of the 16S rDNA amplicon could be due to a lack of amplification of the variable region from the 16S gene that allows the correct taxa assignment. With the targeted PCR amplification, the use of more specific primers allowed confirmation of their presence. Moreover, the fact that *Proteobacteria* has been identified as a characterised taxon for the longer FWT supports the microbiome changes towards one more likely to bear *Enterobacteriacieae*. In healthy rabbits, they are not normally present after weaning. However, in cases where researchers did detect some of these bacteria, they reported low relative abundances of 0.01-0.11% (Gouet and Fonty, 1979; Combes *et al*., 2014; Kylie *et al*., 2018). Furthermore, Zeng *et al*. (2015) found only a limited number of *Enterobacteriaceae* in Rex-rabbit caecotrophs. However, more studies are needed with a higher number of animals to confirm these results.

CONCLUSION

The results of this study indicate that, without the effect of crating and transport, feed withdrawal was not particularly stressful for rabbits. The physiological adaptation mechanisms used by rabbits, such as caecotrophy, resulted in a limited impact of FWT on muscle reserves as confirmed by the stabilisation of the glycolytic potential in the *Longissimus lumborum* muscle after a 10-h FWT. Thus, the caecotrophic behaviour of rabbits allows them to be relatively resistant to hunger despite their small size. Alpha and beta diversity indicated a difference in microbiome richness and composition between different FWT. A FWT longer than 18 h is not recommended, as *Enterobacteriaceae* were detected in the faeces of male rabbits after 18 and 22 h of FWT. The limited numbers detected by metataxonomic and PCR analyses suggest a lower risk of faecal shedding for rabbits compared to other animal species such as pigs and poultry. Nonetheless, longer FWT could still present the risk of faecal shedding of those undesirable organisms and carcass contamination during slaughter. To better understand the likelihood of this occurring, a study in commercial conditions is currently under way.

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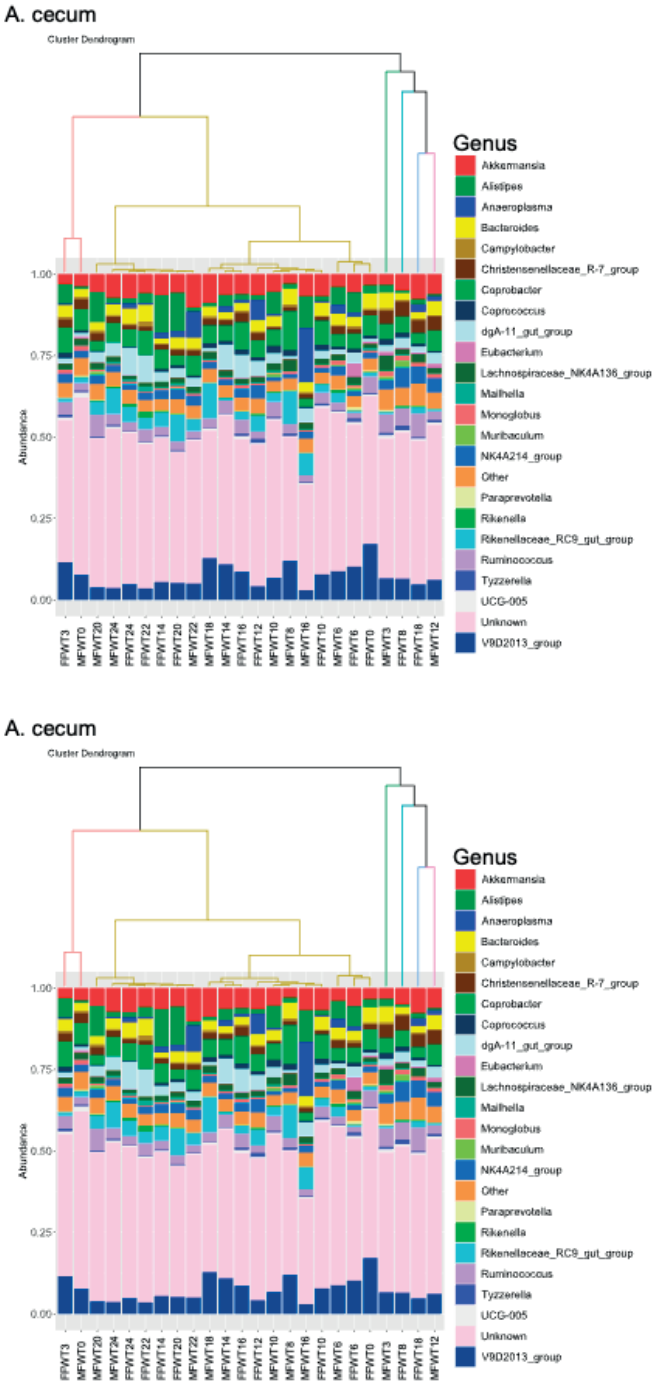
SUPPLEMENTARY MATERIALS

