

Antimicrobial effects of Black Soldier Fly and Yellow Mealworm fats and their impact on gut microbiota of growing rabbits

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Abstract

Background

The study aimed to evaluate the *in vitro* antimicrobial activities of two types of insect fats extracted from black soldier fly larvae (HI, *Hermetia illucens* L.) and yellow mealworm larvae (TM, *Tenebrio molitor* L.) and their effects as dietary replacement of soybean oil (S) on caecal fermentation pattern, and faecal and caecal microbiota in rabbits.

Results

A total of 120 weaned rabbits were randomly allotted to 3 dietary treatments (40 rabbits/group): a control diet (C diet) containing 1.5% of S and two experimental diets (HID and TMD) where S was totally substituted by HI or TM fats. Regarding the *in vitro* antimicrobial activities, HI and TM fats did not show any effects on *Salmonella* growth. *Yersinia enterocolitica* showed lower growth when challenged with insect fats than the controls. The insect fat supplementation in rabbit diets increased the contents of the caecal volatile fatty acids when compared to the control group. A metataxonomic approach was adopted to investigate the shift in the microbial composition as a function of the dietary insect fat supplementation. The microbiota did not show a clear separation as a function of the inclusion, even if a specific microbial signature was observed. Indeed, HI and TM fat supplementation enriched the presence of *Akkermansia* that was found to be correlated with $\text{NH}_3\text{-N}$ concentration. An increase in *Ruminococcus*, which can improve the immune response of the host, was also observed.

Conclusions

This study confirms the potential of HI and TM fats as antibacterial feed ingredients with a positive influence on the rabbit caecal microbiota, thus supporting the possibility of including HI and TM fats in rabbit diets.

Introduction

In rabbit production, a high mortality due to gastrointestinal disorders and epizootic enteropathy is the major health concern. The rabbit's digestive health and physiology, as well as the immune system, are based on its abundant caecal microbiota [1, 2]. The gut microbiota, as well as the factors affecting its composition, are considered as important aspects to maintain digestive health and therefore to enhance rabbit production [1, 3]. The diet is one of the main factors influencing the microbiota and the co-occurrence patterns of the caecal bacterial community [1, 3]. Specifically, dietary fat intake can modulate gut microbiota [4, 5]. It has been reported that some fatty acids (FA) and in particular medium-chain fatty acids (MCFA) act as bacteriostatic (growth inhibiting) or bactericidal (killing) molecules [6, 7]. Then, in addition to modulate the bacterial community, the amount and type of dietary fat can have an effect on

the immune function both at systemic and intestinal levels [3]. Data regarding the effect of fat type and level in rabbit diet on gut health are still very limited and leads to contradictory results [3, 8].

The well-known antimicrobial resistance caused by the misuse of antibiotics drugs in animal production, and the EU ban for their in-feed use [Regulation EC/1831/2003] has led to an increase of the incidence of livestock disease and economic damages. Since several years, research dedicated great efforts to investigate alternatives able to sustain production without causing an increase of antimicrobial resistance and several natural products have been investigated [9, 10].

Recently, insects are receiving great attention as novel alternative feed ingredients because of their excellent nutritive properties [11] and their potential effect on animal health [12, 13]. It has been shown that insects have the ability to modulate the intestinal microbiota with positive effects on poultry growth, health and resistance against pathogens [13–17]. Lipids are a main component of insects (30–40% of the dry matter, DM) [18] and, once extracted during the insect larvae processing, they can be used as an interesting feed ingredient in animal farming [12–13; 19–24]

Black soldier fly (*Hermetia illucens*, HI) and Yellow mealworm (*Tenebrio molitor*, TM) fats are characterized by a different fatty acid (FA) profile. The HI fat is rich in saturated FAs (SFA) and in medium chain FAs (MCFA), mainly lauric acid (C12:0; 48% of the total FA profile) [12], which has an antimicrobial effect on a wide range of microbes [25]. On the other hand, the TM fat is considered as a source of n-6 poly-unsaturated FAs (PUFA), with high linoleic acid content (9% of the total FA profile) [12]. MCFA are effectively absorbed and metabolized on the gastro-intestinal tract and known for their physiological and antibacterial effects on Gram positive bacteria [26–28]. Furthermore, MCFA can exert beneficial effects on intestinal health and microbial growth inhibition [29], as well as a favorable impact on the digestive health of the growing rabbit [30]. Both the short chain FAs (SCFA; propionic acid and butyric acid) and the MCFA (caproic acid and caprylic acid) have a direct *in vitro* antimicrobial activity against *Salmonella typhimurium* [31]. Matsue et al. [25] demonstrated that lauric acid has a high antimicrobial activity against pathogenic Bacteroides and *Clostridium* species, and consequently can modulate intestinal health. Significant *in vitro* gut antimicrobial effects against *D-Streptococci* and *Lactobacilli* have also been attributed to HI prepupae fat [28]. In a recent study, Sypniewski et al. [13] showed that the addition of HI fat to replace soybean oil (S) in turkey diets significantly reduced the presence of potentially pathogenic microbes and decreased gastrointestinal tract (GIT) inflammation by modulating the level of IL-6 and TNF- α .

In the light of what was reported above, the aim of the present study was to evaluate the *in vitro* antimicrobial activities of HI and TM fats and their effects on caecal traits and gut microbiota of growing rabbits.

Material And Methods

Animal ethics statement

The study was performed in accordance with the guidelines of the European and the Italian laws (European Directive 86 609/EEC-Italian law D.L. 116/92), and was approved by the Bioethical Committee of the University of Torino (Italy) (Ref. 386638, 4/12/2017).

In vitro analyses for antimicrobial activity of HI and TM fat

Bacterial strains were selected considering their impact on rabbit and on public health [32–35]: *Salmonella tiphymurium* (CIP 60.62T), *Salmonella enteritidis* (CRBIP 19.329), *Yersinia enterocolitica* (CIP 101.776), *Pasteurella multocida* (CIP 100.610) and *Listeria monocytogenes* (CIP 82.110T); all these were purchased from Institute Pasteur (Paris, France). After overnight incubation following producers' instructions, strains were mixed with TM and HI fats [12] in order to reach a final concentration of 3 Log CFU/mL, verified by immediate plate streaks. Briefly the broths for growth were prepared as follows: 200 µL of Brain Heart Infusion broth (BHI, Oxoid, Fisher Scientific, Rodano, Milano, Italy) were added to 250 µL of insect fat and then to 50 µL of fresh bacterial overnight culture previously quantified by measuring the optical density (PxiMa UV/VIS 5200, Fulltech Instruments, Roma, Italy) and diluted to 4 Log CFU/ml. Only for *Pasteurella multocida*, instead of BHI, Tryptic Soy Broth (TSB, Oxoid) was used. These mixes were incubated 24 hours at 37 °C with horizontal shaking (RPR Horizontal rotator- LE8S, Fisher Scientific, Italy). At regular timings (every 2 hours), three aliquots for each mix were streaked on Tryptic Soy Agar (TSA, Oxoid) (only *Pasteurella multocida*) or on Brain Heart Infusion Agar (BHA, Oxoid). Each strain/fat combination was analyzed in triplicates. Moreover, control tests were prepared: 450 µL of BHI were added with 50 µL of quantified overnight cultures to reach a final concentration of 3 Log CFU/mL of broth (A); a mix of soybean oil (S) and bacterial broth, prepared as described above for insect fats (B). Control tubes were also prepared in triplicates.

