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RESEARCH ARTICLE

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Effect of dietary supplementation with purified wood lignocellulose on performances, caecal fermentation and gut microbiota of growing rabbits

Giulia Zardinoni[a], Angela Trocino[a,b], Marco Birolo[a], Francesco Bordignon[a], Piergiorgio Stevanato[a], Fabrizio Pirrone[a], Andrea Zuffellato[c] and Gerolamo Xiccato[a]

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ABSTRACT

A total of 576 rabbits were housed in 18 open-top elevated pens (32 rabbits per pen) during the fattening period (54–71 d of age) and fed a standard diet or a diet enriched in purified wood lignocellulose and fat formulated to guarantee rabbit optimal digestive health and convenient feed conversion ratio. The impacts on gut microbiota (both hard faeces and caecal content) and caecal fermentation, diet digestibility and rabbit performance were examined. The microbial diversity and the bacterial community structure of hard faeces barely differed from that of caecal content ($p > .05$). No differences in alpha and beta diversity of microbiota were detected between rabbits fed the different diets. Twelve genera, mostly belonging to the family of Lachnospiraceae, increased ($p < .05$) in rabbits fed the diet added with the purified lignocellulose and fat. The increase of insoluble fibre by purified wood lignocellulose reduced the digestibility of fibre fractions (NDF digestibility 19.2% vs. 26.8%; $p < .001$) while increasing protein (73.5% vs. 71.6%; $p < .01$) and ether extract digestibility. Growth performance was not affected by the dietary treatment (average daily weight gain: 40.9 g/d; feed conversion ratio: 4.04). Total losses (dead + underweight animals) averaged at 4.17% without significant differences between the dietary treatments. In conclusion, the addition of purified wood lignocellulose in fat-added diets did not affect rabbit performance and overall farm efficiency was maintained. Some changes in the caecal fermentation profile may indicate a change in microbial pathways rather than composition, where no significant effects on the gut microbiota were observed.

HIGHLIGHTS

- Low levels of pure lignocellulose added to diets along with fat do not compromise the overall farm efficiency.
- No major effects on gut microbiota were observed.
- A few changes of the caecal fermentation profile were recorded.

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Feed efficiency; dietary fibre; fat; caecal fermentation; microbiota

Introduction

Rabbits are herbivorous, monogastric, hindgut-fermenting animals, with a peculiar digestive characteristic, i.e. caecotrophy, which has a key role in nutrient utilisation (Kylie et al. 2018) and gut health (Gidenne, Carabaño, et al. 2020). Under farming conditions, the management of feeding can be challenging due to the high susceptibility of rabbits to digestive diseases (Gidenne et al. 2017; Chen et al. 2018) which can lead to high morbidity and mortality, especially in growing rabbits, at the expense of productive results and farm efficiency (Curone et al. 2022).

Digestive diseases are generally caused by pathogenic agents which can be favoured by suboptimal farming conditions besides dietary unbalances (Bennegadi et al. 2003; Gidenne, Lebas, Licois, et al. 2020). Within nutritional strategies for protecting rabbit health, the manipulation of fibre content and quality is known to have a large effect (Trocino et al. 2013; Gidenne, Lebas, and Fortun–Lamothe 2020; Gidenne, Lebas, Licois, et al. 2020). In fact, plant fibres are primarily fermented in the caecum and proximal colon of rabbits by different microbial communities (Bennegadi et al. 2003; Combes et al. 2017), where any unbalance

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of this microbiota can result in dysbiosis, changes in caecal fermentation patterns, and the proliferation of pathogens that endanger animal health (Fang et al. 2020).

As for quantity, dietary fibre is the main nutrient of diets for rabbits (35–50% as-fed); as for quality, the insoluble fibre, represented by neutral detergent fibre (NDF), is generally the most relevant fraction which accounts for about 65–90% of the total dietary fibre (Trocino et al. 2013). The increase of insoluble fibre (NDF and other fibre fractions) has been recognised by the scientific community as useful for the prevention of digestive disorders of weaning and fattening rabbits (Gidenne et al. 2002; Krieg et al. 2012), as it controls the digesta retention time in the gut and, especially, in the caecum segment, therefore affecting the caecal microbial composition and the corresponding fermentative pattern (Gidenne, Lebas, and Fortun–Lamothe 2020). To maintain digestive health in the post-weaning period, NDF content should be around 34–36%, acid detergent fibre (ADF) not less than 18–19% and acid detergent lignin (ADL) not less than 4.5–5.0% (Gidenne, Lebas, Licois, et al. 2020). However, when the contents of dietary fibre and insoluble fibre fractions increase, the nutritive value decreases, and, due to the chemiostatic regulation of appetite, rabbits increase their feed intake, which is associated with a decreased overall farm efficiency (Gidenne et al. 2017).

In rabbit feeding, manipulation of dietary fibre content is usually obtained by changes in the inclusion rate of fibrous raw materials, whereas, to our knowledge, less information exists about the use of purified fibres (Krieg et al. 2012). In other monogastric and poligastric animals, the dietary inclusion of purified fibres (cellulose and/or lignin) does not represent a barrier to feed digestion (Baurhoo et al. 2008). In poultry, purified lignin has been used to modulate gut health (Fangueiro et al. 2023), favouring the growth of beneficial bacteria and controlling intestinal pathogens (Baurhoo et al. 2007).

Thus, the present study aimed to test the hypothesis that increasing dietary insoluble fibre fractions, through the addition of purified wood lignocellulose, and the energy level, by adding vegetal fat, could promote digestive health and guarantee adequate growth performance and feed efficiency in growing rabbits. To this aim, we compared growth performance, digestibility, caecal fermentative activity and gut microbiota composition (in caecal content and hard faeces) of growing rabbits fed diets including or not purified wood lignocellulose and formulated to be isoenergetic by modulating the fat content.

