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Chlorella and vegetable oil inclusion in diets for growing rabbits: effects on growth, digestibility, plasma metabolites, and caecal fermentations and microbiota

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ABSTRACT

The inclusion of microalgae in livestock diets has been shown to enhance animal productivity, immune response, and meat quality. However, the role of chlorella (*Chlorella vulgaris*) in growing rabbit nutrition has been scarcely explored, with available studies focusing on low inclusion levels (<1%) and their effects on rabbit growth and immune response. This study evaluated the growth performance, nutrient digestibility, plasma metabolites, caecal fermentative activity, and caecal microbiota composition of growing rabbits fed diets with different inclusion levels of chlorella and crude fat. A total of 648 mixed-sex Grimaud crossbred rabbits (33 d of age; 841 ± 140 g live weight) were fed six experimental diets (96 rabbits per diet for the growth trial) based on a bifactorial design with three dietary inclusion levels of chlorella (0, 1, and 2%) and two levels of crude fat (3 and 5%) obtained by the inclusion of soybean oil (1 and 3%, respectively). The trial lasted 38 days until slaughter. From 47 to 51 days of age, 72 rabbits (12 per diet) were submitted to a digestibility trial. At 51 days of age, samples of plasma and caecal content were collected from 36 rabbits (six rabbits per diet) to analyse plasma metabolites, caecal fermentations, and caecal microbiota. Rabbit live weight at 71 days of age (2 700 g, on average), weight gain (48.8 g/d) and feed conversion ratio (3.27) were unaffected by chlorella inclusion, while feed conversion ratio improved (−5%; $P < 0.001$) with an increase of crude fat from 3 to 5%. The digestibility of ADF (23.2 vs 20.9%; $P < 0.05$) and crude fat (83.8 vs 85.6%; $P < 0.01$) improved with the inclusion of chlorella at 2%, as well as the digestibility of crude fat (82.4 vs 86.9%; $P < 0.001$) and gross energy (57.3 vs 58.7%; $P < 0.001$) with crude fat inclusion at 5%. Plasma non-esterified fatty acids decreased (−19%; $P < 0.05$) in diets with 5% crude fat. Neither chlorella nor crude fat inclusion levels affected other plasma metabolites, caecal fermentations, or caecal microbiota. Overall, the inclusion of chlorella up to 2% in diets for growing rabbits did not significantly affect diet nutritional value, animal performance, or caecal activity. On the other hand, increasing crude fat to 5% improved the overall feed efficiency.

Implications

The dietary inclusion of *Chlorella vulgaris*, rich in bioactive compounds, could improve the digestive activity and immune response of growing rabbits, thereby enhancing animal growth and health, while the increase of dietary fat levels could improve feed efficiency. However, information about the effectiveness of these strategies and optimal inclusion levels is limited. This study showed that chlorella inclusion of up to 2% in the diet did not bring significant beneficial effects on rabbit health and performance, whereas the crude fat increase is a viable strategy to increase the efficiency of rabbit production.

Introduction

The global population is projected to reach 9.7 billion by 2050, leading to a 60–100% increase in food demand (Nagarajan et al.,

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2021). Livestock production accounts for 43% of the protein supply and represents a key sector for meeting future protein demand (Yarnold et al., 2019). However, livestock systems are facing several challenges, including climate change, disease outbreaks, and consumer demand for environmentally friendly products. In this context, the selection of alternative feed resources such as microalgae becomes crucial for sustainably meeting protein demand and consumer expectations (Calicioglu et al., 2019).

The inclusion of microalgae in livestock feeds could offer several advantages from both nutritional (Bature et al., 2022) and environmental perspectives (Saadaoui et al., 2021). Microalgae are protein-rich (50–70% CP) and supply essential nutrients other than essential amino acids, such as omega 3 fatty acids, vitamin B_{12} (Saadaoui et al., 2021; Bature et al., 2022) and several bioactive compounds, such as carotenoids, polysaccharides, phenolic compounds, polyphenols, and sterols (Yang et al., 2023). These compounds have been shown to provide various benefits, including improved immune response, enhanced meat quality, and increased productivity and reproductive performance even at low dietary inclusion rates in livestock species (Saadaoui et al., 2021).

In rabbits, several studies have recently been conducted to test low to moderate inclusions of sources of bioactive compounds, such as lemon (*Citrus limon*) powder (1–2% inclusion) (Elwan et al., 2019a), tomato (*Solunum lycopersicum*) powder (Elwan et al., 2019b), moringa (*Moringa oleifera*) leaves (10–30%) (Sun et al., 2018), carob pods (*Ceratonia siliqua*) (2.5–10%) (Hafsa et al., 2017), and black cumin (*Nigella sativa*) seeds (0.3–0.6%) (El-Gindy et al., 2020), showing promising results in terms of enhanced performance and feed efficiency (Hafsa et al., 2017; Sun et al., 2018; Elwan et al., 2019a, b; El-Gindy et al., 2020), antioxidant activity (Hafsa et al., 2017; Elwan et al., 2019a, b; El-Gindy et al., 2020), an improved immune system (El-Hack et al., 2019; El-Gindy et al., 2020), and antimicrobial and anti-inflammatory effects (Singh et al., 2021).

Nevertheless, studies on microalgae inclusion in rabbit diets are limited (Valente et al., 2021). Among microalgae species, some studies evaluated the inclusion of spirulina (*Arthrospira platensis*) (0.06–15%), chlorella (*Chlorella* sp.) (0.05–1%), and Schizochytrium (*Schizochytrium sp.*) on rabbit performance, health, and meat quality. The substitution of soybean meal with spirulina did not affect rabbit growth or improve meat quality (Peiretti and Meineri, 2011) but reduced diet digestibility (Peiretti and Meineri, 2008). On the other hand, the inclusion of Schizochytrium (180–1 800 mg/kg/d) in rabbit diets increased feed consumption and BW (Hammond et al., 2001).

The role of chlorella in growing rabbits is poorly studied, with few existing works focusing mainly on oxidative stress and immune responses, carcass characteristics, and production performance (Abdelnour et al., 2019; Sikiru et al., 2019a, b). Overall, low inclusions (up to 1% of the diet) of chlorella seem to positively influence production performance, whereas its effects on diet digestibility, gut fermentation and microbiota, as well as on nutritional metabolites, are still unexplored. Given the lack of studies examining chlorella inclusions higher than 1% in rabbit diets and considering that inclusions above 2% would substantially increase diet costs, we selected chlorella inclusion levels of 1 and 2% for this study. Additionally, the possible interactions between microalgae supplementation and the nutritional characteristics of the diet, for instance, in terms of dietary fat level, must be studied. In fact, the addition of lipids in the diet through the inclusion of vegetable oils could support the bioavailability of fat-soluble vitamins and of essential fatty acids provided by the chlorella meal (Michalski et al., 2020), having positive effects on nutrient absorption and on improving the overall feed efficiency. In addition, such combinations may positively impact plasma metabolites by modulating cholesterol levels and improving lipid profiles, supporting healthier metabolic functions (Xia et al., 2024). Moreover, the contribution of chlorella meal to dietary fibre and antioxidants may enhance caecal fermentative activity, increasing the production of beneficial short-chain fatty acids, maintaining gut health, and favour the presence of beneficial bacteria (Martins et al., 2022). In rabbits, the addition of crude fat in the diets through the inclusion of vegetable oils or animal fats has been shown to increase dietary energy concentration and improve feed efficiency (Xiccato, 2020), as well as reduce mortality rates and nitrogen excretion (Saiz del Barrio et al., 2021), thereby improving the overall efficiency and sustainability of rabbit farms. On the other hand, high–fat inclusions (>6%) could affect pellet stability (Maertens, 2010) and gut microflora (Casado et al., 2010). In our study, soybean oil was selected as the source of crude fat due to its widespread availability in the global vegetable oil market (Wang, 2011).

