

Dietary supplementation with tannins from tara extracts (*Caesalpinia spinosa*) alters the pig gut microbiota

Filippo Biscarini

filippo.biscarini@cnr.it

National Research Council

Paola Cremonesi

National Research Council

Antonio Natalello

University of Catania

Alessandro Priolo

University of Catania

Giuseppe Luciano

University of Catania

Bianca Castiglioni

National Research Council

Research Article

Keywords: *Cesalpina spinosa*, pig, gut microbiota, diet

Posted Date: September 4th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4990923/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Dietary supplementation with tannins from tara extracts (*Caesalpinia spinosa*) alters the pig gut microbiota

Filippo Biscarini^{1*†}, Paola Cremonesi^{1†}, Antonio Natalello²,
Alessandro Priolo², Giuseppe Luciano², Bianca Castiglioni²

¹*Institute of Agricultural Biology and Biotechnology (IBBA), CNR
(National Research Council), Via A. Corti 15, Milan, 20133, Italy.

²Department of Agriculture, Food and Environment, Università degli
Studi di Catania, Catania, 95131, Italy.

*Corresponding author(s). E-mail(s): filippo.biscarini@cnr.it;

Contributing authors: paola.cremonesi@cnr.it;

[†]These authors contributed equally to this work.

Abstract

In this study, the effect of the dietary supplementation with a novel source of tannins on the gut microbiome of pigs was evaluated. Tannins from different sources are used in pigs as an alternative to antimicrobials and to zinc and copper supplementations. This is the first report on the effects of tannins extracted from tara (*Cesalpinia spinosa*) on the pig gut microbiome. Twenty male finishing pigs were sampled and assigned randomly to the treatment (tannin supplementation) and control groups: rectum swabs were used for 16S rRNA-gene sequencing of the gut microbiota. Based on the gut microbiota composition as a whole the two groups were clearly distinguishable, as shown by Bray-Curtis dissimilarities and by the high accuracy of prediction (0.854) from the OTU-count table with a Random Forest classification model. On the other hand, other typical indicators of microbial diversity, like alpha diversity indices, and the F:B ratio, were not significantly different between treatment and controls, likely because of the small sample size. In conclusion, the dietary supplementation with tannins from tara extracts appears to significantly alter the overall gut microbiome of finishing pigs, although it is still unclear how this reverberates at the level of specific microbiome diversity indicators.

Keywords: *Cesalpinia spinosa*, pig, gut microbiota, diet

047 Introduction

048
049 Natural extracts are increasingly adopted as a valuable alternative to antimicrobials
050 and growth-promoting additives (like zinc and copper) in livestock farming [Kumar](#)
051 [et al \(2014\)](#). These diverse bioactive compounds, such as phytosterols, glucosinolates,
052 carotenoids and polyphenols, have shown antioxidant, anti-inflammatory and antibac-
053 terial effects in farm animals ([Huang et al \(2018\)](#); [Dell'Anno et al \(2020\)](#); [Perricone](#)
054 [et al \(2020\)](#); [Sotira et al \(2020\)](#)). Tannins are the largest class of polyphenol com-
055 pounds from plant extracts, especially among those used in poultry and pig farming,
056 and can be classified into the hydrolysable and condensed subgroups ([Jansman \(1993\)](#);
057 [Caprarulo et al \(2020\)](#)).

058 Poultry and swine nutrition are the most important sectors in which tannins have
059 been used: firstly, adopting tannin-rich feedstuff and, more recently, using tannin
060 extracts from different plants. Several commercial products are available containing
061 tannins extracted from the European chestnut tree (*Castanea sativa*) and the Amer-
062 ican quebracho (*Schinopsis* spp.). Tannins extracted from these plants have been
063 employed in intensive swine farms at different physiological stages (post-weaning and
064 growing/finishing phases) to enhance growth, modulate the intestinal microbiota and
065 decrease the incidence of diarrhoea - particularly during post-weaning [Girard et al](#)
066 [\(2019\)](#); [Girard and Bee \(2020\)](#). However, the effectiveness of tannin supplementation
067 in weaned and finishers seems to be highly related not only to the dose administered to
068 the animals but also to the duration of supplementation, the presence of other sources
069 of tannins in the basal diet, the feed regimen adopted (ad libitum vs restricted), the
070 tannin form (hydrolysable or condensed) and the potential synergistic or antagonistic
071 effects with other supplements [Caprarulo et al \(2020\)](#).

072 These and other aspects of tannin dietary supplementation in pigs still require
073 further investigation to be fully clarified. In particular, there are currently few studies
074 on the *in vivo* modulation of the pig's microbiota by tannins, especially in post-weaning
075 and finishing pigs, as the intestinal populations studied so far are mostly restricted to
076 the main pathogens in weaned piglets [Biagi et al \(2010\)](#); [Girard et al \(2018\)](#); [Girard](#)
077 [and Bee \(2020\)](#). Additionally, tannins extracted from plants other than chestnut and
078 quebracho, such as Tara (*Caesalpinia spinosa*), were investigated only in ruminants
079 for their effects on fatty acids composition and microbiological quality of the lamb
080 meat [Biondi et al \(2019\)](#); [Valenti et al \(2021\)](#). To our knowledge, no results have been
081 so far reported in monogastric animals —specifically in pigs— and on their effects on
082 the gut microbiome.

083 Therefore, the aim of the present study was to evaluate the effect of the dietary
084 administration of tannins extracted from tara (*Caesalpinia spinosa*) on the pig gut
085 microbiota composition. This will help elucidate the following: i) the effect of dietary
086 supplementation with tannins on the growth performance and gut microbiome of fin-
087 ishing pigs; ii) the evaluation of hydrolysable tannins extracted from an additional
088 plant source, tara; iii) a better understanding of the effect of tannin administration
089 on the pig gut microbiome. Results show that tannins from tara extracts do alter the
090 pig gut microbiome, but to a different extent depending on the different aspects of the
091 microbiome.

