

Metabolomic and microbial changes reveal the biotransformation of *Calotropis gigantea* into silage

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Abstract

Background: We recently reported that *Calotropis gigantea* could be used as a potential functional feed additive to specifically inhibit the detrimental rumen protozoa without impairing the fermentation traits. Meanwhile, to ensure the applicability at the farm level, bio-transforming *Calotropis gigantea* (giant milkweed, GM) into silage is of an utmost requisite which constitutes a long-term biological preservation. This study aimed at investigating the metabolite and microbiota profiles that can lead to the bio-transformation of *Calotropis gigantea* into silage, after supplementing fermentative bacteria and sucrose.

Results: After ensiling, several metabolites like 3,4'-dihydroxybenzoic acid ethyl ester, 2-hydroxyethylphosphonic acid, 3,4'-dihydroxy-3',5'-dimethoxypropiofenone, vnilloylmalic acid, sedoheptulose, 2-hydroxy-3,5-dinitrobenzoic acid, L-arginine, putrescine, methyl linolenate and calactin were up-regulated while other like 2'-o-methyladenosine, xanthosine, 2-hydroxy-2-methyl propyl glucosinolate and isopentenyl adenine-7-N-glucoside were down-regulated making GM ensiling a biological process to manipulate the metabolite composition and structure for therapeutic needs. This was possible after the colonization by bacteria species like *Bacteriodes salanitronis*, *B. plebeius*, *B. barnesiae*, *B. vulgatus*, *B. caecicola*, *Prevotella copri*, *Megamonas hypermegale*, *Olsenella sp.* which increased in ensiling samples with *Lactobacillus buchneri* specifically found only in ensiled and inoculated samples. The "biosynthesis of secondary metabolites" was the KEGG pathway induced by the highest number of studied GM metabolites. PICRUST2 identified the "brite hierarchies" as the more expressed microbial functional group and "human diseases and organismal systems" the least expressed one.

Conclusion: These findings provide a fundamental description of the microbiota colonizing the plant GM for a successful ensiling process that induced a remarkable metabolomic changes. The cause and effect relationship predicted several metabolic pathways and the contribution of the microbiota profile to the biosynthesis of functional metabolites. Understanding the specific mechanisms modulated by the colonizing bacteria and fungi underpinning the bio-transformation into silage deserves further studies.

Background

The majority of natural plants are considered a source of novel biologically active compounds that can lead to metabolic interventions as new therapeutics [1-2]. The recent finding of a powerful antimalarial drug, derived from *Artemisia annua* L. (sweet wormwood) [3] is recommended by the World Health Organization to cure malaria disease, caused by the *Plasmodium falciparum* parasite [4]. Using high throughput screening, a variety of phytochemicals can be searched for their chemical, bioactivity and ethnobotany information (<https://phytochem.nal.usda.gov/phytochem/search/list>; 16 August 2021) and for their potential targets of proteins and nucleic acid (<https://db.idrblab.org/ttd/>; 16 August 2021) [5].

In an *in vitro* study, *Calotropis gigantea* (giant milkweed, GM) leaf compounds have been recently found for the first time as inhibitor(s) of the detrimental rumen protozoa *Entodinium*, and of the microbial alpha-glucosidase enzyme, all known as the culprit of feed inefficiency and ruminal acidosis respectively [6-7]. Indeed, exciting results from that study revealed the natural bioactives from GM dried plant leaves could inhibit 41% of the detrimental rumen protozoa *Entodinium* while in contrast, the concentration of some volatile fatty acids (VFA) increased, methane and wasteful ammonia concentration reduced by 12% and 50%, respectively. Partial screening of the plant leaves indicated the presence of 5884.29 µg/g of quercetin 1121.77 µg/g of anthraquinone, 1020.31 µg/g of 3-hydroxybenzoic acid, 451.31 µg/g of myricetin. These phytochemicals have demonstrated antioxidant, anti-inflammatory, antidiabetic [8] and antimicrobial activities, especially against pathogenic gram-negative bacteria [9].

In addition, the plant can also be valorized in the textile industry because the fruits contain high-quality fiber. GM fibers comprise up to 80–90% hollow structures, similar to kapok (*Ceiba pentandra*) fibers [10] thus, they exhibit outstanding hydrophilic or oleophilic properties [11], fewer natural curls in the longitudinal direction, lighter weight [12] resulting in a smoother and softer surface. Moreover, GM leaves are used for fractionating the raw milk and making traditional cheese with positive effects on *E. coli*, yeast and mold load [13]. Therefore, GM is considered a multipurpose plant that can grow on dry land, without fertilizer constraints. The plant is native to South-East and continental Asia introduced in the pacific islands, Australia, Central and Northern South America and Africa [14-15]. In animal husbandry, transforming the fresh forage or fodder into silage enables the storage or preservation of a huge amount of feed without altering the quality. Previous attempts aiming at transforming GM into silage failed, mainly due to the non-strictly anaerobic conditions provided by either the silo or the cans used for ensiling. Therefore this study hypothesized that performing new ensiling can technology designed with the compactor to provide strictly free-O₂ could maintain appropriate conditions for the microbial fermentation of GM. Microbial and metabolomic characterizations will enable an understanding of the underpinning mechanism of GM ensiling processes. In other words, understanding the microbiome mutations colonizing the plant for new biological functions and interactions that lead to successful mechanism of bio-transformation or ensiling processes is of crucial importance.

The metabolite concentration and composition of the ensiled plant can change its initial functional role. Therefore, characterizing the metabolome still unknown remains an utmost requisite not only for the silage quality but also for the animal metabolism, health and welfare [16]. In absence of any successful GM ensiling procedure, testing different treatments either with sucrose and/or inoculant bacteria will enable the identification of the appropriate ensiling additives [17]. The inoculation of lactic acid bacteria (LAB) are often applied as additives before ensiling. Homofermentative strain members of *Lactobacillus* can rapidly produce lactic acid, which lower the silage pH and help to preserve the forage's nutritional value [18]. As far as we know, no studies explored the cause and effect relationships between the microbial composition or structure and some KEGG metabolome pathways after bio-transforming GM into silage.

Results

Nutritional analysis of ensiled and non-ensiled GM

The nutritional traits of GM were not altered with the ensiling processes but some numerical changes were rather observed with the treatments (Table 1). Crude proteins increased with the treatment D1+i and D2+i. No matter the initial DM (D1 or D2), the protein content increased when adding the inoculant compared to when adding the sucrose. DM immediately after ensiling did not change compared to the respective initial DM. The treatment D1+i had the highest protein content and was therefore selected for further metabolomic and sequencing analysis. pH value decreased and was finally maintained within the normal range of acidity for ensiling. Except for D1+S that increased the temperature, the other treatments were maintained. To further understand the effect of the inoculant on ensiling, sample 1, sample 2 and sample 3 standing respectively for ensiled treatment (D1+i), non-ensiled treatment and inoculant were investigated for the metabolomic analysis.