Thus, the present study aimed to test the hypothesis that a low or moderate inclusion of chlorella could promote rabbit performance and digestive health, whereas the addition of fat would maintain a high energy level of diets and a favourable feed conversion ratio. In detail, we compared the growth performance, nutrient digestibility, plasma metabolites, caecal fermentative activity, and caecal microbiota composition of growing rabbits fed diets with different inclusion levels of chlorella (0 vs 1 vs 2%) and with two levels of crude fat (3 vs 5%).

Material and methods

Animals and housing conditions

The trial was carried out at the experimental rabbit farm of the University of Padova (Italy) during March-April. The facility was equipped with an automatic heating system and extraction fans that controlled the air circulation, temperature (19–22 °C), and relative humidity (41–56%). A total of 648 mixed-sex Grimaud crossbred rabbits (33 d of age; 841 ± 140 g live weight) born and weaned at the same experimental facility were selected from healthy litters of primiparous and secondiparous does and individually identified using earmarks. A first group of 576 rabbits was allocated into 72 modules (52.5 cm × 92 cm × 110 cm height) of a park system (elevated pens) with 8 rabbits per module until slaughtering (71 days of age). Each module was equipped with a plastic slat floor (hole dimensions: 70 mm long × 10 mm wide; distance between holes: 7 mm), a feeder for manual feed distribution, and 2 automatic nipple drinkers. A second group of 72 rabbits was allocated to individual digestibility cages for the *in vivo* digestibility trial to determine the nutrient coefficients of total tract apparent digestibility and the nutritive value of the experimental diets (12 rabbits/diet) and, subsequently, the blood plasma metabolites and caecal fermentation and microbiota. The growth performance of these latter rabbits was not included in the dataset analysed for the fattening trial.

Experimental diets

The rabbits were fed six experimental diets (96 rabbits per diet for the fattening trial; 12 rabbits per diet for the digestibility trial) based on a bifactorial design with three dietary inclusion levels of chlorella (C0: 0%; C1: 1%; C2: 2%) and two levels of crude fat (F3: 3%; F5: 5%) obtained by the inclusion of soybean oil (1 and 3%, respectively). The experimental diets were named as follows: C0-F3 (0% chlorella; 3% crude fat), C1-F3 (1% chlorella; 3% crude fat), C2-F3 (2% chlorella; 3% crude fat), C0-F5 (0% chlorella; 5% crude fat), C1-F5 (1% chlorella; 5% crude fat), and C2-F5 (2% chlorella; 5% crude fat). Diets were formulated to be isonitrogenous (15.5% CP) with two levels of digestible energy (9.48 and 9.98 MJ/kg in

diets with 3 and 5% crude fat, respectively) within nutritional recommendations for growing rabbits (De Blas and Mateos, 2020).

The ingredients of the experimental diets and the proximate composition of the chlorella meal (ALLMICROALGAE – Natural Products SA, Pataias, Portugal) and the experimental diets are reported in Table 1. As for the amino acid content of the chlorella meal, lysine was 2.5–3.5 g/100 g of product, methionine 0.4–1.2 g/100 g, cystine + cysteine 0.1–0.8 g/100 g, and threonine 1.5–2.5 g/100 g. To include 2% chlorella in the diet, sunflower meal (30% CP) was reduced by 4% to balance the protein content, whereas 2% dehydrated alfalfa meal (14% CP) was added to balance the fibre content. On the other hand, to increase soybean oil from 1 to 3%, barley meal (10% CP) was reduced by 2%. 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 microminerals (Table 1). No antibiotics or coccidiostats were included in the diets or administered via water during the trial.

The chlorella meal (food grade) was provided as a powder (size of grains $\leq$ 63 μm) stored inside side gusset pouches of 5 kg each. At the feed plant, the chlorella meal was incorporated into the feed through electrostatic adhesion to ensure that it was evenly dispersed throughout the feed mix without clumping or segregation during the pelleting process. Specifically, to obtain diets with 2% of chlorella inclusion, 50 kg of chlorella powder was included in the feed mix to obtain feed batches of 2 500 kg, which were then stored in paper bags of 25 kg each. The vegetable oil was blended with feed during the mixing stage at the feed plant, allowing for even distribution of the oil across the feed components and therefore uniformly included in the final pellets.

To prepare the diets with 1% of chlorella for each level of fat, one bag of C0 diet and one bag of C2 diet were carefully mixed at the experimental farm before administration to the rabbits. The experimental diets were fed from weaning (33 days of age) to slaughter (71 days of age) and were distributed manually both in collective pens and individual digestibility cages. Throughout the trial, the animals had free access to feed and fresh water. Individual live weights were recorded twice a week to closely monitor rabbit growth and promptly detect any abnormal weight changes and health problems, while pen feed intake was measured daily. The health status of the rabbits was monitored daily following the guidelines provided by Gidenne and Feugier (2009). During the trial, a total of 21 rabbits died (3.65%), and an additional 45 (7.81%) were excluded due to health problems (sanitary risk accounting for 11.5% on average) without any significant effect of dietary treatment (data not reported in tables).

In vivo digestibility

The coefficients of total tract apparent digestibility of DM and nutrients and the digestible energy contents of the experimental diets were assessed *in vivo* on 72 rabbits (12 per diet), distinct from those involved in the growth trial, as mentioned above. The rabbits were housed in individual digestibility cages (30 cm × 48 cm × 33 cm height), each equipped with two automatic nipple drinkers and an individual feeder. Below the cages, a basket made of galvanised mesh was installed to collect the faeces. The basket was equipped with a deflector to avoid the contact of the urine with the faeces. The digestibility trial was conducted from 47 to 51 days of age, following the European reference

Table 1
Ingredients (%) and proximate composition (% as fed) of chlorella and experimental diets fed to growing rabbits from 33 days to 71 days of age.