092

Methods

Animals, experimental design, diet and sampling

The experimental trial was approved by the animal welfare committee (OPBA) of the University of Catania (protocol n. 286946). In a commercial farm, twenty castrated male pigs (crossbred PIC x Piétrain) were selected for their body weight (BW: 63.2 ± 3.29 kg) and age (125 ± 5 d). The barrows were then transferred to the University facilities and allocated to individual pens with concrete floor and bedding with wheat straw. Each pen was equipped with water nipples and feeders. The farrows were randomly assigned to two experimental treatments (10 pigs/treatment) and fed ad libitum with a commercial concentrate without supplementation (control group, CTRL) or supplemented with 11.5 g kg^{-1} of tannin extract (tannin-treated group, TREAT) from tara (*Cesalpinia spinosa*). The amount of extract to add was calculated to obtain a supplementation level of 10 g kg^{-1} of total tannins as tara extract contained 86.9% of total tannins according to the method of Makkar et al. [Makkar et al \(1993\)](#). The commercial extract (“Tannino T80”®) was purchased from *Silvateam* (San Michele Mondovì, Cuneo, Italy). The basal diet was composed of (as-fed basis) maize (42%), barley (20%), wheat bran (15.2%), soybean meal (13.4%), fava bean (7.4%) and mineral and vitamin premix (2%). All the ingredients of the basal diet were ground, mixed thoroughly and pelleted (temperature of 40°C). Tannin extract was added before mixing and pelleting. Barrows always had free access to fresh water and their respective diets. The amount of offered and refused feeds was recorded in order to calculate the dry matter intake. Individual body weight was measured on a fortnightly basis to estimate the average daily gain (ADG) and feed conversion ratio. After the 55 days of the feeding trial, individual feces samples were collected directly from the rectum using sterile swabs, immediately inserted into the sterile swab tubes and kept on ice until arrival in the laboratory, where samples were stored at -80°C before 16S rRNA-gene sequencing.

Table 1 Growth performance of pigs in the two experimental groups: body weight (BW) at the end of experiment (day 55), average DMI (dry matter intake), ADG (average daily gain) and FCR (feed conversion ratio). S.e.m.: standard error of the mean. p-values refer to the between-group differences.

	Dietary treatment		s.e.m.	p-value
	CTRL	TREAT		
Final BW (kg)	116	120	2.2	0.248
DMI (g/d)	2862	3018	55.1	0.161
ADG (g/d)	956	1025	30.5	0.268
FCR (DMI/ADG)	3.00	2.99	0.065	0.909

Sequencing

DNA was extracted from 20 rectal swab samples (10 pigs each from the control and treated groups) using a QIAmp DNA Stool kit (Qiagen, Hilden, Germany), following the manufacturer's protocol with minor modifications. The rectal swabs were dissolved in 1 mL Buffer ASL and shaken at 1000 rpm (Mixing Block MB-102, CaRlibiotech S.r.l. Rome, Italy), continuously until the stool samples were homogenized. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was then stored at -20°C until use. Bacterial DNA was amplified using the primers described in literature [Caporaso et al \(2011\)](#) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 μL volumes per sample. A total of 12.5 μL of KAPA HIFI Master Mix 2x (Kapa Biosystems, Inc., MA, USA) and 0.2 μL of each primer (100 μM) were added to 2 μL of genomic DNA (5 ng/ μL). Blank controls (no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95°C for 3 min, followed by 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2x250-base paired-end reads.

Bioinformatics processing

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were joined into single reads using the C++ program SeqPrep [John \(2011\)](#). After joining, reads were filtered for quality based on: i) maximum three consecutive low-quality base calls (Phred < 19) allowed; ii) fraction of consecutive high-quality base calls (Phred > 19) in a read over total read length ≥ 0.75 ; iii) no "N"-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were filtered out. All remaining reads were combined in a single FASTA file for the identification and quantification of OTUs (operational taxonomic units). Reads were aligned against the SILVA closed reference sequence collection release 132, with 97% cluster identity [Yilmaz et al \(2014\)](#), applying the CD-HIT clustering algorithm [Li and Godzik \(2006\)](#). A pre-defined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. OTUs with total counts < 10 in < 2 samples were filtered out. All of the above steps were performed with the QIIME v.1.9 open-source bioinformatics pipeline for microbiome analysis [Caporaso et al \(2010\)](#). The command lines and parameters used to process 16S rRNA-gene sequence data were adapted from Biscarini et al. 2018, S1 Appendix [Biscarini et al \(2018\)](#).

Microbial diversity

The microbial diversity of the pig microbiota was assessed within (alpha-diversity) and across (beta-diversity) samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness and diversity were estimated using the following indices: Chao1 and ACE (abundance-based coverage estimator) for richness, Shannon, Simpson and Fisher’s alpha for diversity [Chao \(1984\)](#); [Chao and Lee \(1992\)](#); [Chao et al \(1993\)](#); [Shannon \(1948\)](#); [Simpson \(1949\)](#); [Fisher et al \(1943\)](#), as well as Simpson E and Pielou’s J (Shannon’s evenness) for evenness [Smith and Wilson \(1996\)](#). Across-sample gut microbiota diversity was quantified by calculating Bray-Curtis distances [Bray and Curtis \(1957\)](#) from OTU counts normalized for uneven sequencing depth by cumulative sum scaling (CSS, [Paulson et al \(2013\)](#)). Among groups (control and treated) and pairwise distances were evaluated non-parametrically using the permutational analysis of the variance approach (PERMANOVA: 999 permutations) [Anderson \(2001\)](#). The details of the calculation of the mentioned alpha- and beta-diversity indices can be found in Biscarini et al. 2018, S2 Appendix [Biscarini et al \(2018\)](#).

Table 2 Alpha diversity indices in pigs treated with tannins (feed supplementation) and controls. p-values come from the original dataset (p.value) and from 1000 bootstrapping replicates of the data (boot_pval). The column boot_diff_pct reports the average bootstrapped difference between the tannin and control groups expressed as percentage of the average index value.

metric	CTRL	TREAT	p.value	boot_diff_pct	boot_pval
chao1	976.4	992.0	0.8706	5.448	0.5127
ace	960.6	993.7	0.7122	5.036	0.4862
fisher_alpha	447.5	465.6	0.6458	4.924	0.4568
observed_otus	759.7	803.2	0.4274	4.757	0.4165
shannon	9.243	9.348	0.3417	0.845	0.3737
simpson	0.998	0.998	0.2898	0.013	0.3428
equitability	0.969	0.970	0.4784	0.089	0.4079
simpson_e	0.685	0.690	0.6967	0.944	0.4779

Statistical analysis

Comparison between experimental groups

The gut microbiota has been sampled once, after 55 days from the beginning of the supplementation experiment. We therefore had one single microbiota measurement per pig, and each pig belonged to either the control or treated group. The linear model used to analyse between-group (treatments) differences in terms of alpha diversity indices, OTU counts and F:B (Firmicutes to Bacteroidetes) ratio had the following form:

$$y_{ij} = \mu + \text{treatment}_j + e_{ij} \quad (1)$$

where y_{ij} is the alpha diversity index value (or OTU counts or F:B ratio) for record i belonging to treatment j ; μ is the intercept, treatment_j is the systematic effect of treatment and e_{ij} is the residual.