Table 1: Nutritional traits of ensiled and non-ensiled GM

Trt	CP (%)	NDF(%)	ADF(%)	Ash(%)	DM*(%)	DM#(%)	pH	Temp. (°C)
NE GM	13.89±0.6 ^{bc}	39.81±3.0 ^{bc}	24.46±1.5 ^{bc}	13.98±0.49 ^a	NA	94.58±0.62 ^a	7.75±0.35 ^a	NA
D1	14.06±0.19 ^b	43.62±3.4 ^a	27.11±0.72 ^a	13.03±0.02 ^b	50.87±2.01 ^b	90.87±0.46 ^{bc}	4.14±0.02 ^c	20.0±1.35 ^b
D1+i	15.43±0.22 ^a	40.65± 3.2 ^b	25.73± 0.92 ^b	13.38±0.13 ^b	51.07±1.73 ^b	91.38±0.83 ^b	4.28±0.03 ^c	19.9±1.35 ^b
D1+S	14.6±0.73 ^b	38.66±3.1 ^c	24.52±1.51 ^{bc}	13.20±0.38 ^b	50.58±1.93 ^b	92.13±1.01 ^{ab}	4.28±0.03 ^c	21.6±1.21 ^a
D1+i+S	14.12±0.18 ^b	39.60±4.9 ^b	25.95±2.91 ^b	13.25±0.07 ^b	50.89±1.88 ^b	91.51±076 ^b	4.26±0.01 ^c	19.9±1.14 ^b
D2	13.46±0.56 ^c	36.58±2.7 ^c	23.89±2.76 ^c	13.60±0.12 ^{ab}	59.87±0.45 ^a	88.46±1.01 ^c	5.38±0.41 ^b	20.6±1.41 ^b
D2+i	15.13±0.17 ^a	/	/	13.33±0.49 ^b	61.17±0.53 ^a	89.88±0.26 ^c	5.35±0.14 ^b	20.4±1.01 ^b
D2+S	13.92±0.43 ^{bc}	41.76±4.4 ^{ab}	26.65±1.12 ^{ab}	12.87±0.16 ^{bc}	61.00±0.49 ^a	89.60±0.44 ^c	5.26±0.35 ^b	19.7±1.02 ^b
D2+i+S	14.02±1.0 ^b	33.01±0.7 ^d	21.17±1.56 ^d	12.67±0.67 ^c	60.77±0.51 ^a	88.65±1.27 ^c	5.41±0.21 ^b	19.9±1.03 ^b

Trt: treatment, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber, Temp.: temperature, DM: dry matter, *: DM immediately after 60 days ensiling, #: DM after 48 h oven drying at 65° C and 4 h at 105° C, NE GM: non-ensiled GM, D1: DM 50 %, D2: DM 60 %, D1+I: DM 50 %+inoculant, D2+I: DM 60 %+inoculant D1+I+S: DM 50 %+inoculant+sucrose, D2+I+S: DM 60 %+inoculant+sucrose, D2+S: DM 60 %+sucrose, D1+S: DM 50 %+sucrose. NA: Not applicable, Different superscripts within columns denote a significant difference, $P \leq 0.05$.

Metabolomic profile of GM, ensiled GM and inoculant bacteria

The flavonoids, lipids, organic acid, nucleotides and derivatives content decreased in the ensiled GM compared to the non-ensiled GM. Lignans and coumarins, terpenoids and steroids were balanced while phenolic acids, amino acids and derivatives, alkaloids and quinones increased (Fig. 1a). The inoculant bacteria metabolites compared to GM, recorded a lower

X-axis indicates the sample name and the Y-axis are the class of metabolites. Sample 1, 2 and 3 are respectively the ensiled GM, the non-ensiled GM and the inoculant bacteria with 3 replications. Group indicates sample groups. Z-Score indicates the relative quantification of each metabolite with red representing higher content and green representing lower content.

content of flavonoids, lipids, phenolic acids, lignans and coumarins, terpenoids, steroids and quinones but a higher amino acid and derivatives compared to the non-ensiled GM (Fig. 1b). A detailed hierarchical cluster analysis of the metabolites at a lower level, before and after GM ensiling is shown in Figure S1.

K-Means and Venn diagram analysis of differential metabolites in the treatment groups

K-Means analysis (Fig. 2) examined the trend of relative quantification changes of metabolite sub-classes with different treatments or sample groups. A total of 10 sub-classes based on metabolic trends across the treatments were represented. Sub-class 1 recorded 77 metabolites with the same tendency along with sample 1 and 2 but the highest relative quantification or abundance in sample 3. Sub-classes 3, 4, 5, 8 and 9 recorded respectively 78, 69, 100, 69 and 64 metabolites with the lowest abundance in sample 3. Sub-class 2 with 282 metabolites and sub-class 5 with 100 metabolites have the highest abundance in sample 2. After fermentation (sample 1), metabolites from sub-classes 3, 4, 6 and 8 have been found with the highest abundance.

The X-axis represents the sample names and the Y-axis represents the normalized relative quantification. "Sub Class" represents a group of metabolites with the same trend and the number represent the number of metabolites in this cluster. K-Means is performed based on the Z-score normalized relative quantification value.

Venn diagram revealed that a major portion of the metabolites were overlapped among the different treatments, whereas few metabolites were stage-specific (Fig. 3). For instance, only 33, 60 and 23 metabolites were specific to samples 3, 2 and 1 respectively. The other interconnected metabolites were detected among all the different samples. GM, ensiled GM and inoculant display non negligible metabolic differences that remained to be characterized at a lower level.

Principal component analysis of the treatment groups

The principal component analysis (PCA) presented three distinct clusters (inoculant bacteria, non-ensiling plant and QC samples) observed along PC1, which represented 52.94% of the variation among the samples (Fig. 4), and suggested the level of metabolome change during ensiling. The ensiling plant and the inoculant bacteria were separated along PC2 with 38.09% of the variation. The separation between the ensiling and non-ensiling plant was not observable in this 2-dimensional PCA, neither along PC1 nor along PC2.