Items	Chlorella meal	Diets					
		C0-F3	C1-F3	C2-F3	C0-F5	C1-F5	C2-F5
Dehydrated chlorella (49% CP)	–	0.00	1.00	2.00	0.00	1.00	2.00
Soybean oil	–	1.00	1.00	1.00	3.00	3.00	3.00
Dehydrated alfalfa meal (16% CP)	–	19.0	19.0	19.0	16.0	16.0	16.0
Dehydrated alfalfa meal (14% CP)	–	11.0	12.0	13.0	11.0	12.0	13.0
Wheat bran (20% of starch)	–	24.8	24.8	24.8	24.8	24.8	24.8
Barley meal (10% CP)	–	10.0	10.0	10.0	8.0	8.0	8.0
Dried beet pulp (8% CP)	–	14.0	14.0	14.0	14.0	14.0	14.0
Sunflower meal (30% CP)	–	17.0	15.0	13.0	20.0	18.0	16.0
Cane molasses	–	1.50	1.50	1.50	1.5	1.5	1.5
Limestone	–	0.65	0.65	0.65	0.65	0.65	0.65
Salt	–	0.40	0.40	0.40	0.40	0.40	0.40
L-lysine HCl (liquid form)	–	0.15	0.15	0.15	0.15	0.15	0.15
DL-methionine	–	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin–mineral premix[1]	–	0.40	0.40	0.40	0.40	0.40	0.40
Proximate composition							
DM	93.4	90.2	89.6	89.9	89.9	90.6	90.7
CP	49.6[2]	15.5	15.4	15.2	15.6	15.7	15.3
Crude fat	3.76	2.9	2.9	3.1	5.0	5.2	4.9
Ash	7.30	8.4	8.0	8.1	8.0	8.2	8.5
Starch	8.61	8.9	7.4	9.3	8.2	8.0	9.3
aNDF	3.49	38.9	38.5	39.0	39.1	38.6	38.4
ADF	0.40	22.0	21.2	21.0	22.0	21.5	21.5
Hemicelluloses	3.33	16.9	17.2	18.0	17.1	17.1	16.8
ADL	n.d.	5.21	4.71	4.81	5.06	4.74	4.81
Gross energy, kcal/kg	–	16.7	16.3	16.6	16.9	17.1	17.0

Abbreviations: aNDF = amylase-treated NDF; C0 = 0% chlorella; C1 = 1% chlorella; C2 = 2% chlorella; F3 = 3% crude fat; F5 = 5% crude fat; n.d.: not determined.
C1-F3 diet was obtained at farm by mixing 0.5 C0-F3 + 0.5 C2-F3. C1-F5 diet was obtained at farm by mixing 0.5 C0-F5 + 0.5 C2-F5.
Amino acid values (UPLC method) were obtained from the product specification document provided by the selling company (ALLMICROALGAE – Natural Products SA, Pataias, Portugal).

[1] Premix provided per kg of feed: vitamin A, 12 000 IU; vitamin D3, 1 000 IU; vitamin E acetate, 50 mg; vitamin K3, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; riboflavin, 4 mg; vitamin B6, 2 mg; vitamin B12, 0.1 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1 mg; choline chloride, 300 mg; Fe, 100 mg; Cu, 20 mg; Mn, 50 mg; Co, 2 mg; I, 1 mg; Zn, 100 mg; Se, 0.1 mg.

[2] Amino acids (g/100 g): Alanine (2.5–3.5), Arginine (2.5–3.5), Aspartic acid (3.0–4.0), Cystine + Cysteine (0.1–0.8), Glutamic acid (3.8–4.5), Glycine (2.0–3.0), Histidine (0.6–1.0), Isoleucine (1.5–2.5), Leucine (3.0–4.0), Lysine (2.5–3.5), Methionine (0.4–1.2), Phenylalanine (2.0–3.0), Proline (1.5–2.5), Serine (1.5–2.5), Threonine (1.5–2.5), Tryptophan (0.03–1.0), Tyrosine (1.5–2.5), Valine (2.0–3.0).

method (Pérez et al., 1995). In brief, total faecal excretion was collected daily at the same time (0900 h). During collection, particular attention was paid to avoid the inclusion of rabbit hair. Each day, the total faecal output (including both hard and soft faeces, if present) per rabbit was collected in the same individual bag and stored at −18 °C.

Blood and caecal content sampling

At 51 d of age, 36 rabbits (6 per diet) were randomly selected among those individually housed in digestibility cages for blood and caecal content collection. Rabbits were euthanised through CO_2 asphyxiation followed by cervical dislocation after confirming loss of consciousness via vestibulo-ocular reflex assessment. Blood was immediately taken from the heart to obtain samples with a more consistent mixture of systemic blood, while minimizing the effects of localised physiological variations. Once collected, the blood was immediately placed into lithium-heparin tubes. The plasma was directly obtained by centrifugation (Thermo Scientific SL, 16R, Thermo Fisher Scientific, Waltham, MS, USA) at 6 339 × g for 10 min (4 °C) and immediately stored at −20 °C. To determine the caecal microbiota, samples of caecal content were aseptically collected from the sacrificed rabbits, placed in 2-ml Eppendorf tubes, and stored at −80 °C until DNA extraction. To determine caecal fermentative activity, after pH measurement (GLP 22 pH meter, Crison Instruments S.A., Barcelona, Spain), another sample of the caecal content was diluted with a 15% HPO_3 solution (25% wt/wt) and stored at −20 °C until analysis (Trocino et al., 2011).

Commercial slaughtering

At 71 days of age, after a 4-h fasting period, all rabbits in the trial were weighed at the experimental farm before loading to determine the final live weight. Then, all rabbits were caged into standard plastic crates (100 cm length × 50 cm width × 30 cm height; eight animals per crate; space allowance of approximately 250 cm^2/kg) and transported for approximately 1 h to a commercial slaughterhouse by an authorised truck. After 1 h of lairage, 216 rabbits, representative of the corresponding experimental groups in terms of average rabbit live weight and SD, were individually weighed, stunned using electro anaesthesia, and slaughtered by jugulation. After 24 h of chilling, the commercial carcasses were weighed to calculate the individual dressing percentage (Blasco and Ouhayoun, 1996).

Chemical analyses

Chlorella meal, experimental diets, and faeces collected during the digestibility trial were analysed to determine the contents of DM (934.01), ash (967.05), CP (2001.11), and starch (amyloglucosidase α-amylase method, 996.11) (AOAC, 2000). The crude fat (ether extract) content was determined following acid hydrolysis (EC, 1998). The fibre fractions, namely amylase-treated NDF (**aNDF**; assayed with a heat−stable α-amylase and expressed inclusive of residual ash, without sodium sulphite), ADF (inclusive of residual ash), and ADL (obtained by solubilisation of cellulose with sulphuric acid), were analysed using sequential procedures and a filter bag system (Ankom Technology, Macedon, NY, USA) according to previous studies (Van Soest et al., 1991; Maertens, 2010). The gross energy of the experimental diets was measured using an adiabatic bomb calorimeter (C200, IKA, Staufen, Germany).

The samples of thawed caecal content were centrifuged at 1 500 × g for 10 min. Caecal ammonia nitrogen was determined in the supernatant using a pH meter (GLP 22) equipped with an ammonia-specific electrode (mod. 9663 combined with the reference electrode mod. 5044; Crison Instruments S.A.). The molar content of volatile fatty acids (**VFAs**) in the supernatant was measured following the method described by Osl (1998) via gas chromatography (Agilent 7820A, equipped with a flame ionisation detector, split–splitness injection system, programmable oven) on a cross bond capillary column DB–FFAP (30 m × 0.25 mm I.D., 0.25 μm film thickness) (Agilent Technologies, Santa Clara, CA, USA).

Blood plasma glucose, urea, creatinine, inorganic phosphorous, and calcium were determined according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1800 Chemistry System autoanalyzer; Siemens Medical Solutions, Tarrytown, NY, USA). Glutamate, glutamine, and free amino groups (**free-NH_2**) were determined according to Larsen and Fernández (2017). D-lactate was determined according to Larsen (2017). Finally, non-esterified fatty acids (**NEFAs**) were determined by the NEFA C ACS-ACOD assay method (Wako Chemicals GmbH, Neuss, Germany) using an ADVIA 1800 Chemistry System autoanalyzer (Siemens Healthcare s.r.L., Milano, Italy).