For the alpha diversity indices, a bootstrapping analysis was conducted: for each index, the 20 values of the two groups (10 Ctrl and 10 Treat) were resampled with replacement and the comparison between treated and control pigs was reevaluated on the resampled data. This procedure was replicated 1000 times, leading to 1000 bootstrapped results (CTRL-TREAT differences, estimated treatment coefficients and p-values from equation 1). From bootstrapped replicates of the analysis, besides p-values also the estimated treatment coefficients and the percent differences between treatment and control were obtained. Percent differences were calculated as: % difference = $\frac{|\text{TREAT}-\text{CTRL}|}{\bar{x}_{\text{index}}}$, where $\bar{x}_{\text{index}}$ is the average value (across groups) for each alpha diversity index.

Random Forest predictions

A random forest (RF [Breiman \(2001\)](#)) model for binary classification was developed to predict the experimental group (treated vs control) based on CSS-normalized OTU counts (at the *genus* level). A 5-fold cross-validation scheme was used to fine-tune the hyperparameters “minimum size of terminal nodes” (in the range [2, 5]) and “number of variables randomly sampled in each tree” (around $\sqrt{m}$, where m is the number of variables —microbial taxa— in the data). As for the total number of trees in the RF model, the default value of 500 was chosen. The Gini index ($G = \sum_{k=1}^K \hat{p}_{mk}(1 - \hat{p}_{mk})$, where $\hat{p}_{mk}$ is the proportion of samples in the m_{th} belonging to class k) was used to decide on splits (the smaller, the better: if all $\hat{p}_{mk}$ ’s are close to zero or one then G is small, indicating node purity: the node mainly contains samples of the same class). From the final RF model after hyperparameter-tuning, classifications were obtained by majority vote over the $B = 500$ classification trees:

$$\hat{f}_{avg}(x_i) = \frac{1}{B} \sum_{b=1}^B I(\hat{f}_b(x_i) = [0/1]) \quad (2)$$

where x_i is the vector of normalised OTU counts for sample (pig) i , and $\hat{f}_b(x_i)$ is the prediction (control/treated) from the classification tree built on the b_{th} bootstrapped data sample.

From the final model in equation 2, variable (OTU) importance was obtained based on the amount that the Gini index is reduced due to splits over a variable (averaged over all B trees). A large value indicates an OTU which is important for the accuracy of classification. More in detail, once the RF model (equation 2) was fitted (using OOB observations to decide on splits), the reduction of the Gini index value around each variable was recorded: each variable was used as splitting criterion in some nodes in each tree, and the Gini reduction over such nodes was summed up and then averaged over the number of trees in the RF model. Variable importance was then rescaled between 0 and 100.

Software and data & code availability

The *R* environment for statistical programming [R Core Team \(2023\)](#) was used for data handling and statistical analysis. Specifically, the *R* packages [tidymodels Kuhn and Wickham \(2020\)](#) and [ranger Wright and Ziegler \(2017\)](#) were used for bootstrapping

analysis and for the Random Forest model. 16S rRNA-gene sequencing data have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute (EBI) with project number PRJEB65759.

Results and Discussion

This paper evaluated the effect of tannin extracts, derived from tara (*Caesalpinia spinosa*), a new hydrolysable source of tannins, on the performance and gut microbiome of finishing pigs. The results of the experiment are hereby presented, structured per topic, and discussed along the way.

Animal performance

Table 1 reports the pigs' growth performance at the end of the experiment (day 55) in terms of final body weight (BW), average dry matter intake (DMI), average daily gain (ADG) and feed conversion ratio ($FCR = DMI/ADG$). BW, DMI and ADG increased in the Tann group, while the FCR remained unchanged, although these differences were not statistically significant, which can be linked to the relatively small sample size of this experiment. However, also previous studies failed to report a significant impact of dietary tannin supplementation on the growth performance of pigs (e.g. Chinese gallnut extracts as replacement for antibiotics Wang et al (2020)). In a review on the dietary supplementation with chestnut and quebracho tannins in pigs, mostly no or mildly positive (increased BW, ADG, FCR) effects on growth performance were detected Caprarulo et al (2021).

Hydrolyzed tannins -when fed in large amounts- are traditionally considered to have anti-nutritional effects in monogastric animals, and can potentially reduce growth performance Huang et al (2018). Nevertheless, in this study no adverse or negative effects of tara extracts on growth were observed. On the other hand, dietary supplementation with tannins at different concentrations has been reported to show positive health effects in terms antioxidant, anti-inflammatory and antibacterial activity, especially at weaning and post-weaning, by modulating the gut microbiota and leading to a decreased incidence of diarrhea Caprarulo et al (2021).

Gut microbiome profiling

From 16S rRNA-gene sequencing, a total of 885 393 high quality reads were found in the pigs' gut microbiota (average 44 269.65 reads per sample). After quality filtering, 19 7326 reads were removed, leaving 688 067 reads for the further analyses (34 403.35 reads per sample, on average). Filtering out OTUs with less than 10 total counts in less than 2 samples left 1 528 OTUs available for downstream analysis.

As reported in Table 2, the alpha diversity (which reflects the within-sample microbial diversity) was analysed in the control and treated groups using eight diversity indices. The average alpha diversity was consistently a tad higher in the treated group (Figure 1, panel A), but not significantly so: none of the p-values from the linear model (equation 1) were below 0.05, which was confirmed from bootstrapping. Figure 1 shows that: i) the distribution of the percent differences between treatment and control is