Materials and methods

Animals and experimental conditions

The trial run in the experimental rabbit farm of the University of Padova (Italy), in a closed shelter. A total of 576 commercial crossbred rabbits (Hycole, SARL Hycole, Marcoing, France) were selected from healthy litters of multiparous does, individually identified by earmarks and allocated in groups in 18 open-top elevated pens (210 cm × 92 cm × 110 cm height; 32 rabbits per pen). The pens were equipped with a plastic slat floor, eight automatic nipple drinkers and four feeders for the manual distribution of feed. At 54 days of age, after a post-weaning adaptation period during which all animals were fed a unique diet (crude protein: 157 g/kg; ether extract: 34,7 g/kg; NDF: 350 g/kg; ADF: 194 g/kg; ADL: 51.0 g/kg), the pens were allocated to two experimental groups fed diet C (control), with standard content of fibre fractions and fat, or diet FF (fibre + fat), with a higher content of insoluble fibre, obtained by the addition of purified wood lignocellulose and fat. Both diets were formulated as fattening diets (Gidenne, Carabaño, et al. 2020) and fed until commercial slaughtering of rabbits, at 71 d of age (Table 1). They were isoproteic and isoenergetic. With respect to the diet C, the diet FF contained 20 g/kg of Arbocel® (J. Rettenmaier & Söhne GMBH + CO KG, Rosemberg, Germany), a commercial product based on purified lignocellulose from wood (cellulose to lignin ratio 2:1), that replaced the same quantity of dehydrated alfalfa meal. Other minor changes (−30 g/kg of barley meal, −20 g/kg dehydrated beet pulp, +30 g/kg sunflower meal and +20 g/kg soybean oil) balanced the protein and energy contents of the experimental diets. With respect to diet C, diet FF had −5.7% soluble fibre, +1.5% NDF, +1.7% ADF, +11% ADL and +31% ether extract, besides a higher content of crude protein (+3.9%) and gross energy (+1.8%) (Table 1). Both diets were pelleted (3.5 mm diameter and 10–11 mm length of pellets) and supplemented with L-lysine-HCl and DL-methionine, vitamins and macro- and micro-minerals (Table 1). They did not contain any antibiotics or coccidiostat. The animals had free access to the diet and fresh water during the whole trial.

Individual live weights were recorded once a week; pen feed intake was daily measured. Health status was daily monitored and total losses due to dead rabbits and rabbits discarded because of illness and/or

Table 1. Ingredients and chemical composition of the control and fibre-fat added diets.

Experimental diets[a]	Control (C)	Fibre + fat (FF)
Ingredients		
Alfalfa meal (CP: 16.5%), g/kg	200	180
Arbocel®[b], g/kg	–	20.0
Wheat bran, g/kg	229	228
Barley meal, g/kg	230	200
Dried beet pulp, g/kg	140	120
Sunflower meal, g/kg	160	190
Soybean oil, g/kg	10.0	30.0
Molasses (50% cane + 50% beet), g/kg	15.0	15.0
Calcium carbonate, g/kg	4.0	6.0
Dicalcium phosphate, g/kg	1.5	0.5
Limestone, g/kg	4.0	4.0
L-lysine HCl, g/kg	1.5	1.5
DL–methionine, g/kg	1.0	1.0
Vitamin-mineral premix[c], g/kg	4.0	4.0
Chemical composition and nutritive value (as-fed basis)		
Dry matter, g/kg	901	906
Crude protein, g/kg	152	158
Ether extract, g/kg	28.8	37.8
Ash, g/kg	64.8	67.9
Starch, g/kg	143	138
Soluble fibre[d], g/kg	80.4	75.8
NDF, g/kg	326	331
ADF, g/kg	181	188
ADL, g/kg	42.6	47.4
Gross energy, MJ/kg	16.7	17.0
Digestible energy, MJ/kg	10.3	10.4
Digestible protein/digestible energy ratio, g/MJ	10.5	11.2

Note: CP: crude protein; NDF: neutral detergent fibre; ADF; acid detergent fibre; ADL: acid detergent lignin.
[a]Diets were formulated for fattening period according to Gidenne, Carabaño, et al. (2020).
[b]Arbocel® (J. Rettenmaier & Söhne GMBH + CO KG, Rosemberg, Germany): purified lignocellulose from wood (cellulose to lignin ratio 2:1).
[c]Premix provided per kg of complete diet: vit. A, 12,000 UI; vit. D3, 1000 UI; vit. E acetate, 50 mg; vit. K_3, 2 mg; Biotin, 0.1 mg; Thiamine, 2 mg; Riboflavin, 4 mg; vit. B_6, 2 mg; vit. B_{12}, 0.1 mg; Niacin, 40 mg; Pantothenic acid, 12 mg; Folic acid, 1 mg; Fe, 100 mg; Cu, 20 mg; Mn, 50 mg; Co, 2 mg; I, 1 mg; Zn, 100 mg; Se, 0.1 mg.
[d]Calculated value.

insufficient live weight at slaughter were calculated (Gidenne and Feugier 2009).

In vivo digestibility

The apparent digestibility coefficients of dry matter and nutrients of the experimental diets were measured *in vivo* on 32 rabbits (16 per diet), other than the ones used for the growth trial. They were kept in individual digestibility cages equipped with wire nets to collect faeces separately from urine. The digestibility trial was performed from 61 to 64 days of age following the procedure of Perez et al. (1995). The digestible energy (DE) and digestible protein (DP) contents of the experimental diets were calculated as follows:

- DE (MJ/kg) = diet gross energy (MJ/kg) × gross energy digestibility coefficient/100
- DP (g/kg) = diet crude protein (g/kg) × protein digestibility coefficient/100.

Sampling of caecal contents and hard faeces

At 70 d of age, 20 rabbits (10 per diet) were randomly selected among those kept individually in the digestibility cages and slaughtered for sampling the caecal content to determine volatile fatty acid, ammonia N and microbiota. Rabbits were euthanized with CO_2 asphyxiation followed by cervical dislocation after the loss of consciousness was assessed through the evaluation of the vestibulo-ocular reflex. Pellets of hard faeces were collected per each animal from the distal part of the gut for microbiota characterisation. Samples of caecal contents and hard faeces were aseptically collected in 2-mL Eppendorf and stored at −80 °C until processing for DNA extraction. The pH of the caecal content was immediately measured with a Crison GLP 22 pH metre (Crison Instruments S.A., Barcelona, Spain) and then diluted with a 15% HPO_3 solution (25% wt/wt) to be stored at −20 °C until further analyses.

Chemical analyses

The diets were analysed for the contents of dry matter (934.01), ash (967.05), crude protein (2001.11) and starch (amyloglucosidase α amylase method, 996.11) by AOAC (2000). The ether extract content was determined after acid hydrolysis (EC 1998). The fibre

fractions, i.e. NDF (without sodium sulphite and inclusive of residual ash), ADF (inclusive of residual ash) and ADL (obtained by solubilisation of cellulose with sulphuric acid), were analysed according to Van Soest et al. (1991) and Mertens et al. (2002), respectively, using the sequential procedure and the filter bag system (Ankom Technology, Macedon, NY, USA). The content of gross energy was measured by an adiabatic bomb calorimeter (C200, IKA, Staufen, Germany).