Caecal microbiota analysis

Using the Qiagen DNA Stool Mini Kit (Qiagen, Hilden, Germany), 250 mg of each caecal content sample was processed to extract DNA according to the manufacturer's instructions. The 16S Ion Metagenomics and Ion Xpress Plus 9 Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilised for preparing the library for the amplification of seven hypervariable regions of the 16S gene (V2, V4, V8, and V3, V6-7, V9). To achieve a final concentration of 100 pM, the amplified libraries were combined and then subjected to processing using the Ion 520TM & Ion 530 TM Kit − OT2 400 bp (Thermo Fisher Scientific). The Ion 520 chip (Thermo Fisher Scientific) was utilised to load the sample pool, and sequencing was carried out using the Ion™ GeneStudio S5 System (Thermo Fisher Scientific).

Statistical analyses and bioinformatics

The individual data of live weight and daily growth rates were analysed by ANOVA using PROC MIXED of SAS (SAS, 2013), with the chlorella inclusion level and crude fat level as the main effects with interaction and the pen as a random effect. Pen daily feed intake and feed conversion, individual coefficients of total tract apparent digestibility, caecal fermentation traits and plasma metabolites were analysed by ANOVA using the PROC GLM of SAS with the chlorella inclusion level, crude fat level and their interactions as the main effects. The Bonferroni test was used to compare means. Differences among means with $P \leq 0.05$ were assumed to be statistically significant.

For 16S gene sequencing, the raw reads were processed and quality-checked using the Microbial Genomics workflow of the Computational Life Science Center (CLC) Genomics workbench (version 22.0.2) (Qiagen, Hilden, Germany). Primers were removed by trimming 20 base pairs on both ends. Samples were filtered based on the number of reads to ensure comparable coverage. The minimum number of reads was set at 100, and the minimum percent from the median was set at 50%. High-quality reads were clustered at a 97% level of similarity into operational taxonomic units (**OTUs**). The SILVA SSU v138.1 database was used as a reference for the taxonomic assignment of OTUs (Quast et al., 2012). Low-abundance OTUs were eliminated (< 20 reads for combined abundance). By applying the Microbial Genomics module of the CLC Genomics workbench (version 22.0.2), an alpha rarefaction analysis was generated to determine if the sequencing depth was acceptable. After normalisation of the data through total sum scaling, all the statistical analyses were performed using the microeco package (v1.10.1) in R software (Liu et al., 2022). The alpha diver-

sity was estimated using the Shannon index, and the Kruskal–Wallis statistical test was performed to identify significant differences (false discovery rate-adjusted, **FDR**; $P < 0.05$). Beta diversity among samples was analysed using principal coordinate analysis (**PCoA**) based on Bray–Curtis distances, to which permutational multivariate analysis of variance (**PERMANOVA**) was subsequently applied to investigate the statistical significance of the sample (FDR-adjusted $P < 0.05$). A differential abundance test on linear discriminant analysis effect size (**LEfSe**) with a linear discriminant score of 2.0 on the microeco R package was performed to detect differentially abundant genera (FDR-adjusted $P < 0.05$) between the experimental groups. The raw sequencing data have been deposited in the European Bioinformatic Institute under the project accession number PRJEB73751.

Results

Rabbit growth performance

The inclusion of chlorella did not affect rabbit growth performance (Table 2): the average live weight of the rabbits at 71 days was 2 700 g, which corresponded to a weight gain of 48.8 g/d, a feed intake of 159 g/d, and a feed conversion ratio of 3.27 in the whole trial. On the other hand, diets with 5% crude fat compared with 3% improved the feed conversion ratio (−5%; $P < 0.001$).

The live weight at the slaughterhouse (2 673 g), cold carcass weight (1 653 g), and dressing percentage (61.8%) were not affected by the dietary treatments (Table 2).

Diet digestibility

Compared with diets without chlorella, diets with 1 and 2% chlorella were associated with reduced ADF digestibility (23.2 vs 20.9%; $P < 0.05$), whereas diets with 2% chlorella also reduced crude fat digestibility (83.8 vs 85.6%; $P < 0.01$). Increasing the dietary fat level improved the digestibility of DM (57.4 vs 58.3%; $P < 0.01$), crude fat (82.4 vs 86.9%; $P < 0.001$), hemicelluloses (39.5 vs 43.2%; $P < 0.01$), and gross energy (57.3 vs 58.7%; $P < 0.001$) (Table 3). Significant probability of interaction between chlorella and crude fat levels was observed for crude fat, hemicelluloses, and gross energy digestibility (Table 3; Fig. 1). Diets with 2% chlorella and 3% crude fat (diet C2-F3) showed the lowest digestibility of crude fat (80.7%; Fig. 1a). On the other hand, diets with 1% chlorella and 3% crude fat (diet C1-F3) were showed the lowest digestibility of hemicelluloses (37.4%; Fig. 1b) and gross energy (56.6%; Fig. 1c).

Plasma metabolites

Compared with diets with 1%, diets with 2% chlorella decreased (−8%; $P < 0.05$) the free-NH_2 content, whereas diets with 5% crude fat reduced (−19%; $P < 0.05$) the NEFA content compared with diets with 3% crude fat. However, neither the inclusion of chlorella nor the level of crude fat affected plasma glutamate (55.2 mM, on average), glutamine (560 mM), D-lactate (11.9 mM), creatinine (35.7 mM), glucose (8.52 mM), urea (4.57 mM), inorganic P (2.21 mM), or calcium (3.48 mM) (Table 4).

Characteristics of digestive organs and caecal fermentations

Neither the inclusion of chlorella nor the level of crude fat affected the proportions of the digestive organs. On average, the full gut was equal to 21% of rabbit live weight, while full stomach and full caecum were equal to 7%, respectively. As for the caecal fermentation profile, diets without chlorella and diets with 3% crude fat tended to increase ($P = 0.09$) VFA (+10% and + 7%, respectively). On the other hand, neither the inclusion of chlorella nor the level of crude fat affected the caecal pH (5.80, on average), total ammonia (2.38 mmol/l, on average), or VFA proportions (Table 5).

Caecal microbiota

A total of 6 772 021 reads were obtained from the sequencing of 36 samples, with an average read count of 189 110 ± 58.9 for each sample. The analysis of high-quality reads resulted in the identification of 56 851 OTUs. The bacterial microbiota composition was not affected by the inclusion of Chlorella and the crude fat level. The beta diversity, represented by the PCoA plot using the Bray–Curtis distance matrix (Fig. 2), did not reveal a clear separation of samples based on the inclusion of chlorella or the level of fat. Consistently, the PERMANOVA statistical test did not reveal any significant differences between the microbiota of the rabbits fed the experimental diets. In terms of alpha diversity, the Shannon index was not influenced by the inclusion of chlorella or the crude fat level (Kruskall-Wallis test; FDR adj. $P > 0.05$) (Fig. 3). In addition, the LEfSe analysis, which was conducted to investigate differentially abundant OTUs between the experimental diets, did not reveal significant differences. Among all the samples, an *uncultured* genus of the Eubacteriaceae family was the most abundant (10−11%), except for the rabbits fed the C0-F3 diet, which presented the *Christensenellaceae R-7-group* as the dominant genus (8.7%) and the *uncultured* genus of the Eubacteriaceae family as

Table 2
Effect of diets containing different inclusion levels of chlorella (*Chlorella vulgaris*) and crude fat on growth performance of growing rabbits from weaning (33 days of age) until slaughter (71 days of age).