Inclusion of HI and TM fats in the diet of growing rabbits

Experimental Design

For this experiment, three dietary treatments were tested in 120 growing rabbits (40 rabbits/diet) from 36 to 78 days of age: a control diet (C) containing 1.5% soybean oil (S), and two diets (HID and TMD) with total replacement of S with HI and TM larvae fat, respectively. The three diets contained an average of 16.6% DM of crude protein (CP) and 18.6 MJ/kg DM of gross energy. Detailed information about the chemical composition of the diets, rabbit farming conditions and live performance are provided in details by Gasco et al. [12]. Ingredients and chemical composition of the diets are shown in Table 1. Briefly, no overall significant differences among experimental groups were observed for growth performance during the trial.

Table 1
 Ingredients (% as fed) and chemical composition (% DM) of experimental diets
 (modified from Gasco et al. [12])

Ingredients	Experimental diets		
	C	HID	TMD
Dehydrated alfalfa meal (17 g CP/100 g)	32	32	32
Alfalfa hay	7.5	7.5	7.5
Wheat bran	23.5	23.5	23.5
Barley meal	10	10	10
Dried sugar beet pulp	16	16	16
Soybean meal (44 g CP/100 g)	7	7	7
Soybean oil	1.5	-	-
<i>Hermetia illucens</i> fat	-	1.5	-
<i>Tenebrio molitor</i> fat	-	-	1.5
Cane molasses	1.2	1.2	1.2
Dicalcium phosphate	0.3	0.3	0.3
Salt	0.4	0.4	0.4
L-methionine (98 g methionine/100 g)	0.1	0.1	0.1
Vitamin-mineral premix ^a	0.5	0.5	0.5
Chemical composition			
Dry matter, %	89.4	89.2	89.6
Ash, % DM	8.58	7.77	7.75
Crude protein, % DM	17.0	16.8	16.3
Ether extract, % DM	4.22	3.92	3.87
Neutral detergent fibre (aNDF), % DM	40.2	41.7	40.5

C, control diet with soybean oil; HID, diet with *Hermetia illucens* fat; TMD, diet with *Tenebrio molitor* fat; DM, dry matter.

^aSupplementation per kilogram of feed: vitamin A, 16000 IU; vitamin D₃, 1600 IU; vitamin E acetate, 30 mg; vitamin B₁, 0.8 mg; vitamin B₆, 1.65 mg; niacin, 40 mg; folic acid, 1 mg; Mn, 30 mg; Fe, 116 mg; Cu, 12.5 mg; Zn, 60 mg; Co, 0.45 mg; Ca, 1.3 mg; Se, 0.3 mg.

	Experimental diets		
Acid detergent fibre (ADF), % DM	21.7	23.0	22.8
Acid detergent lignin (ADL), % DM	4.81	5.09	5.02
Gross energy, MJ/kg DM	18.50	18.50	18.62
<i>C</i> , control diet with soybean oil; <i>HID</i> , diet with <i>Hermetia illucens</i> fat; <i>TMD</i> , diet with <i>Tenebrio molitor</i> fat; <i>DM</i> , dry matter.			
^a Supplementation per kilogram of feed: vitamin A, 16000 IU; vitamin D ₃ , 1600 IU; vitamin E acetate, 30 mg; vitamin B ₁ , 0.8 mg; vitamin B ₆ , 1.65 mg; niacin, 40 mg; folic acid, 1 mg; Mn, 30 mg; Fe, 116 mg; Cu, 12.5 mg; Zn, 60 mg; Co, 0.45 mg; Ca, 1.3 mg; Se, 0.3 mg.			

Fatty acid profile of insect lipids and experimental diets

The FA profiles of insect lipids and feeds were determined as reported by Gasco et al. [12]. The fatty acid methyl esters (FAME) were separated, identified and quantified. The results were expressed as g/kg of FAME (Table 2).

Caecal fermentation traits

At slaughtering (78 days of age), a total of 30 rabbits (10 animals per treatment) were randomly selected and eviscerated. The pH, caecal ammonia and volatile FAs (VFA) profile of the caecal content were determined as reported by Tazzoli et al. [36].

DNA Extraction And 16S rRNA High-throughput Amplicon Target Sequencing

In order to observe the development and dynamic of bacterial communities, hard feces were collected from 10 rabbits per group at 36, 51 and 77 days of age, whereas the caecal contents (n = 10 per group) were taken during the slaughtering at 78 days of age. Samples from the same group, the same collection site and day were pooled together in sterilized polyethylene bags, and stored at -80 °C until examination.

DNA from feces and caecal samples were extracted by using a commercial kit (QIAamp Fast DNA stool Mini Kit, QIAGEN®, Hilden, Germany) following the instructions reported by the manufacturer with slightly modification. The DNA were quantified by NanoDrop™ 2000 Spectrophotometer (Fisher Scientific, Italy) and standardized at 5 ng/uL and used a template in the PCR amplifying the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. [37].

The PCR amplicons were cleaned using according to Illumina (San Diego, CA, USA) guidelines. The sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

Bioinformatics And Statistical Analysis

After paired-end sequencing, raw reads were analyzed as previously reported [15]. Sequences were first joined using FLASH software [38] with default parameters and filtered for low quality bases (at Phred < Q20) by QIIME 1.9.0 software [39]. Reads shorter than 300 bp were discarded by using Prinseq [40]. The USEARCH software version 8.1 was used for chimera filtering and Operational Taxonomic Units (OTUs) were clustered at 97% of similarity threshold by UCLUST algorithms [41]. Representative sequences of each cluster were mapped against the Greengenes 16S rRNA gene database version 2013 for taxonomic assignment. In order to avoid biases due to the different sequencing depth, OTU tables were rarefied at 3996 sequences/sample. The OTU tables display the higher taxonomy resolution that was reached. When the taxonomy assignment was not able to reach the genus level, the family or phyla were displayed. Alpha diversity indices were calculated using the diversity function of the vegan package [42]. Weighted and unweight UniFrac distance matrix and OTU tables were used to find differences between samples by Anosim and Adonis statistical test through the function *vegan* in R environment. Pairwise Wilcoxon test was used as appropriate to determine significant differences in alpha diversity or OTU abundance. A Generalized Linear Model was used in order to test the importance of continuous or discrete variables available (sampling time and insect inclusion) on the relative abundance of bacterial genera or family. The interactions between the levels of the fixed factors were evaluated by pairwise comparisons. Not-normally distributed variables were presented as median (range interquartile) and box plots represented the interquartile range between the first and the third quartiles, with the error bars showing the lowest and the highest value. Pairwise Spearman's non-parametric correlations were used to study the relationships between the relative abundance of microbial taxa in caecal samples and VFA variables. The correlation plots were visualized in R using the coplot package of R.