The thawed samples of caecal content were centrifuged at 6,339 $\times g$ for 10 min. Caecal ammonia N was determined on the supernatant with a pH metre (Crison GLP 22) and its ammonia specific electrode (mod. 9663, reference electrode mod. 5044) (Crison Instruments S.A.). Volatile fatty acids (VFA) molar contents were measured on the supernatant with gas chromatography (Agilent 7820 A, flame ionisation detector, Split–splitness injection system, programmable oven) on a cross bond capillary column (DB–FFAP, 30 m $\times$ 0.25 mm I.D., 0.25 μm film thickness) (Agilent Technologies, Santa Clara, CA, USA) (Osl 1988).

DNA extraction from caecal content and hard faeces

The first step of total bacteria extraction involved the use of a Tissue Lyser (Qiagen, Hilden, Germany) for the homogenisation of samples. In particular, 300 mg of hard faeces or caecal content were added to 700 μL of RTL buffer (guanidine thiocyanate 0.12 M), together with one metallic bead in a 2 mL Eppendorf tube for 6 min at 30 Hertz, twice. In the second step, samples were subjected to enzymatic treatment with 22,5 μL of Lysozyme (Thermo Fisher Scientific, Waltham, MA, USA) (30 min at 37 °C) and 30 μL of Proteinase K (Thermo Fisher Scientific) (30 min at 50 °C). At the end of the enzymatic treatment, samples were incubated at 75 °C for 5 min and then centrifuged for 5 min at 20,000 $\times g$, after which the supernatant was collected. The 800 μL of lysate were transferred in an S-block with 200 μL isopropanol and 25 μL MagAttract magnetic beads suspension (Qiagen) and loaded into the instrument Biosprint 96 (Qiagen) for the DNA purification.

During this third step, the samples were subjected to four washing cycles with different solutions: RPW buffer (guanidine hydrochloride, 1.31 M; Qiagen), ethanol (96%) and tween solution at 0.02%. Then, DNA was eluted into 100 μL of nuclease-free water. The final amount of extracted DNA was measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) using the Qubit™ DNA HS Assay Kit Fluorometer (Thermo Fisher Scientific).

16S rRNA gene amplification

The library preparation for the amplification of seven hypervariable regions of the 16S gene (V2, V4, V8 and V3, V6-7, V9) involved the use of the 16S Ion Metagenomics and Ion Xpress Plus 9 Fragment Library Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Amplified libraries were pooled to reach a final concentration of 100 pM and processed with the Ion 520™ & Ion 530 ™ Kit – OT2 400 bp (Thermo Fisher Scientific). The sample pool was loaded on the Ion 520 chip and the sequencing run was performed with Ion™ GeneStudio S5 System (Thermo Fisher Scientific).

Statistical analyses and bioinformatics

The individual data of live weight and daily weight gain were analysed by ANOVA (Analysis of Variance) using PROC MIXED of SAS (SAS Institute 2013) with the diet as the main effect and the pen as a random effect. Pen feed intake and feed conversion ratio, individual coefficients of total tract apparent digestibility, and caecal fermentation traits were analysed by ANOVA *via* the PROC GLM of SAS with the diet as the main effect. The Bonferroni test was used to compare means. Differences among means with $p \leq .05$ were assumed to be statistically significant.

As for 16S gene sequencing, raw reads were processed and analysed using the Microbial Genomics workflow of the CLC Genomics workbench (version 22.0.2) (Qiagen). Primers were removed by trimming 20 base pairs on both ends. Samples were filtered based on the number of reads, in order to have comparable coverage. The minimum number of reads was set at 100 and the minimum percent from the median at 50%. High-quality reads were clustered at a 97% level of similarity into Operational Taxonomical Units (OTUs). SILVA SSU v138.1 database was used as a reference for the taxonomic assignation of OTUs (Quast et al. 2012). Low abundance OTUs were eliminated (<10 reads for combined abundance). Rarefaction analyses of diversity measures of the number of total OTUs were calculated, to check the quality depth of sequencing. Alpha diversity was estimated using the Shannon entropy index, and the Kruskal–Wallis statistical test was performed to identify statistical differences ($p \leq .05$). Beta diversity among samples was analysed using the Principal Coordinate Analysis

(PCoA) based on the Bray–Curtis distances, to which the Permutational MANOVA (PERMANOVA) was subsequently applied in order to investigate the statistical significance of the sample. Differential abundance analysis with a Wald test was used to highlight significant differences ($p \leq .05$) within bacteria at phylum and genus taxonomic levels.

Results

In vivo performance and health status

The growth performance of rabbits during the fattening period was consistent with the standard of the genotype and did not change according to the dietary treatment (Table 2). The live weights of rabbits at 71 d averaged 2833 g, which corresponded to a daily weight gain in the late fattening period of 40.9 g/d, a feed intake of 165 g/d and a feed conversion ratio of 4.04 (Table 2).

During the trial, a total of 17 rabbits (9 fed diet C and 8 fed diet FF) died and other 7 underweight rabbits (4 fed diet C and 3 fed diet FF) were excluded at the end of the trial because they had not reached the minimum commercial slaughtering weight (2.2 kg), i.e. total losses rate averaged at 4.17% without any significant effect of the dietary treatment (Table 2).

Digestibility of diets and caecal fermentation profile

The digestibility coefficients of dry matter (59.9% vs. 61.1%; $p < .05$) and fibre fractions (NDF: 19.2% vs. 26.8%; $p < .001$) were significantly lower whereas the digestibility coefficients of crude protein (73.5% vs. 71.6%; $p < .01$) and ether extract (87.2% vs. 83.6%; $p < .001$) were higher in rabbits fed diet FF compared to those fed diet C (Table 3).

As for the caecal fermentation profile measured, neither total VFA nor ammonia concentrations changed with the diet, whereas the rate of acetic acid was lower (73.2 vs. 76.2 mmol/100 mmol VFA; $p = .058$) and that of butyric acid higher (21.2 vs. 17.8 mmol/100 mmol VFA; $p = .051$) in rabbits fed the diet FF compared to those fed the control diet (Table 4). The C_3 to C_4 ratio was similar in the two experimental groups.

16S rRNA gene microbiota analysis

The 16S multi-amplicon-sequencing produced 5,530,321 raw reads, which resulted in a total of

Table 3. Coefficients of digestibility (%) and nutritive value of fattening diets measured from 61 to 64 d of age.