Items	Chlorella (C)			Crude fat (F)		RMSE	*P*-value		
	0%	1%	2%	3%	5%		C	F	C × F
Rabbits, n	174	168	168	253	257				
Pens, n	24	24	24	36	36				
Initial live weight (33 d)[1], g	839	834	842	840	836	137	0.83	0.73	0.95
Final live weight (71 d)[1], g	2 723	2 672	2 702	2 676	2 722	278	0.42	0.16	0.54
Weight gain[1], g/d	49.4	48.1	48.8	48.1	49.4	5.52	0.40	0.10	0.48
Feed intake[2], g/d	159	160	158	161	157	10.8	0.89	0.16	0.22
Feed conversion ratio[2]	3.22	3.33	3.25	3.34	3.18	0.17	0.08	< 0.001	0.37
Rabbits, n	72	72	72	108	108				
Live weight at slaughter, g	2 682	2 652	2 685	2 653	2 693	222	0.61	0.19	0.69
Cold carcass weight, g	1 660	1 642	1 657	1 638	1 667	140	0.79	0.22	0.53
Dressing percentage, %	61.9	61.8	61.7	61.7	61.9	1.55	0.97	0.44	0.13

[1] Individual data.
[2] Average pen data.

Table 3
Effect of different inclusion levels of chlorella (*Chlorella vulgaris*) and crude fat on coefficients of total tract digestibility and nutritive value of experimental diets fed to growing rabbits.

Items	Chlorella (C)			Crude fat (F)		RMSE	*P*-value		
	0%	1%	2%	3%	5%		C	F	C × F
Rabbits, n	24	24	24	36	36				
DM, %	57.6	57.9	58.0	57.4	58.3	0.72	0.43	< 0.01	0.70
CP, %	75.1	75.5	74.3	74.7	75.3	1.06	0.11	0.16	0.43
Crude fat, %	85.6[b]	84.6[ab]	83.8[a]	82.4	86.9	0.79	< 0.01	< 0.001	< 0.001
Starch, %	98.1	97.8	98.4	98.0	98.2	0.49	0.07	0.34	0.09
aNDF, %	30.5	30.0	30.8	30.0	30.8	1.61	0.63	0.24	0.63
ADF, %	23.2[b]	20.7[a]	21.1[a]	22.3	21.1	1.79	< 0.05	0.11	0.10
Hemicelluloses, %	39.9	41.6	42.6	39.5	43.2	2.45	0.11	< 0.01	< 0.05
Gross energy, %	57.9	57.9	58.2	57.3	58.7	0.76	0.61	< 0.001	< 0.05
Digestible protein (DP), g/kg	11.7	11.7	11.3	11.5	11.7	–	–	–	–
Digestible energy (DE), MJ/kg	9.72	9.69	9.78	9.48	9.98	–	–	–	–
DP to DE ratio, g/MJ	12.0	12.1	11.6	12.1	11.7	–	–	–	–

Abbreviations: aNDF = amylase-treated NDF.
[a,b] Values with different superscript letters significantly differ ($P < 0.05$).

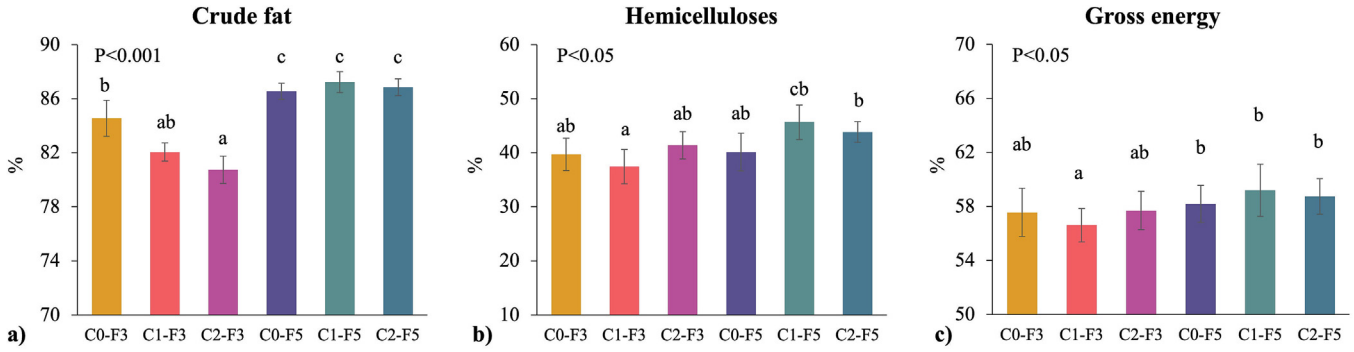

Fig. 1. Significant probability of interaction between dietary chlorella (C0, 0%; C1, 1%; C2, 2% chlorella) and crude fat (F3, 3%; F5, 5% crude fat) levels on the total tract digestibility coefficients of crude fat (a), hemicelluloses (b) and gross energy (c) in diets for growing rabbits.

Table 4
Effect of diets containing different inclusion levels of chlorella (*Chlorella vulgaris*) and crude fat on plasma metabolites of growing rabbits (51 days of age).

Items	Chlorella (C)			Crude fat (F)		RMSE	*P*-value		
	0%	1%	2%	3%	5%		C	F	C × F
Rabbits, n	12	12	12	18	18				
Glutamate, mM	55.2	64.2	46.2	57.9	52.5	25.1	0.23	0.53	0.32
Glutamine, mM	534	537	609	552	573	124	0.27	0.62	0.58
Free-NH_2 groups, μ eqv./L	4 727[ab]	4 782[b]	4 421[a]	4 604	4 682	348	< 0.05	0.54	0.08
D-lactate, mM	12.6	12.6	10.5	11.6	12.1	3.54	0.23	0.70	0.70
Creatinine, mM	35.3	34.2	37.5	34.3	31.4	7.45	0.13	0.27	0.64
Glucose, mM	8.31	8.56	8.70	8.54	8.50	0.45	0.10	0.81	0.37
Urea, mM	4.89	4.32	4.50	4.76	4.38	0.98	0.37	0.25	0.34
Inorganic P, mM	2.17	2.20	2.27	2.22	2.20	0.22	0.61	0.76	0.64
Calcium, mM	3.44	3.56	3.45	3.47	3.49	0.35	0.65	0.82	0.84
NEFA, μ eqv./L	168	183	192	197	166	43.4	0.43	< 0.05	0.24

Abbreviations: NEFAs = Non-esterified fatty acids.
[a,b] Values with different superscript letters significantly differ ($P < 0.05$).

subdominant (6.3%). *Ruminococcus* was the third most abundant genus in all rabbits (7.9–8.9%), followed by the *Lachnospiraceae NK4A136 group* (5.8–7.8%) and the NK4A214 group of the Oscillospiraceae family (3.8–5.9%) (Fig. 4).