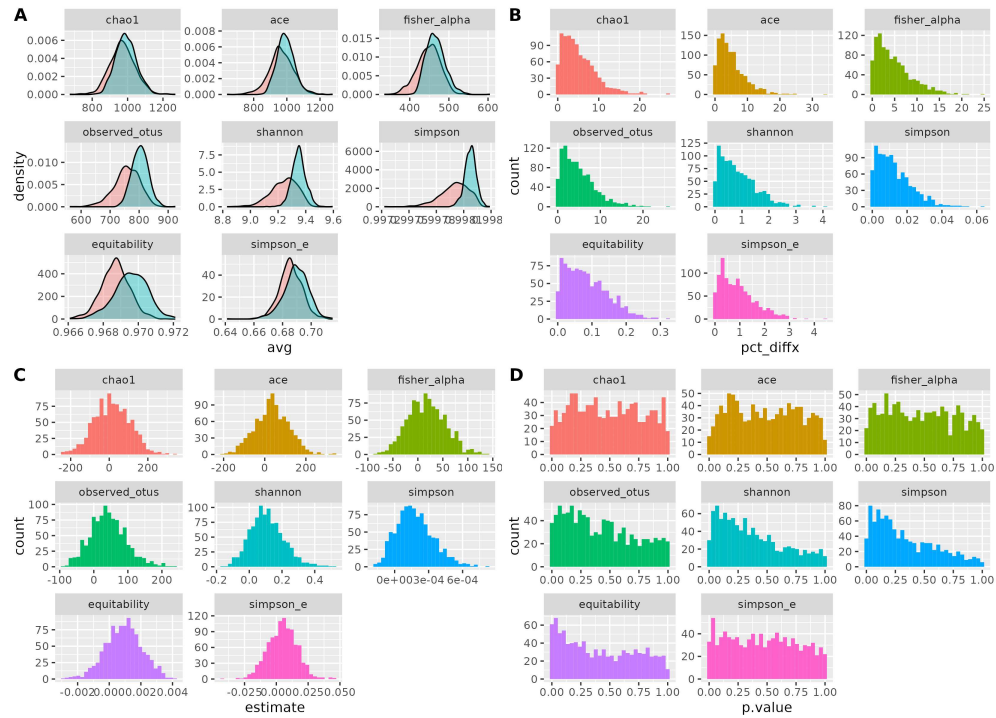


Fig. 1 alpha diversity results from bootstrapping analysis (1,000 replicates) for the eight diversity indices considered: A) density distribution of index values in the two experimental groups (CTRL: pink; TREAT: blue); B) histogram distribution of CTRL-TREAT percentage difference; C) histogram distribution of estimated treatment (tannin supplementation) coefficients from the linear model; D) histogram distribution of p-values from the liner model.

squashed towards zero (panel B); ii) the treatment coefficient from the linear model is distributed around zero, implying lack of effect (panel C); iii) the distribution of p-values is flat in $[0, 1]$, indicating absence of significant differences.

The non-metric multidimensional scaling plot of pairwise Bray-Curtis distances (Figure 2: 2D plot, left; 3D plot, right) showed clear separation of the treated and control clusters, which turned out to be statistically significant from PERMANOVA ($p\text{-value} = 0.004482$). This means that from beta diversity treated samples were easily distinguishable from controls.

Gut microbiota composition

The gut microbiota from the pigs included in this experiment was further investigated in terms of the relative abundance of bacterial taxa. In both the control and tannin groups, most of the reads belonged to the *Firmicutes* (49.8% and 50.3%) and *Bacteroidetes* (30.2% and 31.4%) phyla (Figure 3, left). At the phylum level, the pig gut microbiome also contained *Spirochaetes*, *Proteobacteria* and *Cyanobacteria*, which accounted for about 10% of the total microbiome in both experimental groups.

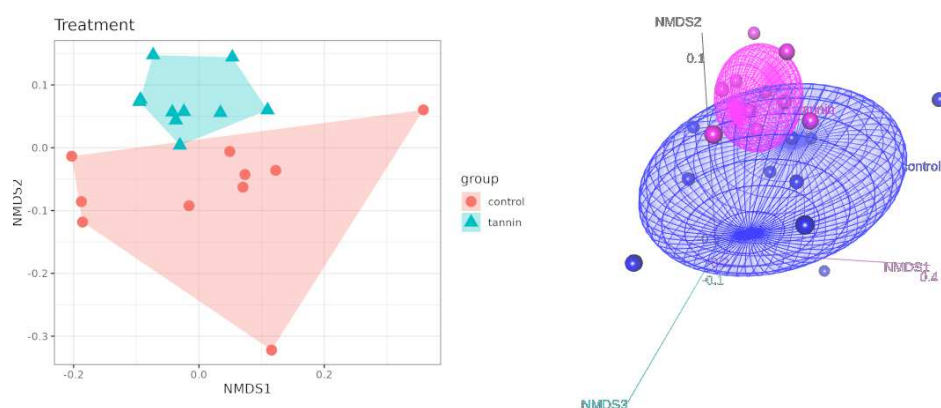


Fig. 2 beta diversity: 2D (left) and 3D (right) plots of pairwise Bray-Curtis distances, based on the OTU counts from the pig gut microbiota, between the treated (tannin supplementation) and control groups.

The median Firmicutes-to-Bacteroidetes (F:B) ratio from normalised counts was higher in the tannin-treated group compared to controls (1.67 vs 1.62), but not significantly so (p -value = 0.626) (Figure 3, right). The F:B ratio is known to play a role in adipogenesis and the fat metabolism: it has been found to be associated with blood and tissue fat content and obesity in humans (Turnbaugh et al (2006); Indiani et al (2018)), and to be correlated with milk-fat yield in Holstein cows Jami et al (2014). The F:B ratio is also considered to be involved in maintaining the normal intestinal homeostasis in humans as well as in animals Stojanov et al (2020). Polyphenols (like tannins) have a stronger action against *Firmicutes* than against *Bacteroidetes* in the gut microbiome, thereby altering the F:B ratio: eating food with high polyphenols content can potentially reduce body weight in obese people Rastmanesh (2011).

Table 2 reports the 73 OTUs from the pig gut microbiota which, from model 1, were significantly different (p -value < 0.01) between treated (feed supplementation with tannins) and control pigs. Of these, 27 OTUs showed a FDR (False Discovery Rate Benjamini and Hochberg (1995)) for the treated-control difference < 5% OTUs. Some of the microbial taxonomies found to differ between tannin-treated and control pigs have been previously reported in literature. For example, in the present study *Lachnospiraceae* NK4A136 group, *Prevotella* 1, *Prevotellaceae* NK3B31 group, *Ruminiclostridium* 6, *Ruminiclostridium* 9 and *Ruminococcus* 1 have been found to be consistently more abundant in the tannin group compared to the control group, similarly to a previous work Choy et al (2014) where tannins extracted from grapes increased the abundance of *Lachnospiraceae*, *Clostridiales* and *Ruminococcaceae*. Also in Tretola et al. (2019 Tretola et al (2019)) tannin supplementation resulted in a greater proportion of the family *Ruminococcaceae* in the pig gut microbiota. These bacterial taxa have been recognised as major producers of short-chain fatty acids

(SCFAs) in the human and animal gut microbiota. *Lachnospiraceae* and *Ruminococcus* are major butyrate and propionate producers and are known to be modulated by polyphenols Choy et al (2014).

In our experiment, dietary supplementation with tannins showed no effect on the order *Lactobacillales*, unlike other studies that found reduced or increased abundance of lactobacilli depending on the type of tannin feed supplementation (Choy et al (2014); Tretola et al (2019)). Lactic acid bacteria are generally considered to be protective against gastroenteric pathogens such as *Clostridium difficile*, *Campylobacter jejuni*, *Helicobacter pylori* and rotavirus Tretola et al (2019). Contrary to Miragoli et al. (2021 Miragoli et al (2021)), that found a reduction of *Prevotella* levels after feeding pigs during the weaning period with a mixture of quebracho and chestnut, an increment of *Prevotellaceae* was observed with the use of tara tannins in this work.