Dynamic distribution of the top 10 metabolites content

The top 10 up-regulated and down-regulated expressed metabolites were distributed in 3 classes or categories of fold-change $\log_2$ FC values (Fig. 5). 3,4'-dihydroxybenzoic acid ethyl ester, 2-hydroxyethylphosphonic acid, 3,4'-dihydroxy-3',5'-dimethoxypropiphenone, vnilloylmalic acid constitute the first category with an up-regulation rate ranging from 13 to 14 $\log_2$ FC. Sedoheptulose, 2-hydroxy-3,5-dinitrobenzoic acid and L-arginine were expressed at a rate of 16 to 17 $\log_2$ FC while putrescine, methyl linolenate and calactin were expressed at the highest rate of 18 to 19 $\log_2$ FC. Meanwhile, among the top 10 down-regulated expressed metabolites, 2'-o-methyladenosine was the most down-regulated metabolite with -19.5 $\log_2$ FC. Xanthosine, 2-hydroxy-2-methyl propyl glucosinolate and isopentenyl adenine-7-N-glucoside have a similar down-regulated $\log_2$ FC value around -17. The other down-regulated metabolites were around -16 (Fig. 5). The chemical formula, class and CAS numbers of the top 10 differential metabolites are presented in Table S1.

X-axis refers to $\log_2$ FC values of top differential metabolites, the Y-axis refers to metabolites. Red bars represent up-regulated differential metabolites and green bars represent down-regulated differential metabolites.

Spearman correlation analysis

The inter-relationship between metabolites has been studied through a Spearman rank's correlation (Fig. 6) and a Pearson's correlation (Fig. S3) that revealed a synergistic or antagonistic relationship among the metabolites. The negative relationship between the down-regulated and the up-regulated metabolites on the one hand, and a positive relationship either between the up-regulated metabolites or between the down-regulated metabolites were observed. When the metabolism of 3,4'-dihydroxy-3',5'-dimethoxypropiofenone increased, the one of xanthosine decreased while L-arginine rather increased.

KEGG enrichment analysis of differential metabolites

Biological pathways and functions are made possible with the contribution of a set of metabolite populations that vary with the pathways (Fig. 7). Therefore, the highest number of metabolites (70), were involved in the "biosynthesis of secondary metabolites" pathway. ABC transporters, biosynthesis of cofactors and biosynthesis of amino acids have the same number of metabolite contributions (30). A small number of metabolites (5) contributed to the thiamine metabolism, sphingolipid metabolism, isoquinoline alkaloid biosynthesis and carbon fixation in photosynthetic organisms. The distribution of metabolites inducing different KEGG pathways related to metabolism, genetic information processing and environmental information processing is detailed in Figure S2.

Microbiota composition and structure in ensiled and non-ensiled GM plant

The bacterial profiles differed to a greater extent at lower (e.g., specie) than at higher (e.g., class) taxonomic ranks with a decrease of the relative abundance at a lower taxonomic rank (Fig. 8). The relative abundance of the majority of identified bacteria increased with ensiling. Indeed, at the class level, *Bacteroidia*, *Clostridia*, *Negativicutes*, *Coriobacteriia*, *Anaerolineae* and others increased with ensiling while *Cyanobacteria* and *Alphaproteobacteria* decreased. At the family level, *Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Rikenellaceae*, *Prevotellaceae*, *Oxillospiraceae*, *Acidaminococcaceae* and others increased in ensiled samples but *Rhizobiaceae* and *Sphingomonadaceae* decreased. At the genus level, *Bacteroides*, *Faecalibacterium*, *Rikenellaceae_RC9_gut_group*, *Phascolarctobacterium*, *Megamonas*, *Prevotella* increased with ensiling while *Aureimonas* and *Pseudomonas* decreased. At the specie rank, *Bacteriodes salanitronis*, *B. plebeius*, *B.*

barnesiae, *B. vulgatus*, *B. caecicola*, *Prevotella copri*, *Megamonas hypermegale*, *Olsenella sp.* and others increased with ensiling processes. *Sphingomonas paucimobilis* was found only in the non-ensiled plant while *Lactobacillus buchneri* was specific to the ensiled plant. The ensiled samples were free of fungi and mold populations meanwhile, a diversity was observed in non-ensiled samples with *Lasiodiplodia margaritacea* the most represented specie belonging to the *Batrachosphaeriaceae* family and *Dothideomycetes* class (Fig. 9).

Only the top 10 taxons of abundance are displayed on the bar chart. The remaining proportions to get 100% on each bar were unclassified taxons.

Only the top 10 taxons of abundance are displayed on the bar chart. The remaining proportions to get 100% on each bar were unclassified taxons.

Phylogenetic analysis of the ensiled and non-ensiled microbiota

To further understand the relationships among the bacteria, a phylogenetic tree was constructed using representative sequences of bacterial genera (Fig. 10a) and fungi and mold genera (Fig. 10b). The tree enabled to observe the phylogenetic distance between the studied microbiota. Among the *Firmicutes*, four sub-groups of bacteria were genetically individualized with *UCG-004* their common ancestor. Individual like *Shuttleworthia* was similar with *agathobacter* than with *Erysipelotrichaceae_UGG-003*.

Within the *Proteobacteria* group, *Pseudomonas* is the parent of all the members but is also very close to the member of another group, *Desulfobacteriota* (*Desulfovibrio*). The members of *Bacteroidota* can be separated into three sub-groups of similar genotypes. *Methanosaeta* had the highest phylogenetic distance among all the studied bacteria. The fungi and mold tree was predominated by the *Ascomycota* members, with *Tremateia*, *Kodamaea*, *Metschnikowia*, and *Candida* genetically very far from the other *Ascomycota* but closer to the *Basidiomycota* members. *Mucoromycota* was the least represented group.

Functional prediction

The relative abundance of KEGG pathways was predicted using PICRUSt2, based on representative sequences and categorized according to groups of functional roles (Fig. 11). The more expressed functional group was related to the "brite hierarchies" while the least one was related to "human diseases and organismal systems". The relative abundance of the pathways related to "signal transduction and membrane transport" increased with the non-ensiling while "translation" and "replication and repair" increased with ensiling. Within the human diseases functional group, the ensiling process reduces the relative abundance of "infectious viral diseases", "neurodegenerative diseases" and "cancer overview". Moreover, within the metabolism group, the relative abundances of "xenobiotics biodegradation and metabolism", "metabolism of cofactors and vitamins", "energy metabolism" and "metabolism of other amino-acids" increased in the non-ensiled plant. "Nucleotide metabolism", "glycan biosynthesis and metabolism" and "carbohydrate metabolism" were increased with ensiling.