Discussion

Effect of chlorella inclusion

Previous studies in growing rabbits (Hassanein et al., 2014; Abdelnour et al., 2019) showed that low dietary supplementation of chlorella (from 0.05 to 0.15% of the diet) did not influence the final live weight, growth rate, feed intake or feed conversion ratio, as we confirmed in our study with chlorella included at moderate rates (1–2% of the diet). Conversely, other reports showed that dietary supplementation of chlorella at concentrations between 200 and 500 mg/kg of rabbit live weight (approximately 0.02–0.05% of the diet at the beginning of the trial to 0.04–0.11% at the end of the trial) can improve the final live weight and feed conversion ratio of growing rabbits (Sikiru et al., 2019a). Overall, the positive effects of low chlorella inclusion in rabbit diets appear to be related more to an enhanced immune response (Abdelnour et al., 2019) and antioxidant activity (Abdelnour et al., 2019; Sikiru et al., 2019b) than to improved growth performance. In

Table 5
Effect of diets containing different inclusion levels of chlorella (*Chlorella vulgaris*) and crude fat on the characteristics of digestive organs and caecal fermentative activity of growing rabbits (51 days of age).

Items	Chlorella (C)			Crude fat (F)		RMSE	*P*-value		
	0%	1%	2%	3%	5%		C	F	C × F
Rabbits, n	12	12	12	18	18				
Live weight (LW), g	2 200	2 168	2 094	2 136	2 172	218	0.49	0.63	0.41
Full gut, % LW	21.6	21.1	21.1	21.5	21.0	1.42	0.64	0.26	0.92
Full stomach, % LW	6.58	6.42	6.40	6.66	6.29	0.84	0.83	0.20	0.65
Full caecum, % LW	6.76	6.48	6.68	6.73	6.55	0.62	0.53	0.38	0.55
Caecal content traits									
pH	5.72	5.86	5.83	5.85	5.76	0.16	0.10	0.11	0.41
Ammonia-N, mmol/l	2.52	2.37	2.26	2.02	2.75	1.32	0.89	0.11	0.29
Total VFA, mmol/l	75.3	67.7	69.6	73.3	68.4	8.32	0.09	0.09	0.76
Acetate, mol/100 mol VFA	79.2	79.6	78.6	79.6	78.6	2.82	0.70	0.32	0.27
Propionate, mol/100 mol VFA	3.50	3.12	3.16	3.46	3.06	1.00	0.62	0.25	0.18
Butyrate, mol/100 mol VFA	16.4	16.2	17.1	15.9	17.3	2.67	0.67	0.15	0.08
Valerate, mol/100 mol VFA	0.37	0.39	0.44	0.40	0.40	0.09	0.18	0.88	0.64
Propionate-to-butyrate ratio	0.23	0.20	0.19	0.23	0.18	0.08	0.56	0.12	0.06

Abbreviations: VFAs = Volatile fatty acids.

terms of growth performance, contrasting findings were reported with the dietary inclusion of spirulina in growing rabbits, which improved growth at very low inclusion rates (from 0.06 to 0.15%) (Aladaileh et al., 2020; Alazab et al., 2020) but had no effect on growth at inclusion rates ranging from 3 to 15% (Peiretti and Meineri, 2008; Aladaileh et al., 2020). Similar to chlorella, spirulina also seems to have beneficial effects, especially on rabbit immunity (Mahmoud et al., 2016; Aladaileh et al., 2020), antioxidant activity (Mahmoud et al., 2016; Aladaileh et al., 2020; Hassan et al., 2021), and meat quality (Mahmoud et al., 2016; Dalle Zotte et al., 2013).

The beneficial effects of chlorella supplementation have also been attributed to changes in animal metabolites (Coelho et al., 2021). For instance, chlorella supplementation in drinking water (200–500 ppm) decreased the serum content of cholesterol, triglycerides, and low-density lipoproteins while increasing the high-density lipoprotein content in laying hens reared under heat stress conditions (Moradi et al., 2016). Our analysis of nutrition-related plasma metabolites revealed a reduction in free-NH_2 with chlorella inclusion at 2%, which could be attributable to the measured reduction in digestible protein levels.

In terms of digestibility, previous studies have reported no effects on nutrient and energy digestibility with the inclusion of chlorella (0.08–0.15% inclusion) (Hassanein et al., 2014) or spirulina at low (0.08–0.15%) (Hassanein et al., 2014) or high (5to 10%) (Peiretti and Meineri, 2008) levels. Compared with those of the other diets, the digestibility of DM, CP, gross energy, and fibre fractions decreased only when the proportion of spirulina reached 15% (Peiretti and Meineri, 2008). In our study, crude fat digestibility decreased with chlorella inclusion, as previously observed in spirulina-supplemented diets (3%) for companion dwarf rabbits (Dalle Zotte et al., 2013), which could depend on the slight decrease in crude fat in the diets coming from other sources (i.e., oils or fats). Other studies hypothesised that crude fat digestibility could also decrease because of the increase in the polyunsaturated fatty acid (**PUFA**) proportion in rabbit diets due to the inclusion of chlorella (Dalle Zotte et al., 2013), which is a PUFA-rich ingredient (Bature et al., 2022). Notably, when considering the interaction between chlorella and crude fat levels, the reduction in crude fat and gross energy digestibility with chlorella inclusion was not observed in diets with 5% crude fat (Fig. 1a, c), suggesting a potential benefit of increasing fat levels in the diets to reduce the negative impact of microalgae inclusion on nutrient digestibility. In fact, the increase in dietary lipids could have enhanced the bioavailability of fat-soluble vitamins and of essential fatty acids provided by the chlorella meal (Michalski et al., 2020), having positive effects on nutrient absorption.

Despite the demonstrated impact of microalgae on the gut microbiota of humans and animals (de Medeiros et al., 2021; Patel et al., 2021), no research to date has explored the influence of microalgae inclusion on the gut microbiota of rabbits. In our study, the microbiota of the rabbit caecum was primarily composed of genera belonging to the phylum Firmicutes and the class Clostridia, which represent the most prevalent bacterial populations in the rabbit intestine (Hu et al., 2021). Specifically, 16S sequencing analysis revealed the presence of genera within (1) Eubacteriaceae, associated with bacteria involved in butyrate production; (2) Christensenellaceae, which generates acetic and butyric acids and is beneficially linked to animal health, feed efficiency, body mass index, fibre digestion, and protein metabolism in goats (Sallam et al., 2023); and (3) the Ruminococcaceae family, particularly the genus Ruminococcus, which plays a crucial role in the degradation and fermentation of dietary polysaccharides in the gut ecosystem (La Reau and Suen, 2018). In the present trial, the inclusion of chlorella in the diet did not affect the caecal microbiota. Similarly, other studies found no differences in bacterial diversity or richness in the faeces (Cabrita et al., 2023) or cecum (Martins et al., 2018; Lee et al., 2023) microbiota of dogs (0.5–1.5% chlorella inclusion), broilers (0.5%), or piglets (5%) (Cabrita et al., 2023) fed diets supplemented with microalgae.