The statistical analysis for data related to *in vitro* antimicrobial activity and caecal traits was performed using IBM SPSS Statistics V25.0.0 software by means of ANOVA, followed, if significant, by Duncan test post-hoc. Regarding the antibacterial activities of insect fats, bacterial counts was evaluated at each time point and the average results compared across the different conditions: pure bacterial broth, bacterial broth mixed with soybean oil, bacterial broth mixed with TM fat and bacterial broth mixed with HI fat. Significance was declared at $P \leq 0.05$. A statistical trend was considered for $P \leq 0.10$.

Results

In vitro antimicrobial activities of insect fats

Overall, the bacterial counts, when quantified broths were challenged with insect fats, were lower than the controls for 3 out of 5 pathogenic species tested, whereas only the two strains of *Salmonella* did not

show any significant difference in counts when compared to control tubes counts (therefore these data are not reported in Table 3). Considering the other species, for all of them results highlighted that HI fat caused a delay in bacterial growth, implying a bacteriostatic effect (Table 3).

Table 3

Distribution of average cell counts (Log (CFU)/per mL) over time for the three bacterial species for which inhibition was recorded (means \pm SEM; n = 3).

Growth Conditions	<i>Pasteurella multocida</i>					
	T4	T6	T8	T10	T12	T24
Control (A)	4.09 (0.19)	4.14 (0.06)	4.76 (0.32) - A	4.80 (0.34) - A	5.69 (0.49) - A	6.82 (0.44) - A
Control (B)	4.11 (0.17)	3.96 (0.12)	4.17 (0.26) - A	4.60 (0.40) - A	5.44 (0.57) - A	6.54 (0.39) - A
TSB + TM fat	3.95 (0.44)	3.75 (0.39)	3.08 (0.35) - B	3.21 (0.30) - B	2.91 (0.86) - B	3.90 (1.21) - B
TSB + HI fat	3.92 (0.2)	3.48 (0.17)	3.61 (0.19) - B	2.80 (0.59) - B	2.67 (0.49) - B	2.06 (0.36) - C
ANOVA	N.S	N.S	F = 4.99; P = 0.01	F = 5.07; P = 0.01	F = 6.36; P < 0.01	F = 13.75; P < 0.01
<i>Yersinia enterocolitica</i>						
Control (A)	4.36 (0.16) - A	5.19 (0.28) - A	6.76 (0.61) - A	7.32 (0.37) - A	8.18 (0.37) - A	9.95 (0.30) - A
Control (B)	4.10 (0.21) - A	5.05 (0.37) - A	5.91 (0.41) - A	6.83 (0.48) - A	7.35 (0.68) - A	9.88 (0.33) - A
BHI + TM fat	4.05 (0.31) - A	4.39 (0.52) - AB	4.98 (0.74) - AB	5.04 (0.94) - AB	5.93 (1.41) - AB	8.09 (2.13) - A
BHI + HI fat	3.34 (0.10) - B	2.69 (0.64) - B	3.01 (1.2) - B	3.02 (1.32) - B	2.94 (1.24) - B	3.98 (2.18) - B
ANOVA	F = 3.28; P = 0.05*	F = 6.38; P < 0.01	F = 4.95; P = 0.01	F = 7.71; P < 0.01	F = 6.46; P < 0.01	F = 5.42; P < 0.01
<i>Listeria monocytogenes</i>						
Control (A)	4.58 (0.35)	5.58 (0.39)	6.47 (0.41) - A	7.41 (0.41) - A	8.28 (0.46) - A	10.00 (0.26) - A
Control (B)	4.52 (0.32)	5.45 (0.42)	6.26 (0.54) - A	6.94 (0.61) - A	7.95 (0.59) - A	9.96 (0.28) - A

HI, *Hermetia illucens*; TM, *Tenebrio molitor*; TSB, Tryptic soy broth; BHI, Brain hearth infusion; N.S., non-significant; SEM, standard error of the means.

*Significant results (without approximation $P = 0.046$); **Results in trend of significance ($P = 0.051$).

In the same columns, different letters identify significantly different results at post-hoc test (Duncan).

Control (A) indicates the broth culture without oil/fat in it; Control (B) indicates a broth culture grown in BHI/Tryptic soy agar and soybean oil.

Growth Conditions	<i>Pasteurella multocida</i>					
	T4	T6	T8	T10	T12	T24
BHI + TM fat	4.20 (0.25)	4.59 (0.79)	5.98 (0.46) - A	6.76 (0.70) - A	7.63 (0.61) - A	9.48 (0.03) - A
BHI + HI fat	4.00 (0.92)	3.66 (0.84)	4.1 (0.63) - B	4.48 (0.61) - B	4.51 (0.62) - B	4.85 (0.97) - B
ANOVA	N.S	N.S	F = 3.30; P = 0.05**	F = 3.98; P = 0.03	F = 6.85; P < 0.01	F = 29.92; P < 0.01
<i>HI</i> , <i>Hermetia illucens</i> ; <i>TM</i> , <i>Tenebrio molitor</i> ; <i>TSB</i> , Tryptic soy broth; <i>BHI</i> , Brain hearth infusion; <i>N.S.</i> , non-significant; <i>SEM</i> , standard error of the means.						
*Significant results (without approximation $P = 0.046$); **Results in trend of significance ($P = 0.051$).						
In the same columns, different letters identify significantly different results at post-hoc test (Duncan).						
Control (A) indicates the broth culture without oil/fat in it; Control (B) indicates a broth culture grown in BHI/Tryptic soy agar and soybean oil.						

After 24 hours of incubation, counts of *Pasteurella multocida* broths challenged with HI fat showed a mean log difference of -4.48 and - 4.76 when compared to control broths with soybean oil (control B) and no oil addition (control A), respectively. Similar results were observed for *Yersinia enterocolitica* showing a mean log difference values of -5.9 and - 5.97, with control B and A; respectively. Finally, also *Listeria monocytogenes* counts led to a similar pattern of results, showing mean log difference values of -5.11 and - 5.15 when comparing counts of broths challenged with HI fat and the control B and A. All these differences were statistically significant (Table 3).

On the other hand, results related to TM fat showed that only *Pasteurella multocida* was effectively inhibited in growth: the mean log difference between controls and TM challenged broths showed values of -2.64 and - 2.92, respectively for control B and control A. These values are lower than what reported above for broth challenged with HI fat (Table 3). Finally, differences in bacterial counts between HI fat challenged broths and controls were statistically meaningful from the 4th hours of incubation on only in the case of *Yersinia enterocolitica*, whereas for the other species the bacteriostatic effects were achieved only starting from the 8th hours of incubation. TM fat challenged broths showed significant effect only in *Pasteruella mutocida* after 8 hours of incubation and was maintained until the end of the experiment (see details in Table 3).

Caecal Fermentation Traits

The caecal fermentation traits are reported in Table 4. The supplementation of insect fat increased the total VFA contents when compared to the control group (85.3 v. 83.9 v. 72.4 mmol/L; $P < 0.05$), whereas

the pH, the caecal ammonia-N content, the molar proportion of the different VFAs and their ratio were not influenced by the supplementation of insect fats ($P > 0.05$).