Diets	Control (C)	Fibre + fat (FF)	*p* Value	RSD
Rabbits, n	16	16	–	–
Dry matter, %	61.1[b]	59.9[a]	<.050	1.22
Crude protein, %	71.6[A]	73.5[B]	<.010	1.62
Ether extract, %	83.6[A]	87.2[B]	<.001	1.20
Starch, %	98.7[B]	98.6[A]	<.001	0.09
NDF, %	26.8[B]	19.2[A]	<.001	2.49
Hemicellulose, %	36.6[B]	32.7[A]	<.001	2.24
ADF, %	18.1[B]	7.4[A]	<.001	2.76
Cellulose	23.5[B]	13.0[A]	<.001	2.72
Gross energy, %	61.8	61.1	.130	1.17

Note: RSD: residual standard deviation; NDF: neutral detergent fibre; ADF; acid detergent fibre; DP/DE: digestible protein/digestible energy ratio.
[a,b]Values with different superscript letters within the same line are significant different ($p < .05$). [A,B]Values with different superscript letters within the same line are significant different ($p < .01$).

Table 4. Fermentative profile of caecal content in growing rabbits at 70 days of age.

Diets	Control (C)	Fibre + fat (FF)	*p* value	RSD
Rabbits, n	10	10	–	–
pH	6.10	6.20	.430	0.16
Ammonia-N, mmol/L	3.29	4.06	.220	1.31
Total VFA, mmol/L	54.80	55.40	.890	9.56
C_2 (mmol/100 mmol VFA)	76.20	73.20	.058	3.28
C_3 (mmol/100 mmol VFA)	4.67	4.10	.300	1.15
C_4 (mmol/100 mmol VFA)	17.80	21.20	.051	3.44
C_5 (mmol/100 mmol VFA)	1.06	1.17	.450	0.29
C_6 (mmol/100 mmol VFA)	0.18	0.36	.170	0.28
C_3 to C_4 ratio	0.27	0.21	.170	0.10

Note: RSD: residual standard deviation; VFA: volatile fatty acid.

Table 2. Growth performance of rabbits from 54 to 71 d of age.

Diets	Control (C)	Fibre + fat (FF)	*p* Value	RSD
Rabbits, n	275	277	–	–
Pens, n	9	9		
Initial live weight[a], g	2140	2141	.95	206
Final live weight[a], g	2825	2844	.37	257
Daily weight gain[a], g/d	40.30	41.40	.09	7.35
Daily feed intake[b], g/d	163	167	.40	8.03
Feed conversion ratio[b], g feed/g gain	4.06	4.04	.84	0.20
Mortality, %	3.13	2.78	.80	–
Underweight rabbits[c], %	1.39	1.04	.70	–
Total lost rabbits, %	4.52	3.82	.68	–

Note: RSD: residual standard deviation.
[a]Individual data.
[b]Average pen data.
[c]Final live weight < 2.2 kg.

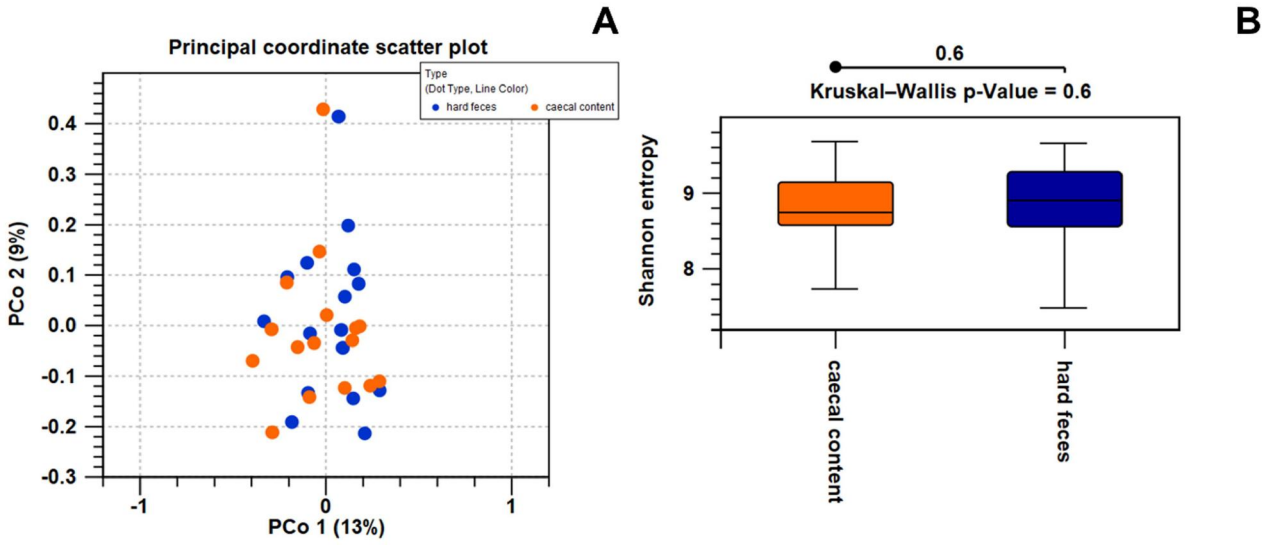

Figure 1. Beta-diversity (principal coordinate analysis) (A) and alpha diversity (Shannon entropy index) (B) at OTUs (Operational Taxonomical Units) level in the sample's communities between caecal content and hard faeces of growing rabbits sampled at 72 days of age.

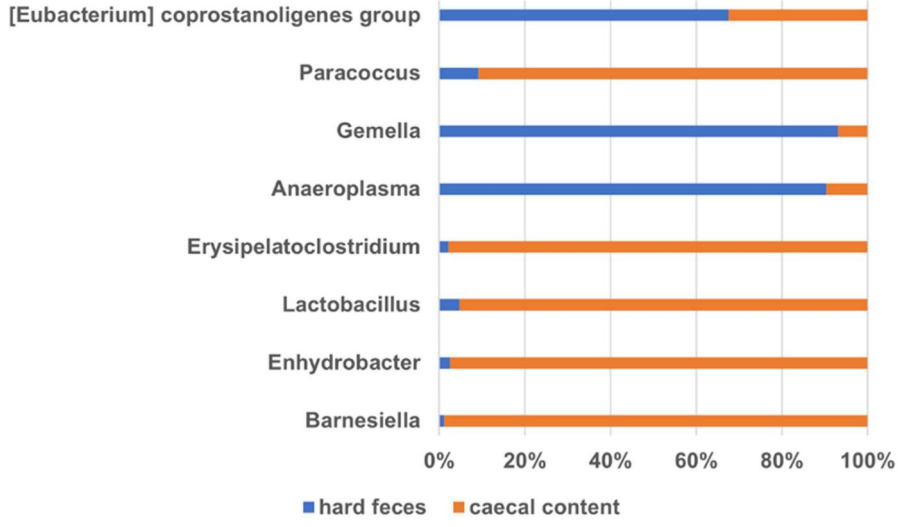

Figure 2. Stacked bar chart indicating the percentage of relative abundance of significantly different ($p \leq .05$) genera in the sample's communities of caecal content and hard faeces.