Overall, the moderate inclusion levels (1–2%) of chlorella as beneficial bioactive compound supplement did not demonstrate a significant impact on rabbit growth, metabolomics, and digestive activity. This could be related to the absence of stressful environmental conditions during our trial, such as high temperatures, where these compounds have been demonstrated to be effective in improving growth and overall health in rabbits (Bashar et al., 2023) and chickens (Chaudhary et al., 2023) under heat stress. On the other hand, the inclusion of microalgae at higher rates in rabbit diets, as a substitute for soybean or sunflower meals, could be economically impractical, given their high costs.

Effect of crude fat level

High-energy diets obtained by partially replacing cereals with fat are recommended during the fattening phase to reduce feed intake, improve feed conversion, and minimise mineral excretion in rabbits (De Blas and Mateos, 2020; Xiccato, 2020). In our study, crude fat at 5% increased the digestible energy level, which reduced

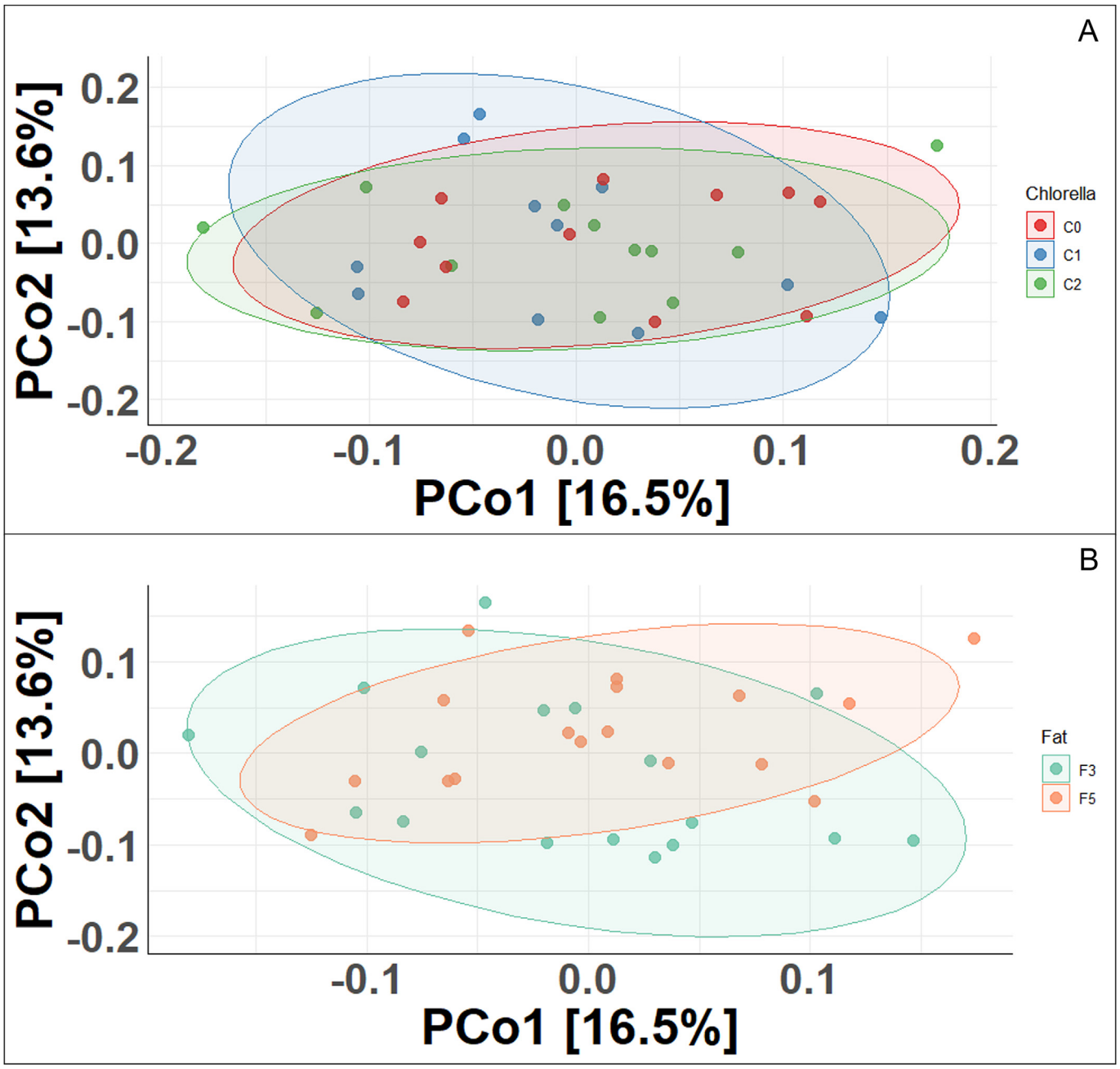

Fig. 2. Effect of dietary (A) chlorella (C0, 0%; C1, 1%; C2, 2% chlorella) and (B) crude fat (F3, 3%; F5, 5% crude fat) levels on Beta diversity of microbial populations in the caecal content of growing rabbits. Principal coordinate analysis (PCoA) plot using a Bray-Curtis distance matrix.

feed intake and led to a reduction in the feed conversion ratio, in agreement with previous findings (Read et al., 2015; Ayyat et al., 2021). In fact, rabbits regulate their feed intake according to the digestible energy content of their diet to satisfy their requirements (Xiccato and Trocino, 2020). When digestible energy increases, the feed conversion ratio generally improves, either without significant effects on growth (Maertens, 2010; Tazzoli et al., 2013) or with improvements in daily weight gain (Knudsen et al., 2014; Read et al., 2015), as observed in our study.

An increase in digestible energy in the diet could also significantly influence caecal fermentations (Birolo et al., 2022) and increase rabbit digestive efficiency (Knudsen et al., 2014), but these effects are strictly dependent on the manipulation of dietary fibre and highly fermentable carbohydrates (Trocino et al., 2013) rather than changes in dietary fat content. In our study, where the crude fat content increased through soybean oil inclusion, the caecal VFA content did not change.

Nevertheless, the dietary inclusion of crude fat (2–6%) has been shown to enhance the digestibility not only of the fats themselves but also of other nutritional components of the diet (Santomá et al., 1987), as we observed in our study, where DM, ether extract, hemicelluloses and gross energy digestibility improved with increasing added fat. In previous findings, the digestibility coefficients of crude fat and CP increased with the inclusion of fats such as pork lard (2% inclusion) (Dalle Zotte et al., 2020) or linseed oil (3% inclusion) (Ibrahim et al., 2017) in growing-rabbit diets. On the other hand, digestive performance is not affected by the source of fat used in rabbit diets (Dalle Zotte et al., 2018; Gasco et al., 2019).