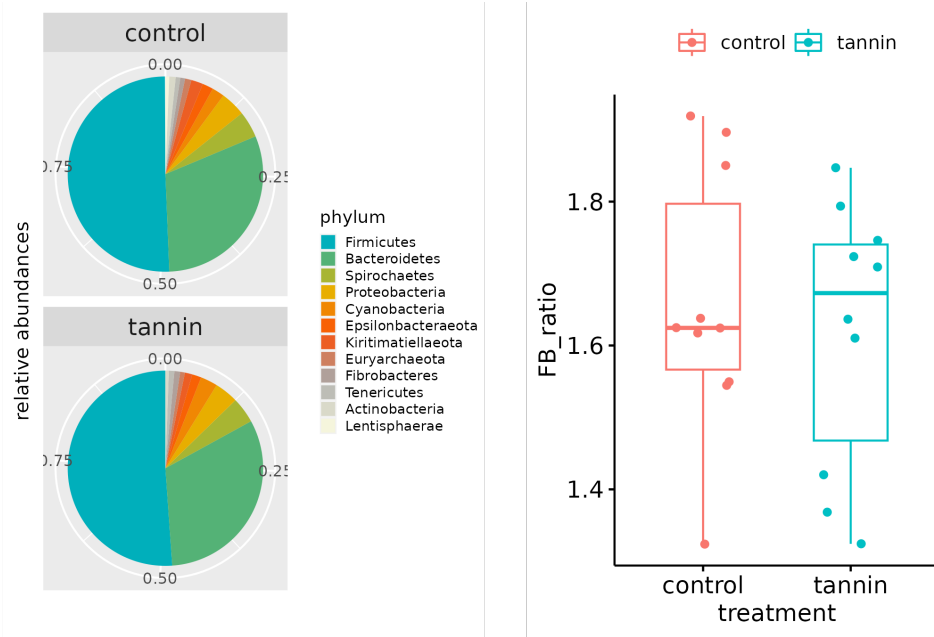


Fig. 3 phylum relative abundance (left) and F:B ratio (right) in the pig gut microbiota of the treated (tannin supplementation) and control groups.

Random Forest 'omic prediction of experimental groups

After fine-tuning of the hyperparameters, the final RF model (equation 2) used 37 randomly selected variables in each tree and a minimum of two observations in terminal nodes. The RF prediction of the experimental group (TREAT/CTRL) based on the gut microbiome data showed a remarkable predictive ability, despite the small sample size: the error rate measured on the OOB (out-of-bag) observations was 0.146

(accuracy: 0.854). Given the high prediction accuracy (especially if we factor in that $N = 20$), OTUs relevant for the prediction can be considered to be microbial taxa that discriminate the gut microbiota with and without dietary tannin-supplementation. Important variables (OTUs) were obtained from the reduction of the Gini index value (the splitting criterion). The top 20 OTUs from the RF model are reported in figure 4, alongside with their abundance in the two groups: these matched perfectly (100% match) with the significant OTUs ($p\text{-value} < 0.01$) from Table 2, and had a 70% match (14 out of 20) when $FDR < 0.05$ was considered.

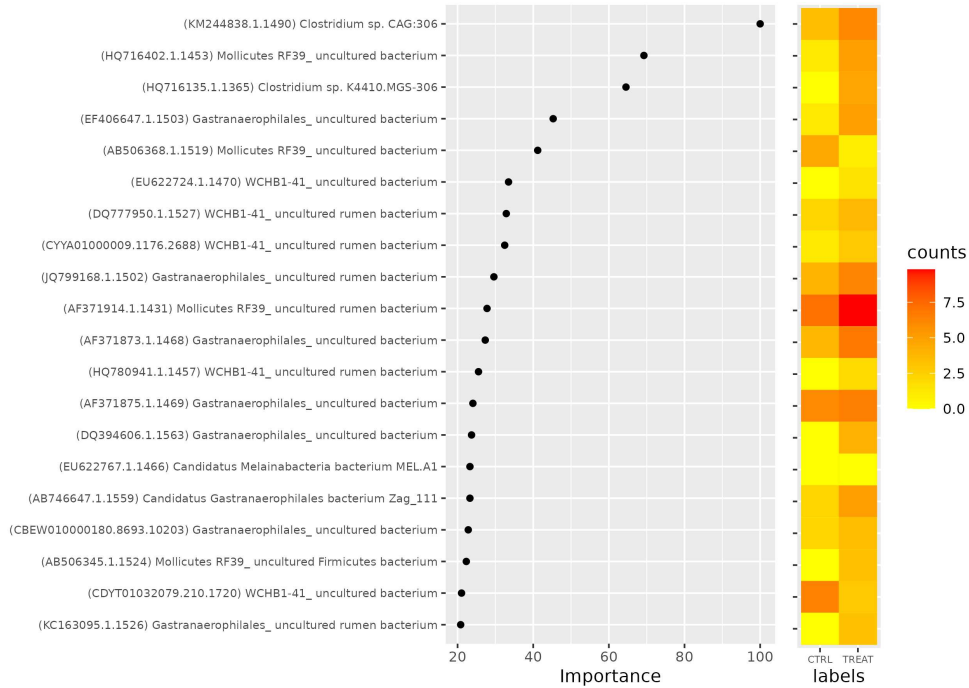


Fig. 4 variable importance plot from the Random Forest predictive model (top 20 variables are presented). Normalized counts in the control (CTRL) and tannin-treatment (TREAT) groups are reported alongside. Importance on the x-axis is rescaled in $[0, 100]$

Conclusion

This is the first report of the effects of tannins from tara (*Caesalpinia spinosa*) on the pig gut microbiome. Tara is an additional source of hydrolysable tannins as a feed supplement for the basal diet of pigs, whose effect on the gut microbiome has not yet been investigated. Results show that tara tannins have a relevant effect on the composition of the gut microbiota in pigs, as shown by the clear clustering of samples based on the microbiome data and by the high prediction accuracy of the random

507

508

509

510

Table 3 OTUs from the pig gut microbiota which are significantly different between treated (feed supplementation with tannins) and control pigs. Average counts in the two groups are reported alongside the p-values from analysis of variance. Significance was called at p-value < 0.01. FDR: false-discovery rate.