3,338,060 valid contigs after different filtering steps and chimaera removal. These final sequences were clustered into 928 OTUs. Each sample had a mean number of sequences per sample of 162,457 ± 31.5.

The Shannon index did not reveal any significant difference between hard faeces and caecal samples in microbial diversity (Figure 1(A)). Additionally, the principal coordinate analysis did not show a clear separation of the two sets of samples (Figure 1(B)). The differential abundance analysis revealed only 7 genera with abundances significantly different between hard faeces and caecal samples (Figure 2). No differences were detected at the phylum level.

The overall microbiota compositions of caecal contents and hard faeces are reported in Figure 3. The phylum of Firmicutes (86%) was the most abundant in both types of samples. Further phyla were characterised as Patescibacteria (5%), Proteobacteria (3%), Bacteroidota (3%) and Desulfobacterota (1%). The phylum of Firmicutes was represented especially by the class of Clostridia, followed by Bacilli, Saccharimonadia and Bacteroidia. The families of Ruminococcaeae and Lachnospiraceae were mostly represented in the Clostridia class in all samples. Within the dominant families, we also detected the presence of bacteria belonging to the RF39 order of the Bacilli class. Then,

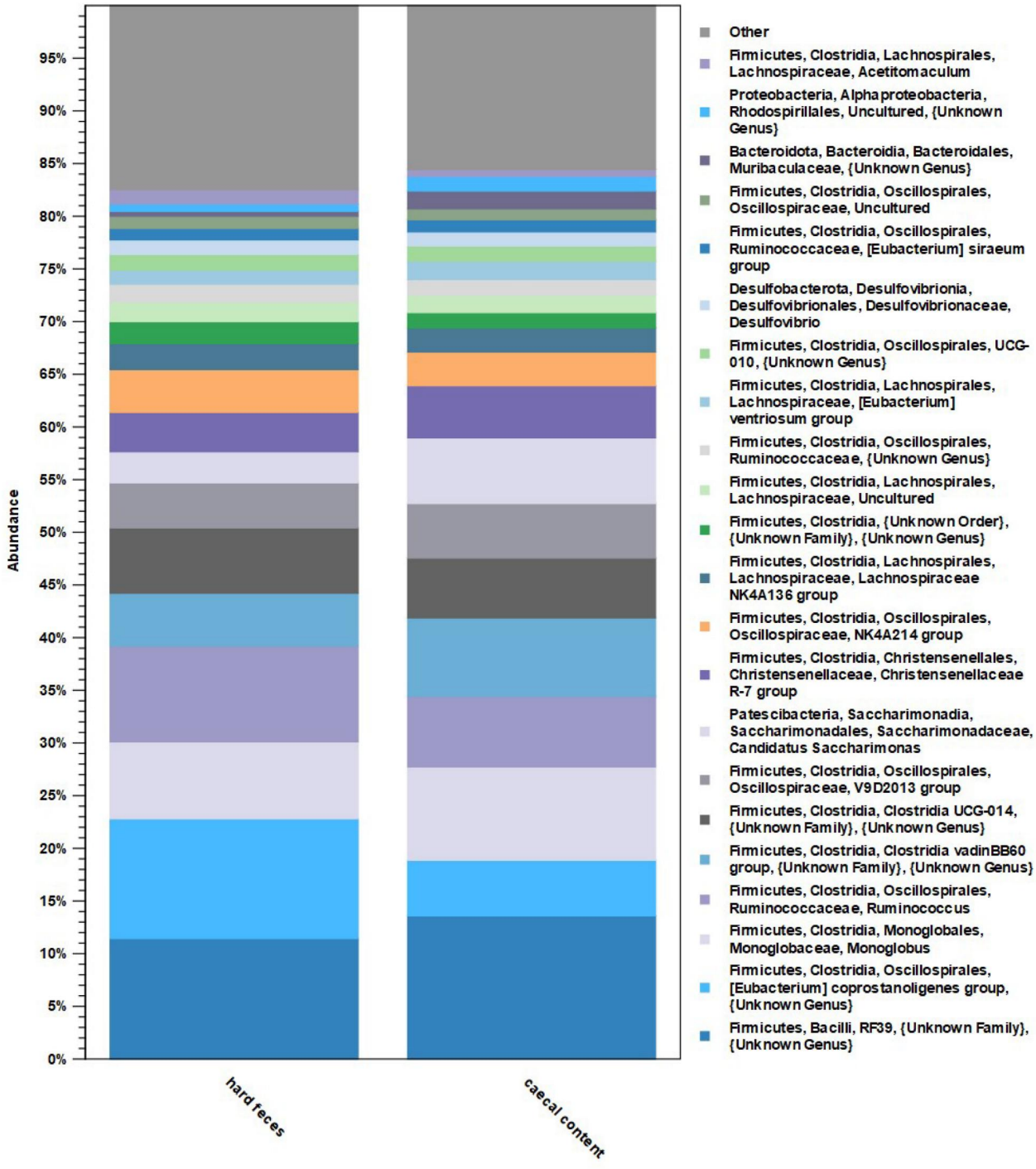

Figure 3. Microbial composition of caecal content and hard faeces indicated as a percentage of the population. Each bar represents the average relative abundance of each bacterial taxon within a group. The 20 most abundant features for each type of sample are shown.