Cause and effect relationships: Network correlation between the microbiota and the differential plant metabolites

A network correlation analysis enabled to characterize the interaction between the most changing metabolites and the microbiota profiles (Fig. 12). *Sphingomonas paucimobilis* is positively correlated to isopentenyl adenine-7-N-glucoside and 5-hydroxymethylfurfural. *Bacteriodes vulgatus* was positively correlated to L-arginine and 3,4'-dihydroxy-3',5'-dimethoxypropiofenone and negatively correlated to medioresinol-4'-O-(6'''-acetyl)glucoside, 8-(benzyloxy)-1-naphthol, 2-(2-phenylethyl)chromones and xanthosine. *Bacteriodes plebeius* and *Prevotella copri* are positively correlated to L-arginine, 3,4'-dihydroxy-3',5'-dimethoxypropiofenone and vniiloylmalic acid. The dynamic of most of the metabolites was explained by the contribution of *Lactobacillus buchneri*, *Bacteriodes salanitronis*, *Bacteriodes barnesiae*, *Bacteriodes caecicola* and *Olsenella sp.* that were correlated to most of the studied metabolites and could serve in their synthetic forms as inoculant to manipulate the metabolic profile of GM plant. Fungi and mold contributed very little to the metabolites dynamic, except for *Filobasidium oeirense* and *Rhizopus arrhizus* which both contributed to up and down-regulation.

In the network diagram, bacteria are represented by turquoise circles, fungi are represented by bright coral circles, and metabolites are represented by cornflower blue circles; positive correlations are represented by red solid lines, and negative correlations are represented by gray solid lines; the size of the circle indicates connectivity, and the thickness of the line indicates correlation. The correlation filtering threshold: $|\text{cor}| > 0.5$, $p\text{-value} < 0.05$

Discussion

By building a new technology of strictly anaerobic can silage and supplementing *Lactobacillus* bacteria to GM, we investigated the metabolome and microbiota profile that led to a successful ensiling of GM. The higher metabolites content found with ensiled GM might be the result of the cumulative effect from the inoculant bacteria metabolites while the lower content might be explained by the microbial breakdown or biodegradation. Xu et al. [16] also found increased amino-acids and derivatives with sainfoin silage supplemented by the fermentative bacteria additives, *Lactobacillus plantarum*. The changes observed on the metabolic profile indicated a remarkable bio-transformation of the metabolic structure either as biodegradability or as biosynthesis of the compounds. Therefore, ensiling GM plant can be seen as a perspective to naturally manipulate the metabolome according to specific therapeutic functionality.

The top 10 up and down-regulated metabolites content may induce specific phenotypes according to the biological responses linked to the metabolite dynamics and networking. Therefore, 5-hydroxymethylfurfural which was down-regulated by -15.82 was reported as genotoxic, an indirect mutagen, carcinogen and causes DNA-damage inhibiting DNA polymerase γ and is nephrotoxic [19] while the up-regulated 2-(2-phenylethyl)chromones are correlative with anti-diabetes, anticancer, antioxidant, antibacterial and anti-inflammatory activities [20]. Supplementing L-arginine enhances endothelial nitric oxide production and improves endothelial function in the setting of atherothrombosis through increased intracellular uptake [21]. 7-O-galloyl-D-sedoheptulose, exhibited lowering effect of elevated triglyceride, oxidative stress and advanced glycation endproduct (AGE) formation in the kidney of db/db mice [22]. The effects of 7-O-galloyl-D-sedoheptulose were mediated through modulation by renal sterol regulatory element binding proteins and nuclear factor-kappa B expression. The other metabolites have very limited information on their bioactivity and deserve to be further investigated. Meanwhile, glucosinolate could be a 2-hydroxy 2- methylpropylglucosinolate derivative that has been previously reported to interfere with iodine uptake (thiocyanate ion) and the synthesis of thyroid hormones triiodothyronine (T3) and plasma thyroxine (T4) (5-vinylloxazolidine-2 thione), leading eventually to hypothyroidism and enlargement of the thyroid gland (goitre). Clinical signs of toxicity observed in food-producing animals include growth rate reduction, performance decreasing (milk and egg production), impaired reproductive activity, liver and kidney functions leading to nitrile formation [23].

The biosynthesis of secondary metabolite pathways which was regulated by high amount of metabolites suggested that there should be a synergistic role among the metabolites that lead to the biosynthesis of more metabolites. The ABC transporters are one of the key

pathways involving proteins that mediate import and export processes acting as ion channels or regulators ending in biochemical fluxes of purification, assimilation or excretion [24]. Previous research on ABC transporters showed that they were involved in many biological functions, such as transport of ions, amino acids, nucleotides, polysaccharides, and peptides; bacterial drug resistance; pheromone secretion; and detoxification of heavy metals [25-26-27].

Sphingomonas paucimobilis is a strictly aerobic Gram-negative bacteria that could not be found in strictly anaerobic ensiling conditions. *S. paucimobilis* is a more important pathogen than previously thought [28]. Meanwhile, *Bacteroides salanitronis* is an anaerobic species that could only be found in ensiling conditions and had been reported to positively impact poultry farming [29]. *Bacteroides* are involved in many metabolic activities in the human colon including fermentation of carbohydrates, utilization of nitrogenous substances, and biotransformation of bile acids and other steroids [30]. *Lactobacillus buchneri* was definitely derived from the inoculant supplementation during ensiling that is known to produce lactic acid, unfavorable for the aerobic taxons. Even though *Lactococcus lactis* was applied, it could not be found during the analysis and so its activity to the ensilage process is likely to be minimal. *Lactococcus* was also found lower in ensiled Napier grasses compared with the fresh plant [31]. The success of ensiling process was indicated by the absence of molds and fungi. There is no available data describing the microbiota colonization of GM but Wang et al. [32] showed that the predominant microorganisms of another plant *Pyropia haitanensis* were bacteria (98.98%) and the phylum with the highest abundance were *Proteobacteria* (54.64%), *Bacteroidetes* (37.92%), *Erythrobacter* (3.98%) and *Hyunsoonleella jejuensis* (1.56%). Microbiota with high genetic similarity observed based on phylogenetic tree may play a mutually reinforcing role in the plant colonization but the one with dissimilarities may justify the need for diverse functions in the ensiling processes. However, stronger pieces of evidence are needed to justify this phenomenon. A stable microbial community is a key factor that contributes to maintaining the growth, development and biological functions in plants. Bacterial community comprising members of *Proteobacteria*, *Firmicutes*, and *Actinobacteria* are functionally closely related to the host plant [33]. Very limited molecular research has been done on GM and the characterization of genes function especially after ensiling, has not been previously investigated. Translation is one of the key metabolic pathways controlling the biosynthesis of proteins and the expressed traits.