Table 4
Effect of lipid sources on caecal traits and fermentation parameters (n = 10 rabbits/group).

	C	HID	TMD	SEM	P-value
pH	6.1	5.9	5.9	0.01	0.15
N-NH ₃ (mmol/L)	2.2	3.0	3.1	0.23	0.25
Total VFA (mmol/L)	72.4 ^b	85.3 ^a	83.9 ^a	2.31	0.03
Acetic acid (C2; mmol/100 mmol VFA)	77.8	78.1	76.6	0.43	0.30
Propionic acid (C3; mmol/100 mmol VFA)	5.3	5.0	5.4	0.19	0.71
Butyric acid (C4; mmol/100 mmol VFA)	16.2	16.1	17.2	0.42	0.53
Valeric acid (C5; mmol/100 mmol VFA)	0.5	0.4	0.5	0.03	0.59
Caproic acid (C6; mmol/100 mmol VFA)	0.3	0.3	0.3	0.03	0.45
C2/C3 ratio	15.2	16.0	14.6	0.54	0.58
C2/C4 ratio	4.9	5.0	4.6	0.16	0.63
C, control diet with soybean oil; HID, diet with <i>Hermetia illucens</i> fat; TMD, diet with <i>Tenebrio molitor</i> fat; VFA, volatile fatty acids.					

Caecal And Fecal Microbiota Characterization

The total number of high-quality paired end sequences obtained from 16S rRNA sequencing reached 13.448.661 raw reads. After the filtering, 7.801.336 reads passed the filters applied through QIIME, with a median value of 59.114 ± 52.946 reads/sample, and a mean sequence length of 441 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (93%), indicated also satisfactory coverage for all the samples. We applied a Generalized Linear Model (GLM) for the alpha-diversity in the fecal samples in order to test the effect of the treatment across time. The Chao1 index increased ($P < 0.01$) in TM and HI groups when compared to the C group ($P < 0.05$), while the Shannon index was affected by the sampling time only. However, the number of observed species significantly increased in HI group in comparison with the other diets ($P < 0.05$) (Fig. 1). No significant differences were observed when comparing the alpha diversity index as a function of the different diets in the caecal samples.

Going more deeply in the microbiota comparison, Adonis and analysis of similarity (ANOSIM) statistical tests based on weight and on unweight UniFrac distance matrix showed significant differences among samples as a function of the sampling time ($P < 0.05$). However, the dietary inclusion of insect fat did not show any significant effect in the microbiota composition of the faecal samples ($P < 0.05$). Figure 2

summarizes the distribution of the microbiota across time and samples and displays a simple microbiota composition dominated by the presence of *Bacteroides*, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae* and *Ruminococcus*. Comparing the relative abundance of the main OTUs across the fecal samples grouped as a function of the dietary supplementation by the GLM function, it was possible to observe that HI inclusion level increased the relative abundance of *Akkermansia* ($P < 0.05$) and *Ruminococcus* ($P < 0.01$) and reduced the presence of *Citrobacter* ($P < 0.05$). Both HID and TMD increased the relative abundance of *Clostridiales* ($P < 0.01$) and *Desulfovibrionaceae* ($P < 0.01$), while reducing the relative abundance of *Lachnospira* ($P < 0.05$) and *Phascolarctobacterium* ($P < 0.01$) when compared to the C diet (Fig. 3). Taking into account the caecal samples (Fig. 4), no differences were observed regarding the microbial composition and distribution. However, the Pairwise comparisons using the Wilcoxon rank sum test showed that TM inclusion reduced the relative abundance of *Klebsiella*, *Lachnospira* and *Parabacteroides* and *Odoribacter* compared to the C and HID (Fig. 5, $P < 0.05$). The correlations between VFA contents and microbiota are summarized in Fig. 6. In details, strong positive correlations between *Lachnospira* and propanoic acid, *Akkermansia*, between *Clostridiales* and $\text{NH}_3\text{-N}$, and between *Phascolarctobacterium* and acetic, propanoic and caproic acids were detected ($P < 0.05$).

Discussion

The current study has been conducted to evaluate the *in vitro* antimicrobial properties of HI and TM fats and their effect as an alternative lipid sources in rabbit diets. The possible utilization of insect fats in animal diets has been poorly investigated so far, and few papers are available, focusing on antimicrobial activities *in vitro* or *in vivo*, but no data are present in literature on pathogen growth rate in presence of insect fats. Mustafa et al. [43] showed that the oil extracted from the melon bug (*Aspongopus vidulatus*) was able to inhibit the growth of bacterial species by using the agar well diffusion method, with only Gram positive bacteria (*Staphylococcus*, *Bacillus* and *Enterococcus*) being susceptible to crude oil extracts. *Salmonella paratyphi* was also tested and no inhibition was recorded, similarly to what was observed in the present research with other serovars. A recent study of Spranghers et al. [28] on the *in vitro* effects of fats extracted from HI (from prepupae fed to weaned piglets) pointed out that D-*Streptococci* and *Lactobacilli* were the only bacterial populations that reduced their load after being challenged with insect fats, whereas no effects were recorded on Gram negative bacteria. The data presented in our paper suggest that fats extracted from TM and HI are able to delay bacterial growth of both Gram positive and Gram negative pathogenic bacteria, even if the susceptibility changes with the considered species. Interestingly, no bactericidal effect was observed, thus indicating the possibility of bacterial cells to repair to damages that may be induced from FAs or monoglycerides [6].

The antimicrobial effect of TM fat was lower than that of HI fat. These results may be related to the different concentration of SFA. Indeed, HI fat used in this study was composed by 79% of SFA (25% in TM fat), and the major component was lauric acid, already reported as very effective against many bacterial species [6; 28]. On the other hand, the antibacterial effects of UFA reported in literature (Yoon et al., 2018) were not detected in this study, probably in relation to the prolonged incubation time and the temperature

of 37 °C, which may have been responsible for the reduction/impairment of the activity of these molecules that characterize TM fat (75% of the FA v. 21% of HI fat). This hypothesis may also explain why soybean oil did not show any activity, considering its higher level in MUFA and PUFA among all fats used in this work.

The present observations highlighted the possibility of using insect fats in feed formulation, considering that they may be important for controlling growth of important microbial pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica* and *Pasteurella multocida* that may be important pathogens for rabbits [32;35] or part of the gut flora potentially contaminating rabbit meat during slaughtering [33;34]. Reducing microbial loads in rabbit gut, apart from animal welfare and safety implications, may also be important for food safety management. However, as already emphasized by Spranghers et al. [28], more studies need to be performed *in vivo*, in order to assess the activity of fat in the gut, considering that the bacterial activities and the digestive enzymatic systems of the rabbit (i.e. lipases) may neutralize the FAs, therefore limiting their activity.

As far as the *in vivo* trial is concerned, there was a lack of differences among groups on caecal fermentation traits in our study. Caecal pH and VFA content are the main variables characterizing the extent and the pattern of caecal fermentation, thus constituting an indirect estimate of caecal microbial activity. The dietary HI or TM fat inclusion led to a greater total VFA content in the caecum than that of control diet, thus potentially enhancing gut with a modification of the fermentation patterns and the composition of the caecal microflora.