Oscillospiraceae and Monoglobaceae were representative families in both types of samples. In the hard faeces, we also detected [Eubacterium] coprostanoligenes group in the predominant families. The unknown genus of RF39 order was the main genus in both faeces and caecal content. In the hard faeces, within the dominant genera, we found an unknown genus of [Eubacterium] coprostanoligenes group. Then, *Monoglobus*, *Ruminococcus* and bacteria belonging to the Clostridia UCG-014 and vadinBB60 groups were subdominant genera in all samples with an occurrence between 5 and 9%. As for groups with a relative abundance $\leq$5%, in the samples of caecal content we also detected the presence of *Candidatus Saccharimonas*,

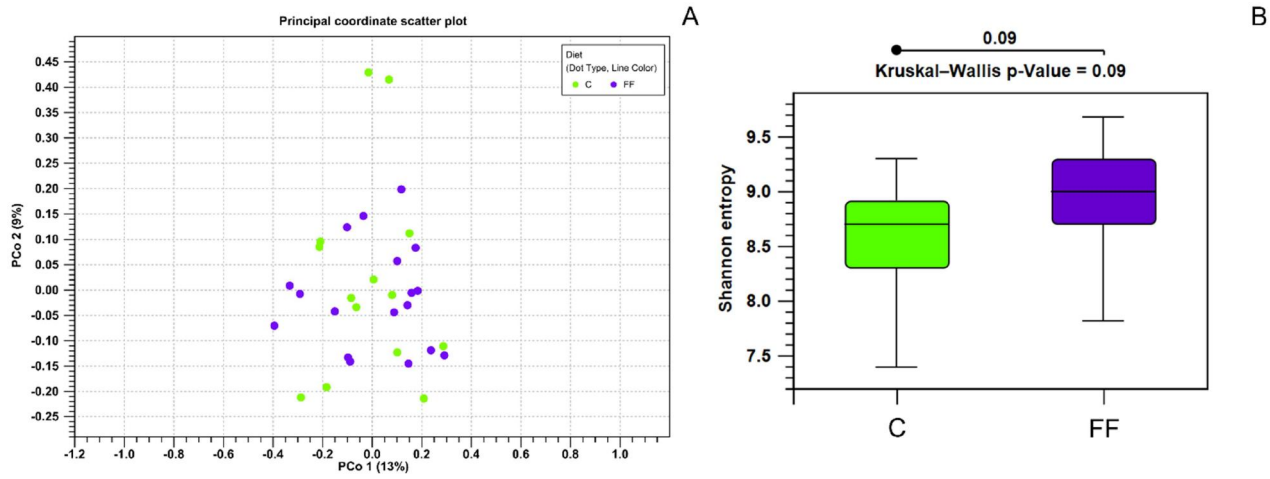

Figure 4. Beta-diversity (principal coordinate analysis) (A) and alpha diversity (Shannon entropy index) (B) at OTUs (Operational Taxonomical Units) level of caecal content and hard faeces microbiota in rabbits fed Control (C) and fibre+fat (FF) diets.

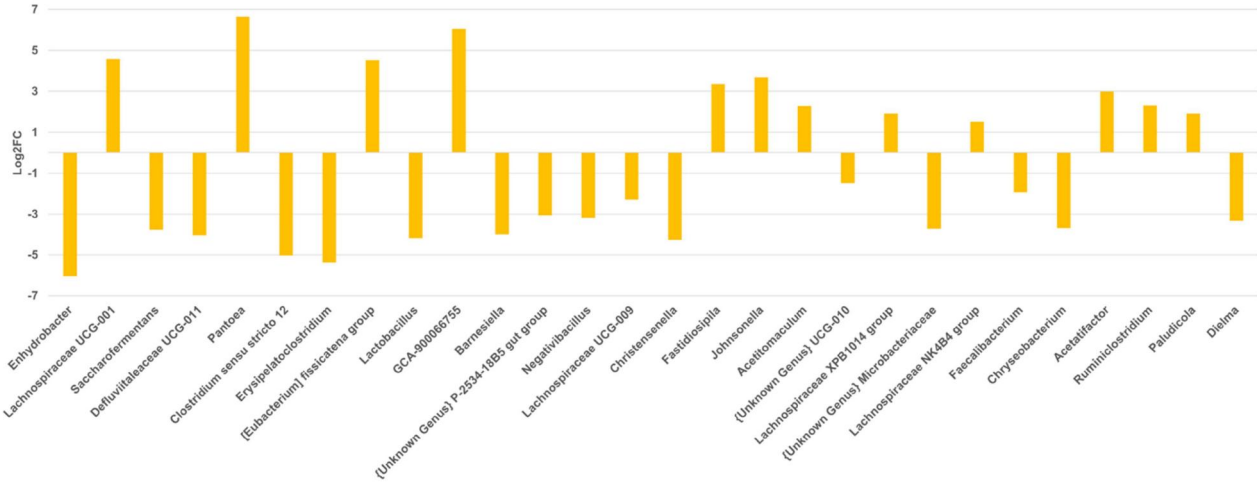

Figure 5. Clustered bar chart with the Log_2 fold change values of significantly different (Wald test. $p \leq .05$) genera between Control (C) and fibre+fat (FF) diets microbial communities. Genera with positive values are overexpressed in animals fed the FF diet. Genera with negative values are overexpressed in animals fed the C diet.

[Eubacterium] coprostanoligenes group and the V9D2013 bacteria group.

The effect of the diet on gut microbial communities of rabbits was evaluated using all samples of both hard faeces and caecal contents, due to the similar microbiota composition in the two types of samples as discussed above.

The principal component analysis on Bray-Curtis distances from the final OTU table did not clearly cluster the samples coming from rabbits fed the two diets (Figure 4); then, the PERMANOVA statistical test did not show any difference in beta diversity according to the diet ($p \geq .05$); finally, the comparison of alpha diversities using the Kruskal-Wallis's test for the Shannon entropy index did not reveal any significant difference in microbial diversity or richness according to the diet. On the other hand, the Walt test for the differential abundance analysis revealed that 29 genera were differently represented in the samples of rabbits fed the two diets ($p \leq .05$) (Figure 5), whereas no difference was recorded at the level of phylum.

In detail, the samples from rabbits fed the diet FF showed an overrepresentation of the unknown genera belonging to *Lachnospiraceae UCG-001*, *Pantoea, [Eubacterium] fissicatena* group, *GCA-900066755, Fastidiosipila, Johnsonella, Acetitomaculum*, *Lachnispiraceae XPB1014* group, *Lachnispiraceae NK4B4* group, *Acetatifactor*, *Ruminiclostridium*, *Paludicola*. Then, the samples from rabbits fed the control diet showed the highest relative abundances of the genera

Enhydrobacter, *Saccharofermentans*, *Defluviitaleaceceae UCG-011*, *Clostridium sensu stricto 12*, *Erysipelatoclostridium*, *Lactobacillus*, *Barnesiella*, *PT-2534-18B5 gut* group, *Negativibacillus*, *Lachnospiraceae UCG-009*, *Christensenella*, Unknown genus of Microbacteriaceae group, *Faecalibaterium*, *Chryseobacterium* and *Dielma*.