511

512

513

514

515

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

531

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552

taxon	control	tannin	p.value	FDR
Erysipelotrichaceae_uncultured_uncultured Solobacterium sp.	0.244	1.456	0.008462	4e-4
Lachnospiraceae_Coprococcus 2_uncultured rumen bacterium	0.822	2.400	0.001037	0.001
Ruminococcaceae_Ruminococcaceae UCG-014_uncultured rumen bacterium	0.000	1.118	0.005559	0.004
Ruminococcaceae_Ruminococcaceae UCG-008_uncultured bacterium	0.901	2.734	0.001114	0.016
Prevotellaceae_Prevotella 9_uncultured bacterium	3.210	5.877	0.000591	0.017
Anaeroplasmataceae_Anaeroplasmataceae uncultured bacterium	0.833	2.992	0.000719	0.017
Lachnospiraceae_Lachnospiraceae NK3A20 group_uncultured bacterium	1.465	0.000	0.000756	0.017
Candidatus Gastranaerophilales bacterium Zag.111	1.768	4.495	0.002451	0.017
Lachnospiraceae_Lachnospiraceae NK4A136 group_uncultured bacterium	1.555	2.866	0.005190	0.017
Prevotellaceae_Prevotellaceae NK3B31 group_uncultured bacterium	4.008	6.263	0.003798	0.019
Lachnospiraceae_Lachnospiraceae FCS020 group_uncultured bacterium	1.005	2.400	0.005693	0.019
Ruminococcaceae_Ruminiclostridium 9_uncultured bacterium	0.000	1.388	0.000078	0.025
Lachnospiraceae_Lachnospira_Lachnospira pectinoschiza	0.170	1.098	0.004110	0.029
Ruminococcaceae_Ruminococcaceae UCG-010_uncultured bacterium	5.220	2.014	0.004824	0.029
Ruminococcaceae_Ruminococcaceae UCG-010_metagenome	2.374	0.864	0.009467	0.029
uncultured bacterium	0.486	2.346	0.000308	0.029
Clostridium sp. CAG:306	0.328	2.142	0.000102	0.030
Ruminococcaceae_Ruminococcus 1_uncultured bacterium	0.000	1.160	0.000137	0.030
Lachnospiraceae_Lachnospiraceae NK4A136 group_uncultured bacterium	0.520	2.782	0.000480	0.030
Lachnospiraceae_Agathobacter_uncultured rumen bacterium	1.303	2.732	0.001662	0.030
Lachnospiraceae_[Ruminococcus] torques group_uncultured bacterium	0.255	2.151	0.001837	0.030
Erysipelotrichaceae_Turicibacter_Turicibacter sp. H121	1.674	3.465	0.006508	0.030
Lachnospiraceae_Lachnospiraceae UCG-004_uncultured bacterium	0.108	1.164	0.002447	0.032
Prevotellaceae_Prevotellaceae NK3B31 group_uncultured bacterium	4.710	6.855	0.007890	0.038
Lachnospiraceae_Marvinbryantia_uncultured bacterium	0.423	2.086	0.000380	0.039
Family XIII_Family XIII AD3011 group_uncultured bacterium	0.407	1.638	0.004650	0.042
Prevotellaceae_Prevotellaceae NK3B31 group_uncultured bacterium	2.372	4.288	0.008202	0.043
Rikenellaceae_dgA-11 gut group_uncultured Bacteroidales bacterium	7.125	5.284	0.000961	0.052
Spirochaetaceae_Treponema 2_uncultured bacterium	9.352	5.042	0.008830	0.052
Lachnospiraceae_Agathobacter_Eubacterium ramulus	0.319	2.328	0.000002	0.053
Ruminococcaceae_Ruminococcaceae UCG-014_uncultured rumen bacterium	0.876	3.967	0.000042	0.053
Family XIII_Family XIII UCG-001_uncultured Anaerovorax sp.	0.434	2.245	0.000273	0.053
Lachnospiraceae_[Eubacterium] xylanophilum group_uncultured bacterium	2.902	4.840	0.004542	0.056
Ruminococcaceae_Ruminococcaceae UCG-014_uncultured bacterium	0.000	1.577	0.000385	0.063
Ruminococcaceae_Ruminococcaceae UCG-002_uncultured bacterium	5.547	3.190	0.008073	0.073
Clostridium sp. K4410.MGS-306	0.722	2.339	0.002009	0.078
Prevotellaceae_Prevotellaceae NK3B31 group_uncultured bacterium	1.743	3.822	0.009553	0.083
Bacteroidales RF16 group_uncultured bacterium	3.165	7.413	0.000431	0.086
Prevotellaceae_Prevotella 1_uncultured bacterium	4.513	7.902	0.002127	0.094
Lachnospiraceae_[Eubacterium] xylanophilum group_uncultured bacterium	1.634	3.237	0.009995	0.094
Ruminococcaceae_Subdoligranulum_uncultured Faecalibacterium sp.	2.588	5.971	0.000000	0.095
Lachnospiraceae_Anaerostipes_uncultured bacterium	1.273	3.044	0.008009	0.096
Ruminococcaceae_Faecalibacterium_uncultured bacterium	1.887	3.604	0.005124	0.120
Lachnospiraceae_[Eubacterium] xylanophilum group_uncultured bacterium	1.825	3.998	0.000413	0.132
Ruminococcaceae_Ruminiclostridium 6_uncultured bacterium	1.105	2.898	0.000637	0.140
Lachnospiraceae_[Eubacterium] xylanophilum group_metagenome	2.874	5.183	0.000244	0.151
Spirochaetaceae_Treponema 2_uncultured rumen bacterium	3.129	7.735	0.000367	0.151
Ruminococcaceae_Ruminococcaceae UCG-014_uncultured bacterium	3.129	5.045	0.001096	0.153
Prevotellaceae_Prevotellaceae NK3B31 group_uncultured bacterium	2.568	5.848	0.001413	0.153
Ruminococcaceae_Ruminococcus 2_uncultured Ruminococcaceae bacterium	1.183	3.189	0.005639	0.153
Prevotellaceae_Prevotella 1_uncultured rumen bacterium	0.558	1.599	0.006565	0.153
Ruminococcaceae_Ruminococcaceae UCG-010_uncultured bacterium	1.731	0.523	0.008434	0.153
Spirochaetaceae_Treponema 2_Treponema saccharophilum DSM 2985	2.825	7.243	0.000094	0.158
Lachnospiraceae_Lachnospiraceae NK3A20 group_Lachnospiraceae bacterium DJF_VP52	1.529	0.000	0.001219	0.158
uncultured bacterium	0.000	1.132	0.003388	0.158
Lachnospiraceae_Lachnospiraceae NK4B4 group_uncultured bacterium	0.566	1.949	0.008631	0.158
Pirellulaceae_p-1088-a5 gut group_uncultured bacterium	4.333	1.607	0.000200	0.173
Corynebacteriaceae_Corynebacterium_Corynebacterium kutscheri	1.541	0.245	0.000979	0.173
uncultured bacterium	1.397	3.302	0.009477	0.185
Ruminococcaceae_Ruminococcaceae UCG-001_uncultured bacterium	0.103	3.163	0.000058	0.194
Ruminococcaceae_Ruminococcaceae UCG-002_gut metagenome	5.162	3.212	0.000091	0.194
Lachnospiraceae_Butyrvibrio_uncultured organism	0.108	2.859	0.000135	0.194
Ruminococcaceae_Ruminococcus 1_uncultured bacterium	0.000	1.588	0.000280	0.194
Ruminococcaceae_Ruminococcus 1_uncultured bacterium	0.639	2.629	0.000392	0.194
Prevotellaceae_Prevotella 1_uncultured rumen bacterium	1.318	3.082	0.002548	0.194
Rikenellaceae_Rikenellaceae RC9 gut group_uncultured bacterium	1.995	3.961	0.005785	0.194
uncultured rumen bacterium	0.117	0.845	0.007145	0.194
Ruminococcaceae_Ruminococcaceae NK4A214 group_uncultured bacterium	4.252	2.875	0.008648	0.194
Muribaculaceae_uncultured bacterium	0.515	4.470	0.000007	0.196
Ruminococcaceae_Ruminococcus 2_uncultured bacterium	0.094	1.109	0.002651	0.203
Ruminococcaceae_Oscillibacter_uncultured bacterium	2.378	3.873	0.004969	0.203
Lachnospiraceae_Lachnospiraceae UCG-004_uncultured organism	0.227	0.981	0.007989	0.203
Prevotellaceae_Prevotella 2_uncultured bacterium	3.276	5.476	0.005150	0.207