Conclusion

Ensiling under a strictly anaerobic condition using an adapted can silage ensures a good quality of *Calotropis gigantea* silage which is a biological approach to preserve the fodder quality for livestock. Several plant metabolites were up-regulated and some known for their side effect were down-regulated. Aerobic bacteria were colonizing the non-silage plant while strictly anaerobic bacteria predominated in ensiled plant with *Lactobacillus buchneri* found only in samples supplemented with inoculant bacteria. A lot of metabolites were identified but some of them are still unknown with regard to their biological function and metabolism.

Materials And Methods

New design of O₂-free compactor cans silage

The cans silage or fermentation tank (Figure S4) were built with a compressor plate (12) set on the top of the tank lid, sliding towards the bottom of the tank. The compression was ensured by a mobile and perforated plate (13) providing pressure and air removal from the tank. The compression was guided by an outer steeling device comprising a handle (1), a screw (2), a N-shaped bracket (3), a nut (4) and a bottom plate. The lower part of the tank is provided with a hole plate (8), a funnel arranged below the hole plate (9), and a water outlet (10) arranged at the bottom of the tank, led by a funnel. The tank can finally be closed with a solid lid (11), not enabling air circulation. The can was finally mounted with all the components (14) including the plant inside, with a real time thermometer (15). The fermentation tank has high compaction density, good airtightness and can retain exudate, the structure is simple, durable, reusable, low cost, easy to operate and handle.

Plant collection and ensiling

GM leaves and stems, previously identified by Prof. Liu Yi, a botanist of the Herbarium Botanical Institute of Kunming, Chinese Academy of Sciences were collected in Honghe, located in the southwest Yunnan Province, China. The plant was selected based on its demonstrated potential as a new feed additive for ruminants [6-7]. In absence of an air-force oven that could help accurately measure in the field the drying temperature of a huge quantity of plants, the sunlight was used for one and two days to get the two different dry matters (50% and 60%) of the plant material used for ensiling. After each dry matter (DM), the leaves and stems were mechanically chopped to a maximum size of 2 cm. Treatments assigned in a complete randomized design consisted of DM 50 %, DM 60%, mixture either with the inoculant (I) or with sucrose (S) (Table 2).

Table 2 Giant milkweed treatments for ensiling

Treatments	DM 50%	DM 60%	Inoculant	Sucrose
D1	+	-	-	-
D1+I	+	-	+	-
D1+S	+	-	-	+
D1+I+S	+	-	+	+
D2	-	+	-	-
D2+I	-	+	+	-
D2+S	-	+	-	+
D2+I+S	-	+	+	+

DM: dry matter, D1: DM 50 %, D2: DM 60 %, D1+I: DM 50 %+inoculant, D2+I: DM 60 %+inoculant D1+I+S: DM 50 %+inoculant+sucrose, D2+I+S: DM 60 %+inoculant+sucrose, D2+S: DM 60 %+sucrose, D1+S: DM 50 %+sucrose. Each treatment had three replications. The inoculant (SiloSolve FC, CHR HANSEN) application rate was as follow: 2g/tons (DSM11037/1K2087 ; *Lactococcus lactis* O-224 ; $\geq 6.02 \times 10^{10}$ cfu/g and DSM225020/ 1K20738; *Lactobacillus buchneri* ; $\geq 6.02 \times 10^{10}$ cfu/g) while sucrose application rate was 1% GM weight, 10g /Kg GM.

Briefly, stock solutions of 1g/400 mL and 1g/mL were respectively prepared for the inoculant and sucrose application. Then, a 10 mL sprayer was used to spray 10 mL of inoculant (2g/tons) all over a sample of 1Kg GM before mixing thoroughly and adding 10 mL of sucrose (10g/Kg) with a sprayer. The treatment without inoculant and/or sucrose was sprayed with 20 mL or 10 mL of pure H₂O/Kg.

A total of 3Kg were filled and an aerobically compacted then closed in each ensiling can for 60 days. The temperature was measured each week using an adapted real-time thermometer device (RC-4HC, Elitech 2528 Qume Dr. Ste 2, San Jose, CA 95131) with a probe directly inserted inside the can.

Nutritional analysis

After 60 days of ensiling, the cans were opened and 100g were sampled, oven-dried for 48h at 65°C, then at 105°C for 4h. The plant was then ground up to pass through a 2 mm mesh for nutritional analysis. Nutritional analysis was conducted using standard methods [33] for dry matter (DM), ash, crude protein, and ether extract. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the methods outlined by Goering and Van Soest [35]. A sample of 20g of fresh silage was squeezed with 180mL distilled water using a blender at a higher speed for 30 sec [16]. The extracted product was filtered through four layers of cheesecloth and pH was measured using a glass electrode pH meter. The extracted juice was aliquoted for microbial DNA extraction and gene sequencing.

Metabolomic analysis

Crude extraction

The ensiled plant with the treatment that recorded the highest protein value (sample 1), indicating the best treatment, the non-ensiled plant (sample 2), and the inoculant bacteria (sample 3), were the three samples further analyzed for metabolomic analysis. The extraction procedure was adapted from Jia et al. [36]. Indeed, the samples were freeze-dried using a vacuum freeze-dryer (Scientz-100F). The freeze-dried samples were pulverized using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. About 50 mg of lyophilized powder was dissolved with 1.2 mL 70% methanol solution and vortexing 30 sec every 30 minutes 6 times, then stored at 4°C overnight. The extract was centrifuged at 12000 rpm for 10 min and filtered using a 0.22µm filter (SCAA-104, ANPEL, Shanghai, China, <http://www.anpel.com.cn/>) before UPLC-MS/MS analysis.