Discussion

Fibre is the main component of the rabbit diet which guarantees the normal functioning of the rabbit's digestive physiology and regulates the composition and diversity of the microbial gut community (Gidenne, Carabaño, et al. 2020; Curone et al. 2022), which finally impacts on rabbit health. Moreover, dietary fibre content and quality affect the productive results as they are related to the energy value of diets and, consequently, feed intake, feed conversion ratio and global farm efficiency (Gidenne et al. 2017).

Thus, the present study aimed at designing diets that could potentially protect rabbit digestive health by increasing insoluble fibres content - through the inclusion of purified wood lignocellulose - and controlling feed intake and, thus feed conversion ratio, by increasing the energy content of diets - through fat addition. To test this hypothesis, the effects on gut microbiota composition and volatile fatty acid production were assessed, as well as the digestibility of diets and the growth performance of rabbits.

On the whole, the chemical composition of the diets used in the present study was consistent with recommendations for fattening rabbits (Gidenne, Lebas, and Fortun–Lamothe 2020), where the ADF and ADL contents should not be less than 170 – 180 g/kg (181 – 188 g/kg in our experimental diets) and 40 – 45 g/kg (42.6 – 47.4 g/kg), respectively, for the digestive health of rabbits.

Previous authors (Krieg et al. 2012) have found that the supplementation of purified lignocellulose as Arbocel® decreased feed intake and daily weight gain in growing rabbits without affecting either mortality or morbidity. Differently, in our trial, no difference was recorded on growth performance and feed conversion ratio between animals fed the two diets. Then, we found that both crude protein and ether extract digestibility increased in rabbits fed diet FF compared with those fed diet C because of the higher inclusion level of sunflower meal replacing the protein from alfalfa meal and because of the higher inclusion of fat, respectively. On the other side, fibre fractions digestibility decreased due to the inclusion of purified lignocellulose partly replacing fibre originating from other fibrous raw materials. In other words, these results show on the whole that in rabbits the dietary inclusion of purified lignocellulose at the tested inclusion rates does not represent a barrier to the digestion of main nutrients coming from the other raw materials, as proved in other species for the inclusion of purified lignin (Baurhoo et al. 2007), whereas the digestibility of the purified cellulose was likely lower than the digestibility of the fibre coming from the raw materials included in the diets, thus decreasing overall fibre fraction digestibility.

Under our conditions, the differences in the digestibility of the different nutrients between the two diets did not produce major differences in microbiota composition at the level of the caecal content and/or hard faeces. In fact, even when comparing diets with larger differences in NDF (420 g/kg vs. 341–354 g/kg, as fed), ADF (270 g/kg vs. 207–211 g/kg, as fed) and ADL content (108 g/kg vs. 54–55 g/kg, as fed) than those tested in our trial, Rodríguez-Romero et al. (2013) did not find differences in the gut microbial composition of growing rabbits fed the different diets. Consistently with our results, other authors did not show a pattern of microbiota clusterization in the caecum of growing rabbits fed diets containing different fibre contents (NDF from 250 to 400 g/kg as fed (Wu et al. 2019); ADL: 29 vs. 48 g/kg DM (Michelland et al. 2011)). On the other hand, when diets differed in fibre content (ADF: 176–210 vs. 136 g/kg DM) and quality (alfalfa meal and peanut vine vs. beet pulp), Liu et al. (2022) found higher microbial diversity and richness in rabbits fed the diets with the higher ADF content. In fact, the dietary inclusion of rapidly fermentable fibres, such as pectins, β-glucans, fructans and gums, has been associated with a decrease in caecum diversity in rabbits (Paës et al. 2022).

On the other hand, under the conditions of the present study, the increase of NDF, ADF and ADL contents in the diet FF compared to the diet C diet did not produce significant effects on the alpha and beta diversity of gut microbiota in growing rabbits at the end of the trial, differently from previous trials where diets with much larger differences in the fibre fractions, i.e. about +35% for NDF, +60% for ADF and +50% for lignin, were compared (Wu et al. 2019; Liu et al. 2022). Moreover, in the present trial, the type of added fibre, purified lignocellulose from wood, could have accounted for the absence of large effects on microbiota diversity and composition. Consistently with our results, Krieg et al. (2012) found that the increase in the diet of pure cellulose as Arbocel®

decreased the total VFA content, but did not affect the fermentation pattern in 40-d-old rabbits.

As for the microbiota gut composition, under our conditions, twelve genera were overrepresented in rabbits fed the diet FF compared to those fed the diet C diet, mostly from the Lachnospiraceae family. According to literature, this change could also be ascribed to the fat addition to the diet: in fact, Lachnospiraceae bacteria have been found to increase in the gut microbiota of rabbits fed diets containing 10% flaxseed oil and 5% fish oil compared to those fed a commercial diet low in added fat (Curone et al. 2022). Then, Lachnospiraceae have been associated with healthy rabbits (Cotozzolo et al. 2020) and a reduction of mortality in young rabbits (Combes et al. 2013), besides an increased production of butyrate (Costantini et al. 2017; Parolini 2019). Also in our trial, a higher proportion of butyrate was measured in the caecal contents of rabbits fed the lignocellulose-added diet compared to those fed the control diet. Usually in rabbits, the production of acetate increases and that of butyrate decreases when the dietary fibre content decreases, but the quality of fibre also plays a role in fermentation activity (Trocino et al. 2013). In our trial, the lower acetate rate in rabbits fed the wood lignocellulose-added diet could be related to the lower inclusion rate of beet pulp and, as a consequence, the lower content of fermentable fibre (i.e. pectins and hemicelluloses) in diet FF compared to the control diet, rather than to the effect of the purified lignocellulose supplementation *per se*. In fact, in growing rabbits, Krieg et al. (2012) found that the total content of caecal total VFA decreased whereas the molar proportions of VFA were not affected when the dietary ratio between lignin and cellulose increased due to the addition of purified wood lignocellulose. On the other hand, changes in the microbiota composition between rabbits fed the two diets could explain the effects on caecal VFA profile.