Peeters et al. [44] previously observed that a high concentration of total caecal VFA in rabbits had a protective effect against enteropathogenic *Escherichia coli* infection. However, the molar proportion of different VFA was not affected by the total replacement of soybean oil with HI and TM larvae fat.

The present study is the first that investigate gut microbiota of growing rabbits fed diets supplemented with insect fats. The results of this study revealed an enrichment of different taxa according to the dietary treatment and a similar microbial diversity and richness between feces and caecum samples. Caecal microbiota is a primary determinant for rabbit health, whereas the fecal microbiota provides an accurate method for studying the evolution of rabbit gut microbiota from weaning to slaughtering [45–46].

Firmicutes, *Bacteroidetes* and *Verrucomicrobia* represented the dominant bacterial phyla in the control and insect fat-fed rabbits of the present study. These findings overall agree with previous researches that identified *Firmicutes* and *Bacteroides* as the main bacterial *phylum* in the gut microbiota [2; 46–48]. In relation to the genera composition, *Bacteroides*, *Bacteroidales*, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae* and *Ruminococcus* families, mainly colonized the caecal and fecal microbiota of the rabbits fed soybean oil or insect fats in the current study. These findings are also in agreement with previous studies, which observed *Bacteroides* [46;48], *Clostridiales* [47] and *Ruminococcus* [46;49] as main the bacterial genera in caecal and fecal microbiota of rabbits.

Regarding the microbial composition, we did not observe any strong effects as a consequence of the dietary inclusion of HI and TM fats. However, a signature in the microbial population was observed. The

fat of TM reduced some taxa such as *Klebsiella*, *Lachnospira*, *Parabacteroides* and *Odoribacter*. On the other hand, the dietary supplementation of HI and TM fats enriched the presence of *Akkermansia*, which is a maintaxa in the gut microbiota of rabbit [45]. It is well reported that *Akkermansia* can be considered a probiotic of new generation able to degrade the mucin in the gut with the production of beneficial molecules like SCFA, thus exerting a significant improvement in the gut barrier and in the maintenance of intestinal health [50–52]. In addition, it was suggested that *Akkermansia* have an important role in the hydrolysis of various dietary polysaccharides, contributing to increase cellulose digestibility as well as methane metabolism [53–55]. The increase of this *taxon* related with the insect fat inclusion suggests an optimal gut environment in our rabbits, even if this observation needs further investigation to be confirmed. In addition, a strong positive correlation between *Akkermansia* and NH₃-N was observed.

Ruminococcaceae family was considered as an important producer of short-chain FAs (mainly butyrate, acetic, and succinic acids) through glucose metabolism and cellulose digestion [56–57]. It was reported that member of this family is an important component of the beneficial microbiota of several herbivores [58–59]. Their presence is related with an improvement of the immune system of the host via intestinal mucus degradation and a prevention of acidosis via lactate degradation [60]. The supplementation of HI fat also increased the presence of *Ruminococcus* (belonging to *Lachnospiraceae* family), that are butyrate producing bacteria. This ability was also confirmed by the strong positive correlation between L-*Ruminococcus*, acetic and propionic acid. The microbial signature drove by the inclusion of insect fat increases the presence of *Bacteroides*, *Clostridium*, *Akkermansia* and *Ruminococcus* and suggests that dietary HI and TM fats may exert a positive influence on the caecal microbiota of the rabbits. It should be pointed out that rabbit fed HI and TM fats showed no significant alterations at histopathological level and no differences on growth performance [12].

Conclusions

In conclusion, the results of the present study provide new information about the *in vitro* antibacterial properties and the *in vivo* effects at the gut level of the replacement of soybean oil with HI and TM larvae fat in rabbit diet. The *in vitro* activities of HI or TM fats against *Pasteurella*, *Yersinia*, known pathogens of rabbit gut, indicate a potential for impairing their growth *in vivo* in rabbits. Furthermore, the dietary inclusion of HI and TM fats stimulate VFA production at caecum and may positively modulate the caecal and fecal microbiota of the growing rabbits. However, further researches are needed to confirm the antimicrobial potentiality of insect fats in rabbit feeding.

Abbreviations

BCFA, brain chain fatty acids; BHI, Brain Heart Infusion broth; C, control diet with soybean oil; DM, dry matter; FA, fatty acid; FAME, fatty acid methyl esters; HI, *Hermetia illucens*; HID, HI diet; MCFA, medium chain fatty acid; MUFA, monounsaturated fatty acids; OUT, Operational Taxonomic Units; PUFA, polyunsaturated fatty acids; S, soybean oil; SCFA, short chain fatty acids; SEM, standard error of the

mean; SFA, saturated fatty acids; TM, *Tenebrio molitor*, TMD, TM diet; TSA, Tryptic soy agar; TSB, Tryptic soy broth; UFA, unsaturated fatty acids; VFA, volatile fatty acids.

Declarations

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Author's contribution

LG, AS, AT, GX, SD, and FG conceived and designed the experiment. SD, DMN, IF, FG, IB, LC and LG collected the experiment data. AT and GX performed the FA analyses and the caecal fermentation parameters. DMN established the microbiological analysis. SD, SM and SDO extracted and quantified the DNA. ACB performed the 16S rRNA amplicon-based sequencing. SD, DMN and IF performed the statistical analysis. All authors interpreted the data. SD, DMN and IF wrote the first draft of the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approved for the version to be published.

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Availability of data and materials

The datasets analysed in the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The trial was designed according to the guidelines of the current European Directive (2010/63/EU) on the care and protection of animals used for scientific purposes, and was approved by the Bioethical Committee of the University of Torino (Italy) (Ref. 386638, 4/12/2017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