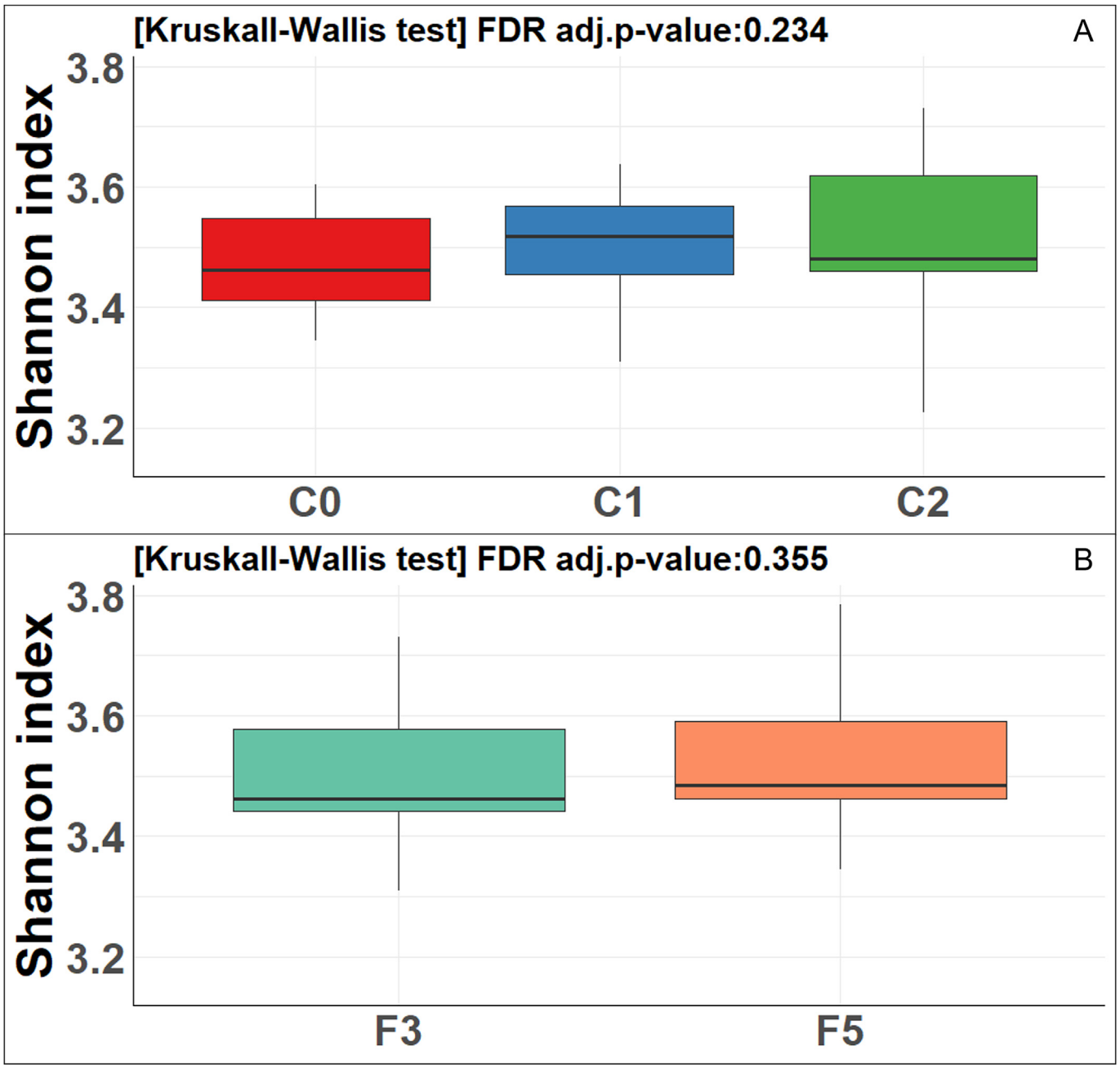

Fig. 3. Effect of dietary (A) chlorella (C0, 0%; C1, 1%; C2, 2% chlorella) and (B) crude fat (F3, 3%; F5, 5% crude fat) levels on alpha diversity (Shannon index, Kruskall-Wallis test, false discovery rate-adjusted *P*-value) of microbial populations in the caecal content of growing rabbits. Abbreviations: FDR adj = false discovery rate-adjusted.

The reduction in feed intake associated with increased digestible energy content of diets, resulting in a decrease in digesta transit time, could also account for the increased digestibility of higher fat diets; however, this was not the case in the present trial, which is consistent with previous observations (Dalle Zotte et al., 2020). As expected, an increase in dietary crude fat reduced plasma NEFA (Jean-Blain and Durix, 1985), while no other changes were observed in plasma metabolites. Overall, despite the expected beneficial effects on performance, the inclusion of crude fat in rabbit diets is typically limited to 2–4% due to its negative effects on feed pelleting (Maertens, 2010). Moreover, high levels of dietary fat (> 6%) appear to have detrimental interactions with caecal microflora, ultimately leading to a reduction in rabbit digestive efficiency (Casado et al., 2010).

In conclusion, in the conditions of the present study, the moderate inclusion levels (1–2%) of chlorella meal as a supplement of beneficial bioactive compounds did not show a significant impact on rabbit growth, metabolomics, and digestive activity. Moderate levels of crude fat (5%) enhanced nutrient digestibility and improved the feed conversion ratio. Future research should focus on validating the results of the present study under field conditions, particularly under suboptimal environmental conditions that could be encountered on commercial farms.

Ethics approval

The study was approved by the Ethical Committee for Animal Experimentation (Organismo Preposto al Benessere Animale) of

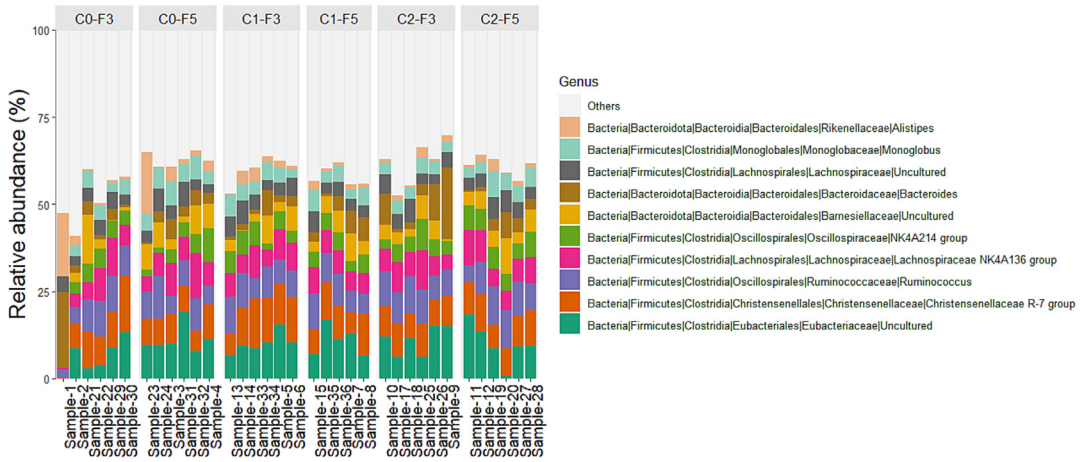

Fig. 4. Effect of diets including different chlorella (C0, 0%; C1, 1%; C2, 2% chlorella) and crude fat (F3, 3%; F5, 5% crude fat) levels on the taxonomy of microbial populations in the caecal content of growing rabbits.

the University of Padova (project 38/2023; Prot. nr. 83737, approved on 16/05/2023). The researchers involved in animal handling were either animal specialists (Ph.D. or MSc in Animal Science) and/or veterinary practitioners. Animals were handled according to the principles of EU Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes.

Data and model availability statement

The raw sequencing data have been deposited in the European Bioinformatic Institute under the project accession number PRJEB73751. The database is available at https://www.ebi.ac.uk/ena/browser/view/PRJEB73751. The other datasets analysed in the current study are available from the corresponding author upon reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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