forest model. It is remarkable that these results have been obtained already with the relatively small sample size of this pilot study. Overall, general properties of the gut microbiome (beta diversity, predictive model) have been clearly affected by the dietary tara supplementation, while specific properties have apparently remained unaltered (e.g. alpha diversity indices). This again is probably related to the small size of this study which, though sufficient to uncover broad properties of the microbiome, is not enough to detect finer-grained aspects like differences in specific diversity indices or taxa.

Although the overall effect of tara tannins on the pig gut microbiome is unquestionable, less clear is whether this effect is beneficial or not. The higher F:B ratio may point to positive effects on the fat metabolism (e.g. leaner pig meat), while the lack of effect on lactobacilli may hint at the absence of a negative repercussion of tara extracts on this beneficial class of microbial taxa. Both effects are however not significant, and additional research is needed to elucidate this further.

Declarations

Ethics approval. The experimental trial was approved by the animal welfare committee (OPBA) of the University of Catania (protocol n. 286946).

Funding. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests. The authors have no relevant financial or non-financial interests to disclose.

Data availability. 16S rRNA-gene sequencing data have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute (EBI) with project number PRJEB65759.

References

- Anderson M (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26(1):32–46. URL <http://onlinelibrary.wiley.com/doi/10.1111/j.1442-9993.2001.01070.pp.x/full>
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)* 57(1):289–300
- Biagi G, Cipollini I, Paulicks BR, et al (2010) Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. *Archives of Animal Nutrition* 64(2):121–135
- Biondi L, Randazzo CL, Russo N, et al (2019) Dietary supplementation of tannin-extracts to lambs: effects on meat fatty acids composition and stability and on microbial characteristics. *Foods* 8(10):469

599 Biscarini F, Palazzo F, Castellani F, et al (2018) Rumen microbiome in dairy calves
600 fed copper and grape-pomace dietary supplementations: Composition and predicted
601 functional profile. *PloS one* 13(11)
602
603 Bray JR, Curtis JT (1957) An ordination of the upland forest communities of Southern
604 Wisconsin. *Ecol Monographs* 27(4):325–349. URL [http://onlinelibrary.wiley.com/](http://onlinelibrary.wiley.com/doi/10.2307/1942268/full)
605 [doi/10.2307/1942268/full](http://onlinelibrary.wiley.com/doi/10.2307/1942268/full)
606
607 Breiman L (2001) Random forests. *Machine learning* 45:5–32
608
609 Caporaso J, Kuczynski J, Stombaugh J, et al (2010) QIIME allows analysis of
610 high-throughput community sequencing data. *Nature* 7:335–336. [https://doi.org/](https://doi.org/10.1038/nmeth.f.303)
611 [10.1038/nmeth.f.303](https://doi.org/10.1038/nmeth.f.303), URL <https://www.nature.com/articles/nmeth.f.303>
612
613 Caporaso JG, Lauber CL, Walters WA, et al (2011) Global patterns of 16s rRNA diver-
614 sity at a depth of millions of sequences per sample. *Proceedings of the national*
615 *academy of sciences* 108(Supplement 1):4516–4522
616
617 Caprarulo V, Hejna M, Giromini C, et al (2020) Evaluation of dietary administration of
618 chestnut and quebracho tannins on growth, serum metabolites and fecal parameters
619 of weaned piglets. *Animals* 10(11):1945
620
621 Caprarulo V, Giromini C, Rossi L (2021) Chestnut and quebracho tannins in pig
622 nutrition: The effects on performance and intestinal health. *Animal* 15(1):100064
623
624 Chao A (1984) Nonparametric Estimation of the Number of Classes in a Popula-
625 tion Author. *Scandinavian Journal of Statistics* 11(4):265–270. [https://doi.org/10.](https://doi.org/10.1214/aoms/1177729949)
626 [1214/aoms/1177729949](https://doi.org/10.1214/aoms/1177729949), URL <http://www.jstor.org/stable/4615964>
627
628 Chao A, Lee SM (1992) Estimating the Number of Classes via Sample Coverage.
629 *Journal of the American Statistical Association* 87(417):210. [https://doi.org/10.](https://doi.org/10.2307/2290471)
630 [2307/2290471](https://doi.org/10.2307/2290471), URL [http://www.tandfonline.com/doi/abs/10.1080/01621459.1992.](http://www.tandfonline.com/doi/abs/10.1080/01621459.1992.10475194)
631 [10475194](http://www.tandfonline.com/doi/abs/10.1080/01621459.1992.10475194)<http://www.jstor.org/stable/2290471?origin=crossref>
632
633 Chao A, Ma MC, Yang MCK (1993) Stopping rule and estimation for recapture debug-
634 ging with unequal detection rates. *Biometrika* 80(1):193–201. [https://doi.org/10.](https://doi.org/10.1093/biomet/80.1.193)
635 [1093/biomet/80.1.193](https://doi.org/10.1093/biomet/80.1.193), URL [https://academic.oup.com/biomet/article-abstract/](https://academic.oup.com/biomet/article-abstract/80/1/193/228245)
636 [80/1/193/228245](https://academic.oup.com/biomet/article-abstract/80/1/193/228245)
637
638 Choy YY, Quifer-Rada P, Holstege DM, et al (2014) Phenolic metabolites and sub-
639 stantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins.
640 *Food & function* 5(9):2298–2308
641
642 Dell’Anno M, Hejna M, Sotira S, et al (2020) Evaluation of leonardite as a feed addi-
643 tive on lipid metabolism and growth of weaned piglets. *Animal Feed Science and*
644 *Technology* 266:114519