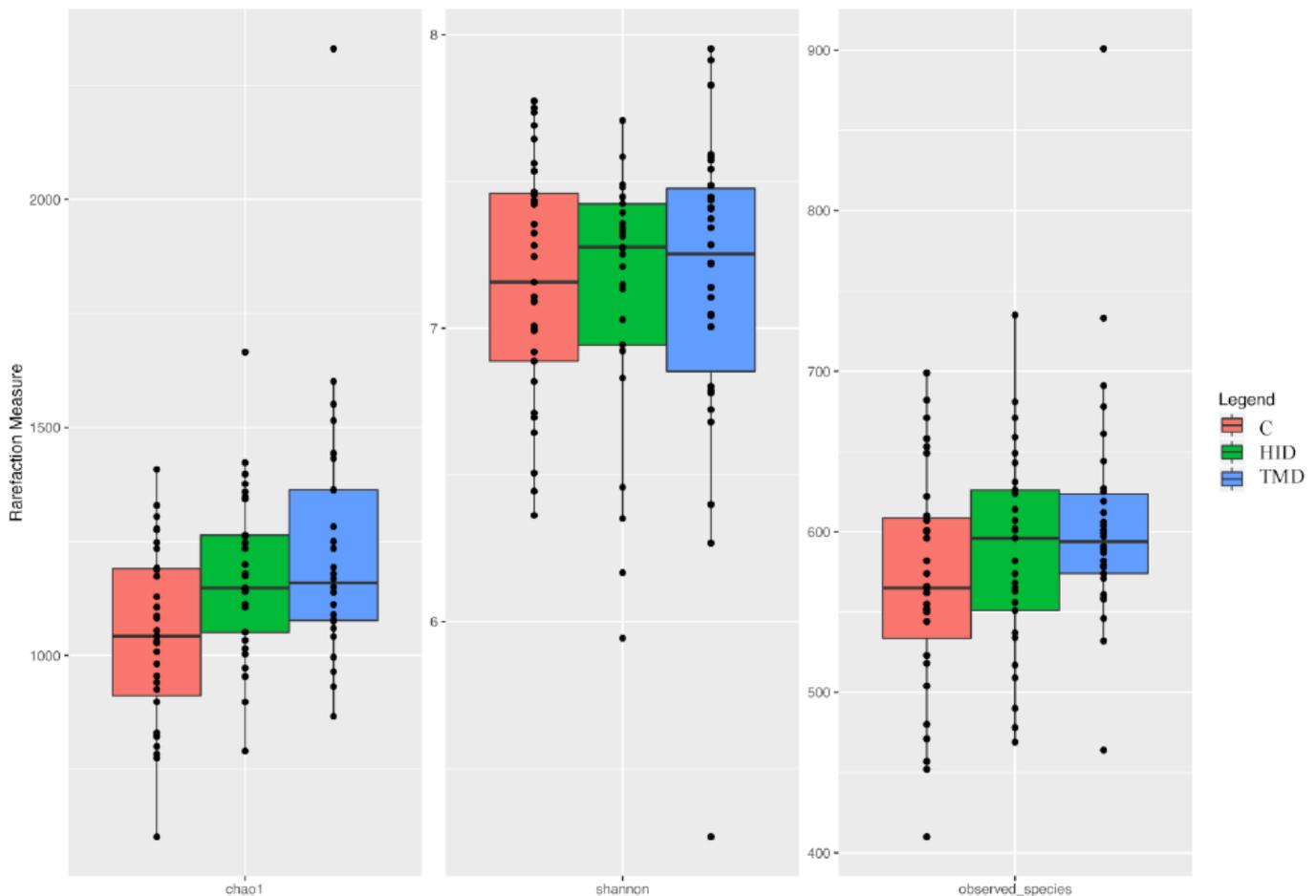


Figure 1

Boxplots showing the alpha diversity rarefaction index across fecal sample of rabbit fed with TM (blue box), HI (green box) and C (red box) diets. Boxes represent the interquartile range (IQR) between the first and third quartile and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartile, respectively.

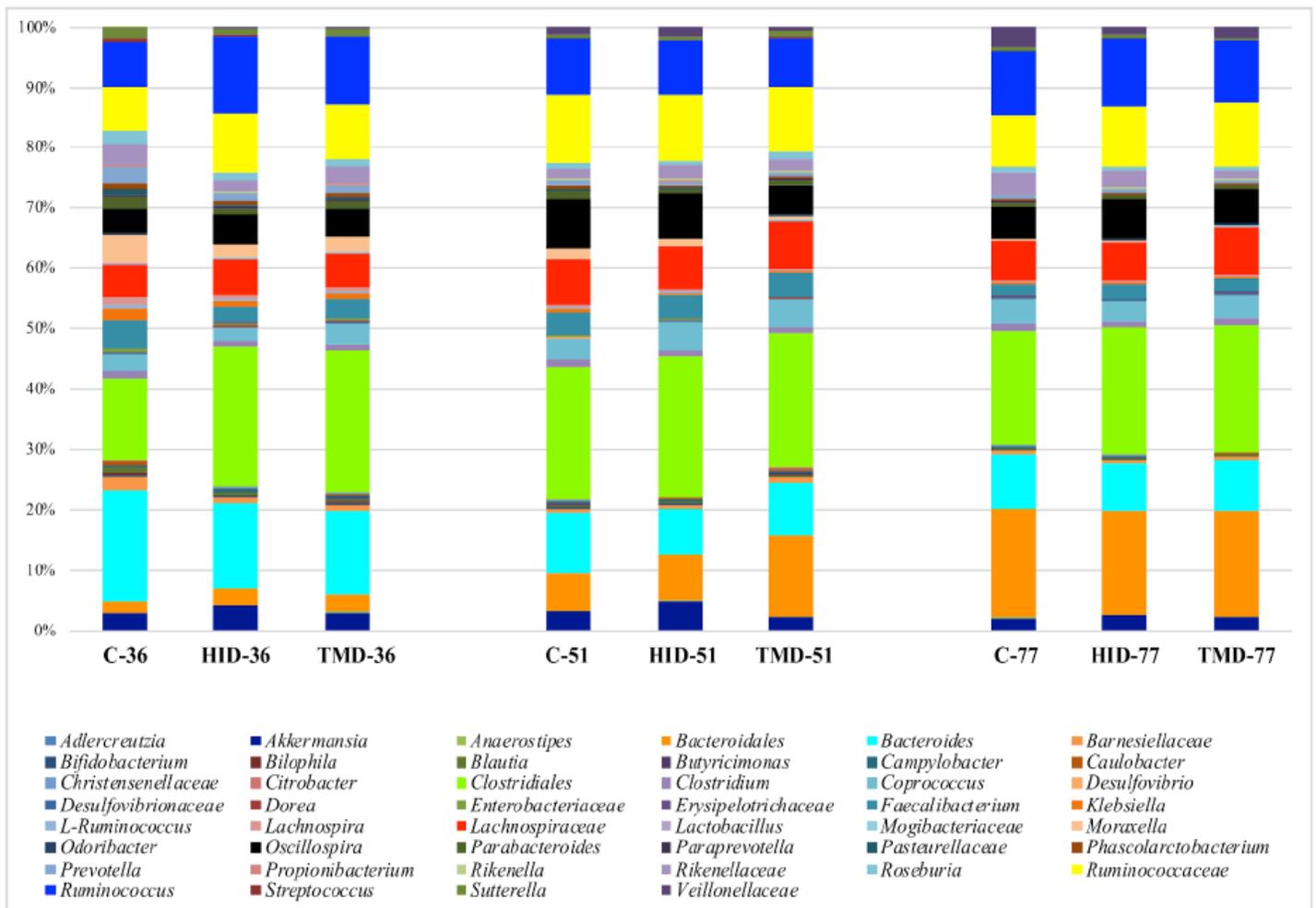


Figure 2

Taxonomic groups detected in fecal samples by means of 16S amplicon target sequencing. Only operational taxonomic units (OTUs) with an incidence above 0.2% in at least two samples are shown. The samples are labelled according to the type of fat supplementation (TM, HI and C diets) and sampling time (36, 51 or 77 days).