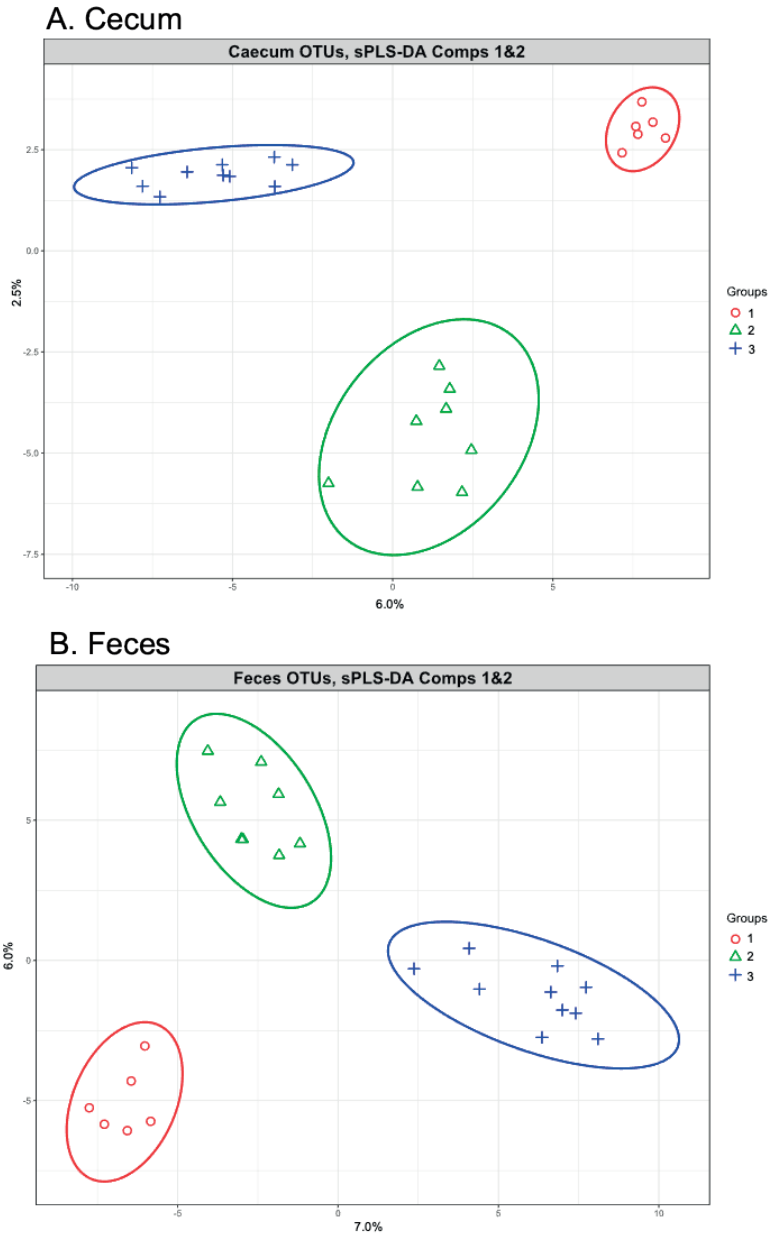
Supplementary Material 1: Nutritional value of the commercial diet[1,2] fed to rabbits used in this study.

Nutrients	Commercial data
Dry matter (%)	94.00
DE (Mcal/kg	2.81
Crude protein (%)	17.02
Crude fat (%)	4.90
Crude fibre (%)	19.25
Calcium (%)	1.06
Phosphorous (%)	0.47
Sodium (%)	0.32
Chloride (%)	0.54
Magnesium (%)	0.32
Potassium (%)	1.18
Sulfur (%)	0.27
Iron (mg/kg)	175.77
Zinc (mg/kg)	49.18
Manganese (mg/kg)	26.27
Copper (mg/kg)	125.00
Iodine (mg/kg)	0.20
Vitamin A (UI/kg)	64.19
Vitamin D (UI/kg)	10.83
Vitamin E (UI/kg)	42.55
Total selenium (mg/kg)	0.20
Added selenium (mg/kg)	0.11
All B vitamins (mg/kg)	1,512.23

[1]Belisle Solution Nutrition, St-Mathias-sur-Richelieu, Quebec, Canada.
[2]Ground alfafa, beet pulp, durum wheat, soybean meal, canola meal, soybean shell, soybean oil, molasses, corn gluten feed.



Supplementary Material 2: Cluster dendrograms with the top 23 identified genus in relative abundance (%) for (A) caecum and (B) faeces samples for each feed withdrawal time. F: female, M: male, FWT: feed withdrawal time.



Supplementary Material 3: PLS-DA analysis for caecum (A) and faeces (B) according to FWT from rabbits slaughtered onsite. Group 1=0-6 h, Group 2=8-14 h and Group 3=16-24 h of FWT. The PLS-DA was created using mixOmics (version 6.18.1; Rohart *et al.,* 2017) in R (R Core Team, 2017; version 4.1.1).

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