Chromatography-mass spectrometry acquisition conditions

Ultra Performance Liquid Chromatography (UPLC) (SHIMADZU Nexera X2, <https://www.shimadzu.com.cn/>) and tandem mass spectrometry (MS/MS) (Applied Biosystems QTRAP 4500, <http://www.appliedbiosystems.com.cn/>) were used to screen the metabolites [37-38]. Liquid phase conditions consisted of chromatographic column: Agilent SB-C18 1.8 µm, 2.1 mm * 100 mm; mobile phase: A phase with ultrapure water (0.1% formic acid added), B phase with acetonitrile (0.1% formic acid added); elution gradient: 0.00 min. The

proportion of B phase was 5%, within 9.00 min, increased linearly to 95%, and remained at 95% for 1 min, 10.00-11.00 min, while the proportion of B phase decreased to 5%, and balanced at 5% up to 14 min. The flow rate was 0.35 mL/min; 40 °C of column temperature and 4 µL of injection volume. The mass spectrum conditions were as follows:

Linear Ion Trap (LIT) and Triple Quadrupole (QQQ) scans were obtained on a triple quadrupole LIT Mass Spectrometer (Q TRAP) (AB4500 Q TRAP UPLC/MS/MS system). This system is equipped with an ESI Turbo ion spray interface and both positive and negative ion modes can be controlled by Analyst 1.6.3 software (AB Sciex). The operating parameters of the ESI source were as follows: an ion source, turbine spray; source temperature 550°C; ion spray voltage (IS) 5500 V (positive ion mode) /-4500 V (negative ion mode); The ion source gas I, gas II and curtain gas were set to 50, 60 and 25.0 psi respectively, and the collision-induced ionization parameter was set to high. Instrument tuning and quality calibration were performed with 10 and 100 µmol/L polypropylene glycol solution in QQQ and LIT mode, respectively. QQQ scan used multiple reaction monitoring (MRM) mode and collision gas (nitrogen) was set to medium. A specific set of MRM ion pairs was monitored at each period based on the eluted metabolites in each period.

Qualitative and quantitative principles of metabolites

Qualitative and quantitative metabolites identification were adapted from the methods described by Fraga et al. [39]. Indeed, using the Metware database, metabolites were qualitatively identified based on their secondary spectrum information. Isotope signals, repeated signals containing K⁺, Na⁺ and NH₄⁺ ions, as well as repeated signals of fragments of other substances with larger molecular weights were removed during analysis. Metabolites were quantified by MRM using QQQ/MS. With MRM mode, the first quadrupole screened the precursor ions (parent ions) of the target substance, and excluded the corresponding ions of other molecular weight substances to eliminate the interference. After ionization induced by the impact chamber, the precursor ions were fragmented, and a characteristic fragment ion was selected through the third quadrupole to exclude the interference of non-target ions, so that the quantification is more accurate and more repeatable. After obtaining the metabolite spectrum analysis data of different samples, the peak area integration was performed on the mass spectrum peaks of all substances, and the integral correction was performed on the mass spectrum peaks of the same metabolite in different samples.

KEGG pathway enrichment analysis was conducted based on the rich factor index calculated for each pathway, which was the ratio of the number of differential metabolites in the corresponding pathway to the total number of metabolites annotated in the same pathway. The greater the value, the greater the degree of enrichment. *P*-value is calculated using the hypergeometric test as shown below:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{m}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

N represents the total number of metabolites with KEGG annotation, n represents the number of differential metabolites in N, M represents the number of metabolites in a KEGG pathway in N, and m represents the number of differential metabolites in a KEGG pathway in M.

Illumina Miseq of the 16S rRNA genes and ITS1 analysis

DNA extraction followed the procedures described by the *Meiji rapure plant DNA Mini Kit*. After the extraction, Qubit was used to detect the DNA concentration, and 1% agarose gel electrophoresis to detect the integrity of the extracted genomic DNA. The commonly used amplicon sequencing primers of V3-V4 regions are 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') for the 16S rRNA [40]. Primers TAREuk454FWD1 (5'-CCAGCASCYGCAGTAATTCC-3') and TAREukREV3 (5'-ACTTTCGTTCTTGATYRA-3') were used for the 18S rRNA [41]. The PCR program was as follows : pre-denaturation at 95°C for 3 min; 95°C for 30s, 55°C for 30s, 72°C for 15s, 25 cycles from the second step; extension at 72°C for 5 min, and storage at 4°C [42]. After PCR amplification, the product was purified with magnetic beads, the concentration of the product was detected by fluorescence quantitative instrument Invitrogen Qubit 2.0, and the size by 1.5% agarose gel electrophoresis. The amplified PCR products were mixed to build a library, and sequencing adapters were added. After PCR amplification, magnetic beads were used to sort out small fragments such as primer dimers, and the library index information was recorded. Reaction conditions were: pre-denaturation at 98°C for 45 s; 8 cycles of 98°C for 15s, 60°C for 30s, 72°C for 30s; extension at 72°C for 10 min, and storage at 4°C. After the library detection is qualified, different libraries are pooled and sequenced on an Illumina MiSeq instrument using paired-end sequencing (2 × 300) (Illumina).

Denoising was performed using the dada2(1.18.0) [43] package in R language, to keep the true sequence before PCR amplification. Chimeras were removed using the de novo method built into dada2. The 16S and 18S sequences were obtained from the SILVA database (release 138, SSURef_NR99). After the removal of the redundant sequences, the taxonomy supported by most sequences was retained.

The plugin feature-classifier fit-classifier-naive-Bayes [44] of QIIME2 [45] was used to get the classifier based on the above SILVA database. Then, the feature-classifier classify-sklearn annotates the sequence. The relative abundance of a taxon was expressed as the proportion (%) of sequences relative to total sequences in respective samples. The FastTree method [46] was used to build the phylogenetic tree based on the representative sequence of the most abundant feature in each genus.

Statistical analysis

The statistical analyses were performed using the “stats” package in R (<https://www.r-project.org>) [47]. Metabolomic profile was analyzed using the hypergeometric test and the relative abundance of the microbiota was analyzed using the t-test. Spearman rank's correlation was calculated to identify the correlation between the identified metabolites using the corrplot package in R (R 3.5.3). Network correlation analysis was calculated to identify the correlation trends between metabolites and microbiota. Significance was declared at $P \leq 0.05$. The abundances of microbial metabolic pathways were compared between ensiled and non-ensiled plant using LEfSe, and significant differences were considered by an LDA score > 2 and P value < 0.05 .

Declarations

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Authors' contribution

AGA, YW and JX designed the study. AGA, GZ and XL collected the plant sample and followed up the experiments. AGA performed bioinformatics and statistical analysis; AGA wrote the manuscript and all other authors revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

No applicable

Ethics approval

The plant was collected and used following standard operating procedures established by the Good Agriculture Practices.