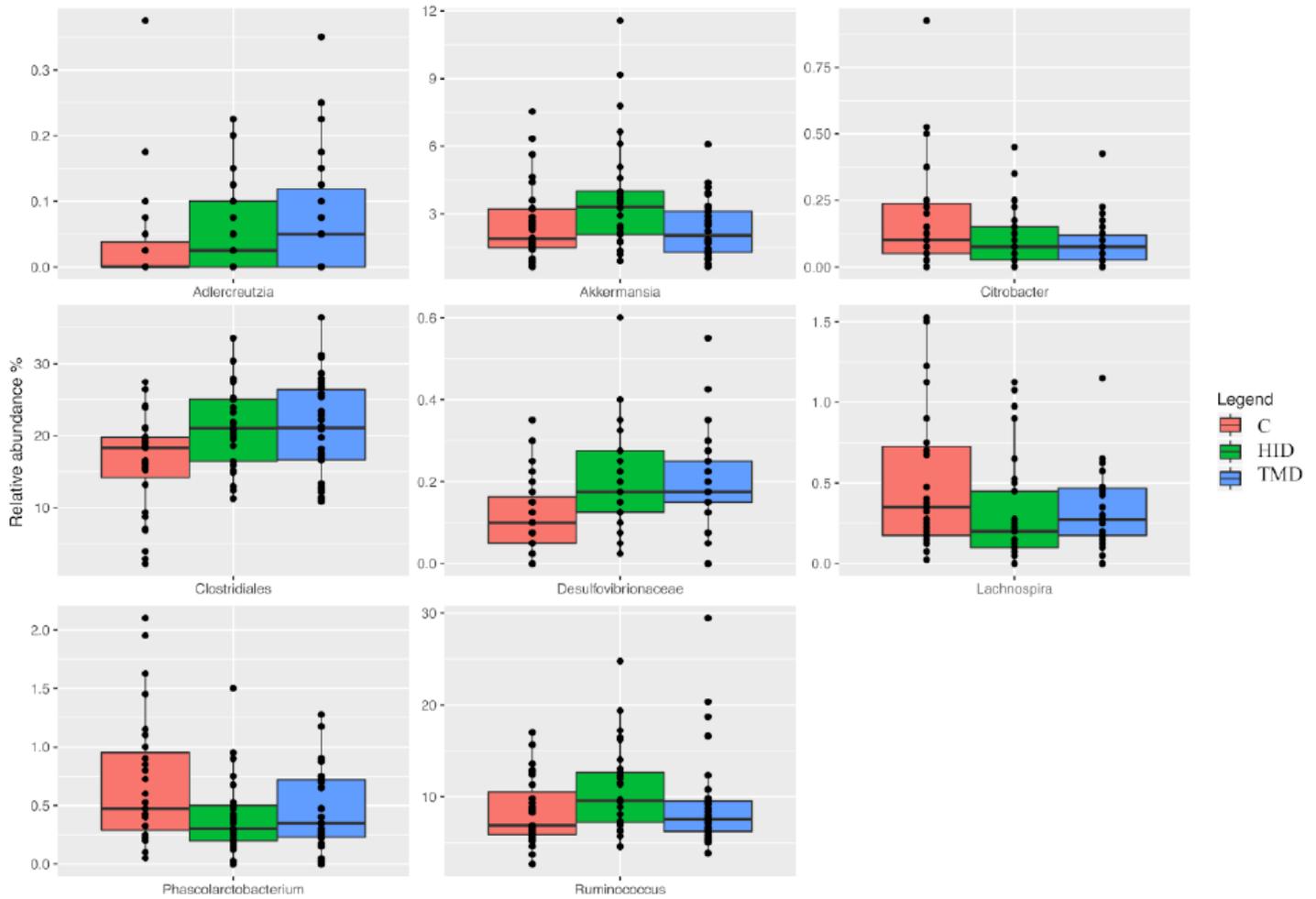


Figure 3

Boxplots showing the relative abundance at genus or family level of the OTUs differentially abundant based on GLM test in fecal samples of rabbit fed with TM (blue box), HI (green box) and C diets (red box).

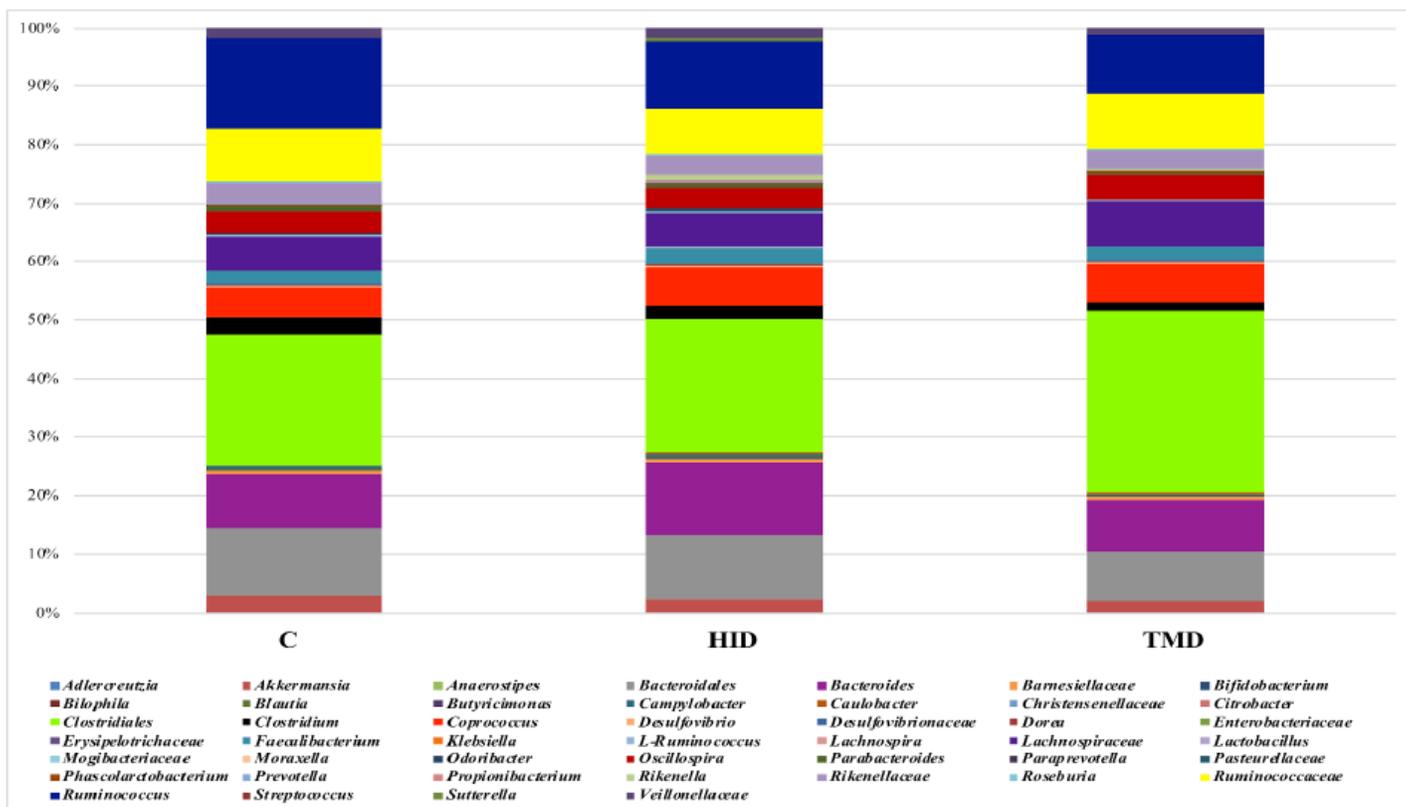


Figure 4

Taxonomic groups detected in caecal samples by means of 16S amplicon target sequencing. Only operational taxonomic units (OTUs) with an incidence above 0.2% in at least two samples are shown. The samples are labelled according to the type of fat supplementation, i.e. TMD, HID and C diets.