Fisher RA, Corbet AS, Williams CB (1943) The Relation Between the Number of Species and the Number of Individuals in a Random Sample of an Animal Population. *The Journal of Animal Ecology* 12(1):42–58. <https://doi.org/10.2307/1411>, URL <http://www.jstor.org/stable/1411><http://www.jstor.org/stable/1411?origin=crossref>

Girard AL, Teferra T, Awika JM (2019) Effects of condensed vs hydrolysable tannins on gluten film strength and stability. *Food hydrocolloids* 89:36–43

Girard M, Bee G (2020) Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. *Animal* 14(1):95–107

Girard M, Thanner S, Pradervand N, et al (2018) Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental etec f4 model. *PLoS One* 13(5):e0197878

Huang Q, Liu X, Zhao G, et al (2018) Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Animal Nutrition* 4(2):137–150

Indiani CMdSP, Rizzardi KF, Castelo PM, et al (2018) Childhood obesity and firmicutes/bacteroidetes ratio in the gut microbiota: a systematic review. *Childhood obesity* 14(8):501–509

Jami E, White BA, Mizrahi I (2014) Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PloS one* 9(1):e85423

Jansman A (1993) Tannins in feedstuffs for simple-stomached animals. *Nutrition research reviews* 6(1):209–236

John JA (2011) SeqPrep v1.1 - Tool for stripping adaptors and/or merging paired reads with overlap into single reads. URL <https://github.com/jstjohn/SeqPrep>

Kuhn M, Wickham H (2020) Tidymodels: a collection of packages for modeling and machine learning using tidyverse principles. URL <https://www.tidymodels.org>

Kumar M, Kumar V, Roy D, et al (2014) Application of herbal feed additives in animal nutrition-a review. *International Journal of Livestock Research* 4(9):1–8

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22(13):1658–1659

Makkar HP, Blümmel M, Borowy NK, et al (1993) Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture* 61(2):161–165

Miragoli F, Patrone V, Prandini A, et al (2021) A mixture of quebracho and chestnut tannins drives butyrate-producing bacteria populations shift in the gut microbiota

691 of weaned piglets. Plos one 16(4):e0250874
692
693 Paulson JN, Stine OC, Bravo HC, et al (2013) Differential abundance analysis
694 for microbial marker-gene surveys. Nature methods 10(12):1200–2. <https://doi.org/10.1038/nmeth.2658>, URL <https://www.nature.com/articles/nmeth.2658><http://dx.doi.org/10.1038/nmeth.2658>, arXiv:NIHMS150003
695
696
697 Perricone V, Comi M, Giromini C, et al (2020) Green tea and pomegranate extract
698 administered during critical moments of the production cycle improves blood anti-
699 radical activity and alters cecal microbial ecology of broiler chickens. Animals
700 10(5):785
701
702 R Core Team (2023) R: A Language and Environment for Statistical Comput-
703 ing. R Foundation for Statistical Computing, Vienna, Austria, URL <https://www.R-project.org/>
704
705
706 Rastmanesh R (2011) High polyphenol, low probiotic diet for weight loss because of
707 intestinal microbiota interaction. Chemico-biological interactions 189(1-2):1–8
708
709 Shannon C (1948) A Mathematical Theory of Communication, The Bell
710 System Technical Journal. The Bell System Technical Journal pp 379–
711 427. URL [https://scholar.google.it/scholar?q=shannon+mathematical+theory+](https://scholar.google.it/scholar?q=shannon+mathematical+theory+of+communication+bell&btnG=&hl=it&as{ }sdt=0{ }2C5)
712 [of+communication+bell{&}btnG={&}hl=it{&}as{ }sdt=0{ }2C5](https://scholar.google.it/scholar?q=shannon+mathematical+theory+of+communication+bell&btnG=&hl=it&as{ }sdt=0{ }2C5)
713
714 Simpson EH (1949) Measurement of diversity. Nature 163:688. [https://doi.org/10.](https://doi.org/10.1038/163688a0)
715 [1038/163688a0](https://doi.org/10.1038/163688a0), URL <http://psycnet.apa.org/psycinfo/1950-02238-001>
716
717 Smith B, Wilson JB (1996) A Consumer’s Guide to Evenness Indices. Oikos 76(1):70.
718 <https://doi.org/10.2307/3545749>, URL [https://scholar.google.it/scholar?hl=it{&}as{ }sdt=0{ }2C5{&}q=A+Consumer{ }27s+Guide+to+Evenness+Indices{&}btnG=http://www.jstor.org/stable/3545749?origin=crossref](https://scholar.google.it/scholar?hl=it&as{ }sdt=0{ }2C5{&}q=A+Consumer{ }27s+Guide+to+Evenness+Indices{&}btnG=http://www.jstor.org/stable/3545749?origin=crossref)
719
720
721 Sotira S, Dell’Anno M, Caprarulo V, et al (2020) Effects of tributyrin supplementation
722 on growth performance, insulin, blood metabolites and gut microbiota in weaned
723 piglets. Animals 10(4):726
724
725 Stojanov S, Berlec A, Štrukelj B (2020) The influence of probiotics on the fir-
726 micutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel
727 disease. Microorganisms 8(11):1715
728
729 Tretola M, Maghin F, Silacci P, et al (2019) Effect of supplementing hydrolysable
730 tannins to a grower–finisher diet containing divergent pufa levels on growth per-
731 formance, boar taint levels in back fat and intestinal microbiota of entire males.
732 Animals 9(12):1063
733
734
735
736

Turnbaugh PJ, Ley RE, Mahowald Ma, et al (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444(7122):1027–31. https://doi.org/10.1038/nature05414 , URL https://www.nature.com/nature/journal/v444/n7122/abs/nature05414.html http://www.ncbi.nlm.nih.gov/pubmed/17183312 , arXiv:t8jd4qr3m [13960]	737 738 739 740 741 742
Valenti B, Campidonico L, Natalello A, et al (2021) Fatty acid metabolism in lambs supplemented with different condensed and hydrolysable tannin extracts. Plos one 16(10):e0258265	743 744 745 746
Wang M, Huang H, Hu Y, et al (2020) Effects of dietary microencapsulated tannic acid supplementation on the growth performance, intestinal morphology, and intestinal microbiota in weaning piglets. Journal of Animal Science 98(5):skaa112	747 748 749
Wright MN, Ziegler A (2017) ranger: A fast implementation of random forests for high dimensional data in C++ and R. Journal of Statistical Software 77(1):1–17. https://doi.org/10.18637/jss.v077.i01	750 751 752 753
Yilmaz P, Parfrey LW, Yarza P, et al (2014) The SILVA and “all-species living tree project (ltp)” taxonomic frameworks. Nucleic acids research 42(D1):D643–D648	754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782