Consent for publication

No applicable

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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Figures

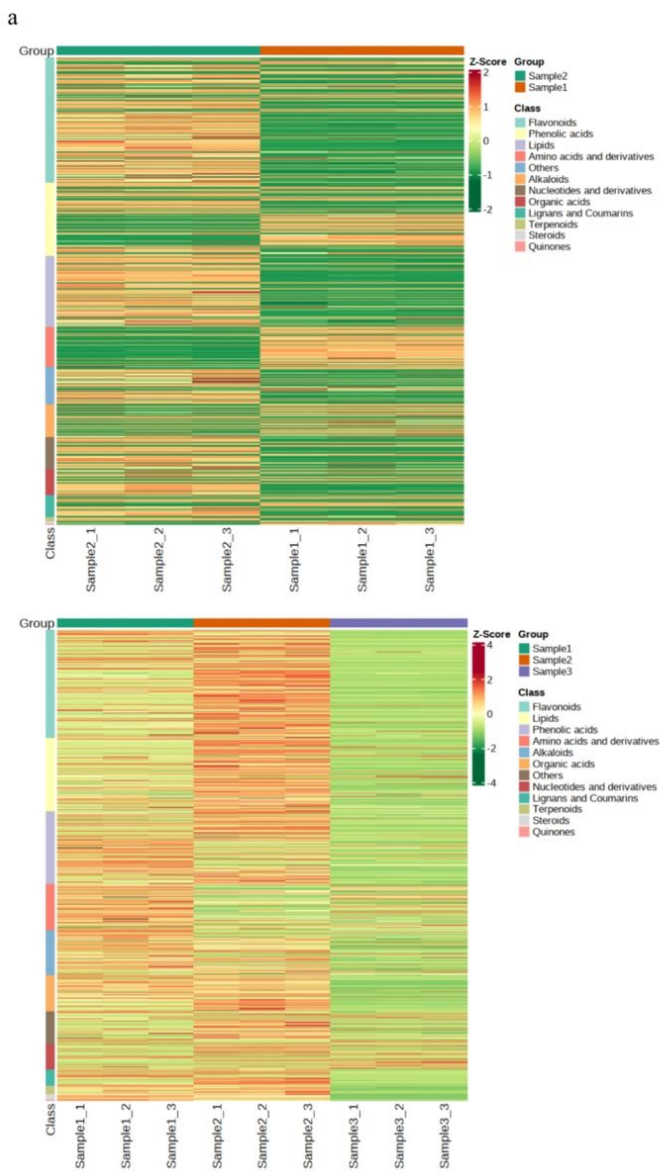


Figure 1

Hierarchical cluster analysis comparing sample 1 and 2 (a) and sample 1, sample 2 and sample 3 (b)