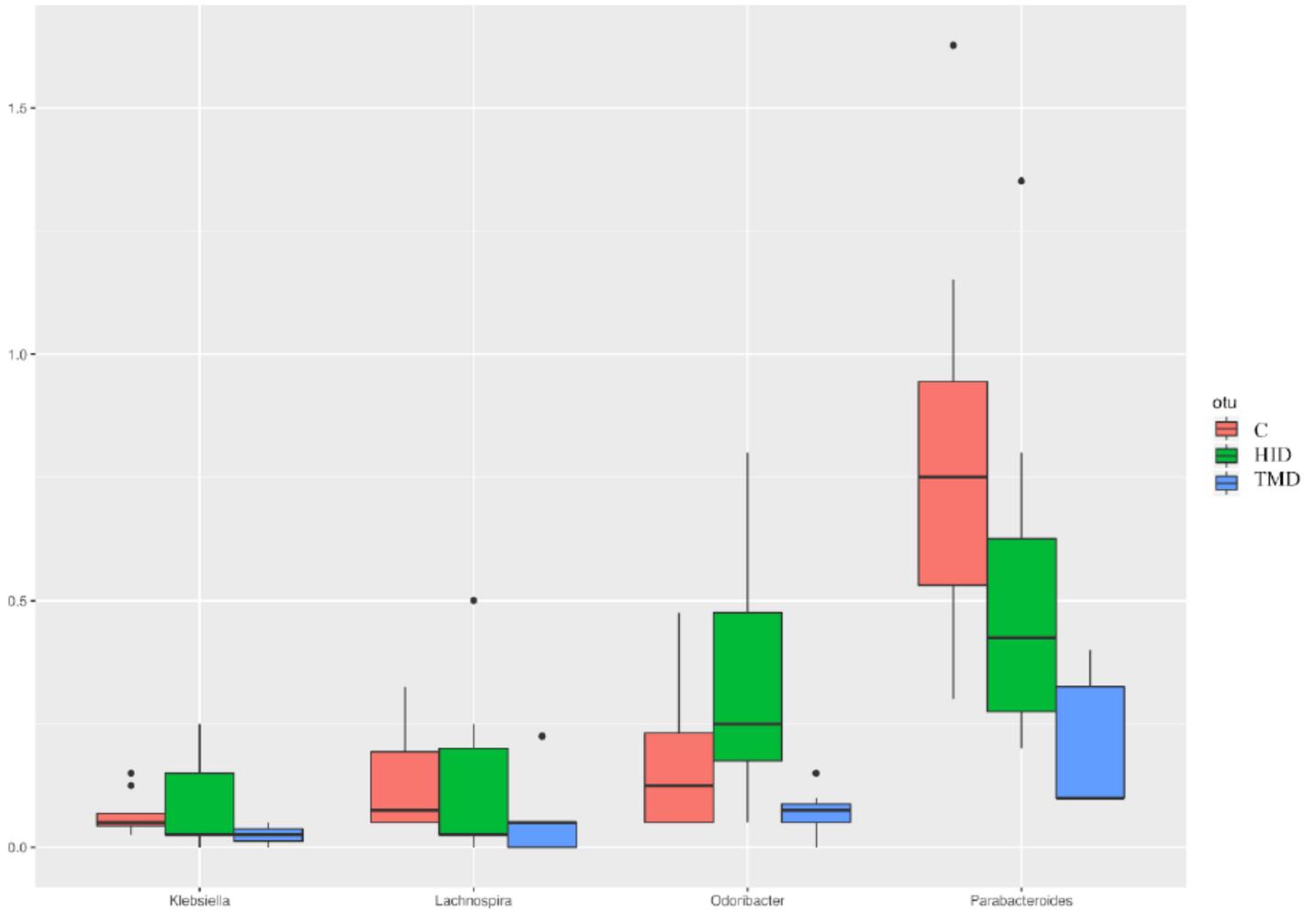


Figure 5

Boxplots showing the relative abundance at genus or family level of the operational taxonomic units (OTUs) differentially abundant based on Wilcoxon rank sum test in caecal samples of rabbit fed with TM (blue box), HI (green box) and C (red box) diets.

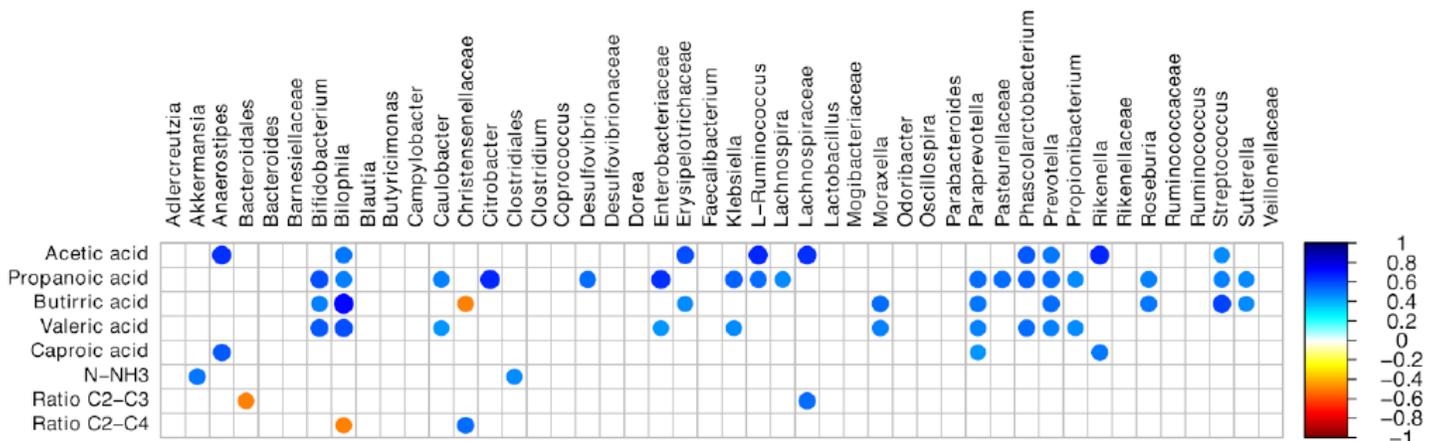


Figure 6

Spearman's rank correlation matrix of caecal operational taxonomic units (OTUs) abundance and volatile fatty acid profile. The colors of the scale bar denote the nature of the correlation, with 1 indicating a positive correlation (blue) and -1 indicating a negative correlation (dark red) between the two datasets.