In our trial, a lower abundance of the genus *Lactobacillus* was detected in rabbits fed with the lignocellulose-added diet compared to the control diet, where bacteria of this genus are recognised as probiotics (Chen et al. 2018). In rabbits, *Lactobacillus* is rarely present in the caecum, due to their lack of adhesive capability (Yu and Tsen 1993) which could have been further compromised when feeding the lignocellulose-added diet (Rodríguez-Romero et al. 2013). Differently, in broilers, purified lignin was effective as a feed additive for modulating gut health (Fangueiro et al. 2023) favouring the growth of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria*, and controlling intestinal pathogens (Baurhoo et al. 2007).

Our findings confirmed the similarity in the microbial diversity and the bacterial community structure of hard faeces and caecal content (Michelland et al. 2010; Velasco-Galilea et al. 2018) as reported also for soft faeces (Michelland et al. 2010), likely because of the strict proximity between they caecum and rectum. Additionally, sampling of caecal content and hard faeces was performed in the afternoon, when feed intake is usually high and hard faeces excretion occurs (Birolo et al. 2020; Gidenne, Carabaño, et al. 2020), whereas soft faeces are excreted during the first hours of the day (Birolo et al. 2020; Gidenne, Lebas, and Fortun–Lamothe 2020).

With regard to the taxonomic characterisation of microbiota in hard faeces and caecal contents, our findings are consistent with previous studies on rabbit gut microbiota (Monteils et al. 2008; Combes et al. 2017). Both in hard faeces and caecal content, Firmicutes are dominant (88%), followed by Patescibacteria (5%), Proteobacteria (3%), Bacteroidota (3%) and Desulfobacterota (1%). Occasionally, the presence of the Tenericutes phylum in caecal content and hard faeces samples has also been reported (Velasco-Galilea et al. 2018; Hu et al. 2021), where this phylum has been classified within the Firmicutes in our study due to the different reference database (i.e. SILVA v138.1 vs. Greengenes and SINA aligners, respectively).

Then, compared to Cotozzolo et al. (2020), we found different abundances at the caecum for Firmicutes (88% in our trial vs. 43%), Bacteroidota (3% in our trial vs. 40%) and Patescibacteria (5% vs. 0%), being this latter detected only in the small intestine by the same authors. Differences between studies may be related to ontogenetic factors, like rabbit genotype and age (Combes et al. 2013), as well as the use of different sequencing regions of 16S rRNA used (Bukin et al. 2019), i.e. seven hypervariable regions of the 16S gene in our study vs. V3-V4 or V4-V5 regions in previous studies.

According to our findings, as for classes, Clostridia and Bacilli were the dominant ones in the bacterial communities of both faeces and caecal content, followed by the Saccharimonadia class within the Patescibacteria phylum. Similarly, Clostridia was the prevalent class, followed by Bacteroidia and Mollicutes, in the caecal content microbiota of rex rabbits (Zou et al. 2016). As for families, consistent with previous studies (Velasco-Galilea et al. 2018), Ruminococcaceae and Lachnospiraceae were dominant in our study, together with a bacteria group of RF39 order within the Bacilli class.

As for genera, we found that *Paracoccus*, *Erysipelatoclostridium*, *Lactobacillus* and *Barnesiella* were overexpressed in the microbiota of caecal content compared to hard faeces. Differently, Zeng et al. (2015) described an overexpression of *Lactobacillus* in the microbiota of hard faeces with respect to the microbiota of soft faeces in rex rabbits. On the whole, we confirmed that the presence of the *Lactobacillus* genus was rather low (<1%) both in the hard faeces and caecal content (Fann and O'Rourke 2001; Hu et al. 2021). As for *Paracoccus* and *Erysipelatoclostridium* genera, to our knowledge, no literature on their abundance and function in the rabbit gut microbiota is available. Then, *Barnesiella* has been also detected in other studies (Kylie et al. 2018) at a relatively low abundance and negatively correlated with the rabbit age in caecal microbiota (Combes et al. 2014); moreover, the family of Barnesiellaceae was overrepresented in soft faeces compared to hard faeces in rex rabbits (Zeng et al. 2015).

The comparison of hard faeces with caecal contents showed an overrepresentation of the genera *Gemella*, *Anaeroplasma* and the unknown genus of [Eubacterium] coprostanoligenes group. The genus *Gemella* embraces a group of Gram-positive or Gram-variable, facultative anaerobic organisms that seem to be resident in the mucous membranes of humans and some warm-blooded animals (Hoyles et al. 2000), but their role is not still clarified in rabbit gut. With regard to the *Anaeroplasma* genus, Zeng et al. (2015) found these bacteria more abundant in the hard faeces of rex rabbits than in soft faeces, whereas Velasco-Galilea et al. (2018) did not find differences between caecal content and hard faeces as in our study. Finally, based on literature (Mukherjee et al. 2020), several strains assigned to the genus *Eubacterium* have been frequently identified in the oral cavity and intestinal tract of mammals.

On the whole, the present study confirmed the similarity in the microbiota composition of caecal content and hard faeces in rabbits, where these latter could be used to evaluate changes in microbiota diversity and richness in the same animal according to age and diet composition as stated by Stanley et al. (2015) in poultry using faecal samples. As shown by the first studies in poultry on novel precision biotic feeding of microbiota for specific end-products regardless of microbiota composition (Bortoluzzi et al. 2023; Yan et al. 2023), further studies should focus on characterising the metabolic bacteria pathways by a metagenomic approach and their harnessing for improving nutritional health.

Conclusions

Under the conditions of our trial in absence of health problems, the dietary inclusion of low rates of purified lignocellulose associated with the addition of fat modified the digestibility of protein, fat and fibre fractions without impairing the diet nutritive value or affecting rabbit daily weight gain and feed conversion ratio. Even if some shifts in the fermentation profile at the caecum were observed, no major effects on gut microbiota were observed in this study. Then, if the dietary inclusion of low rates of purified lignocellulose associated with the addition of fat could be an effective strategy for formulating diets addressed to prevent digestive disorders remains to be proved in the field under the sub-optimal health conditions of commercial farms.

Ethics statement

The study was approved by the Ethical Committee for Animal Experimentation (Organismo Preposto al Benessere Animale) of the University of Padova (project 78/2020; Prot. nr. 447884, approved on 26/11/2020). The researchers involved in animal handling were either animal specialists (Ph.D. or MSc in Animal Sciences) and/or veterinary practitioners, which handled all animals following the principles of EU Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data presented in this study are available upon request from the corresponding author.

Data deposition

Raw sequencing data have been deposited in ENA (European Bioinformatic Institute) under the project accessions number: PRJEB70893.

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