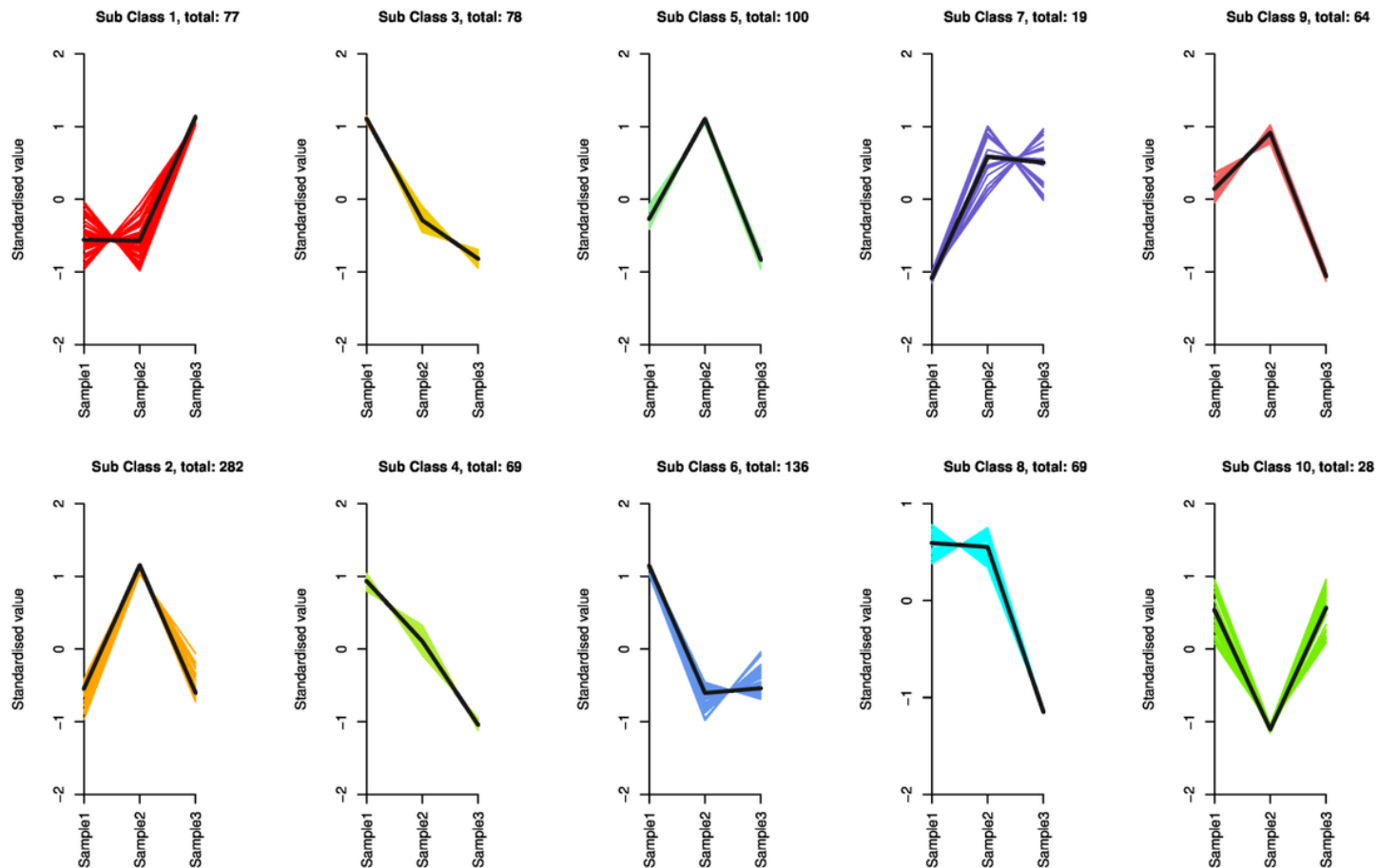


Figure 2

K-Means diagram of differential metabolites

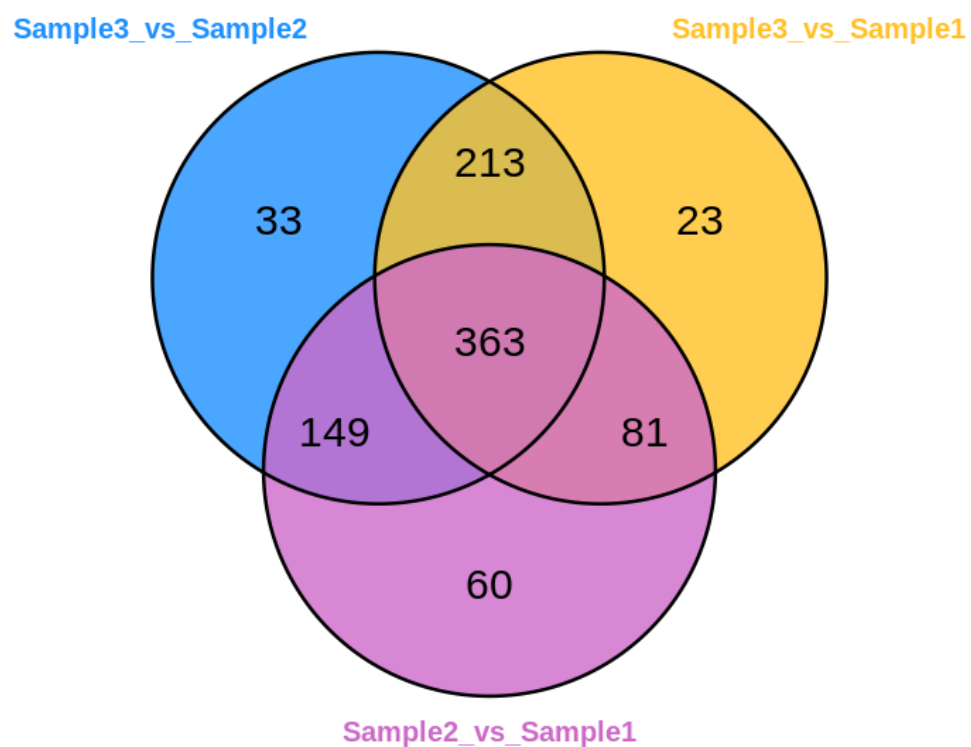


Figure 3

Venn diagram of differences among groups. Each circle in the figure represents a comparison group, the number in overlapped parts represents the number of common differential metabolites between comparison groups, and the number in non-overlapped parts represents the number of unique differential metabolites in comparison groups.

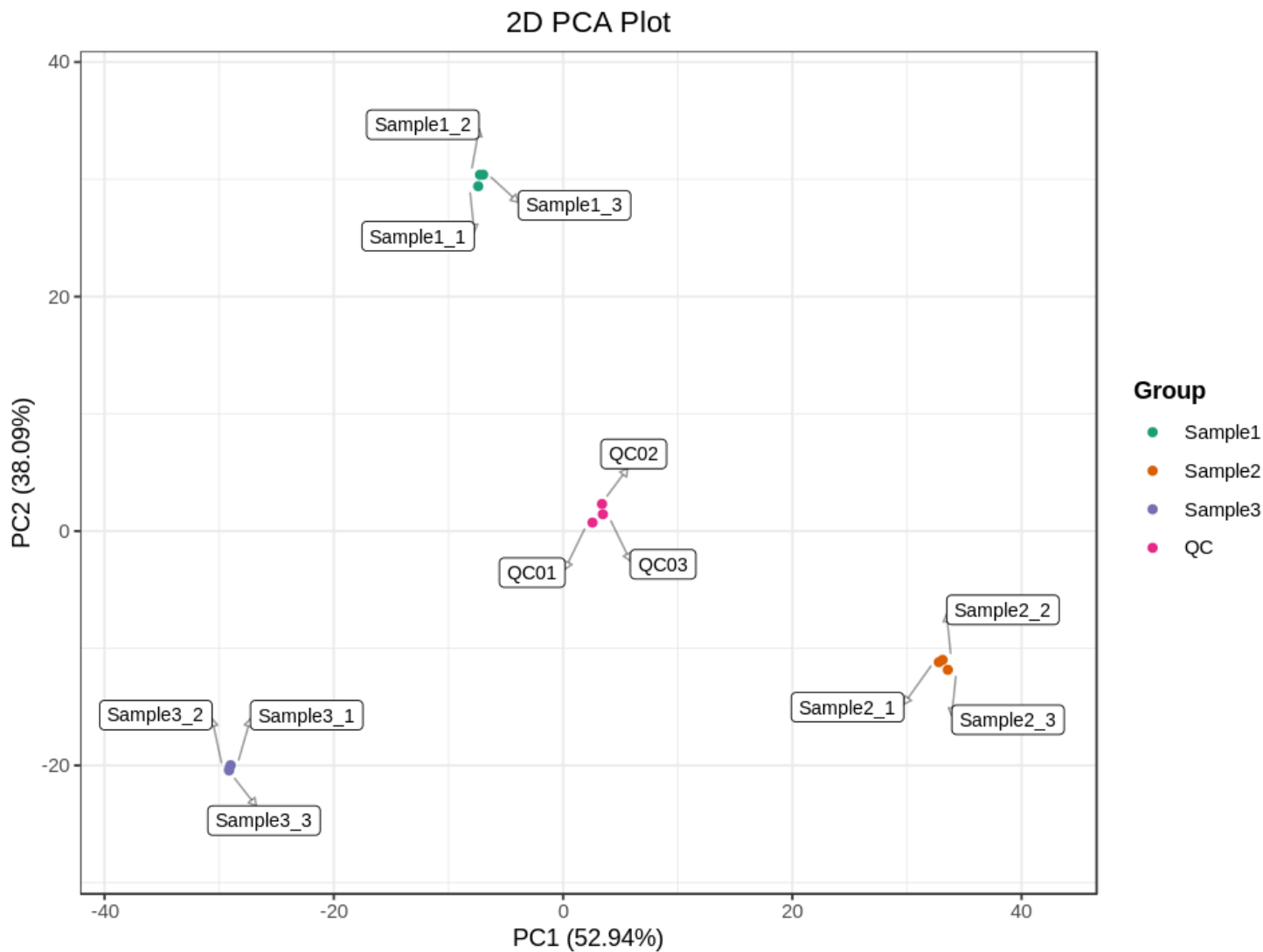


Figure 4

PCA score diagram of quality spectrum data of each group of samples including quality control samples (QC). PC1 represents the first principal component, PC2 represents the second principal component. Percentage represents the interpretation rate of the principal component to the data set. Each dot in the figure represents a sample, and samples in the same group are indicated in the same color.

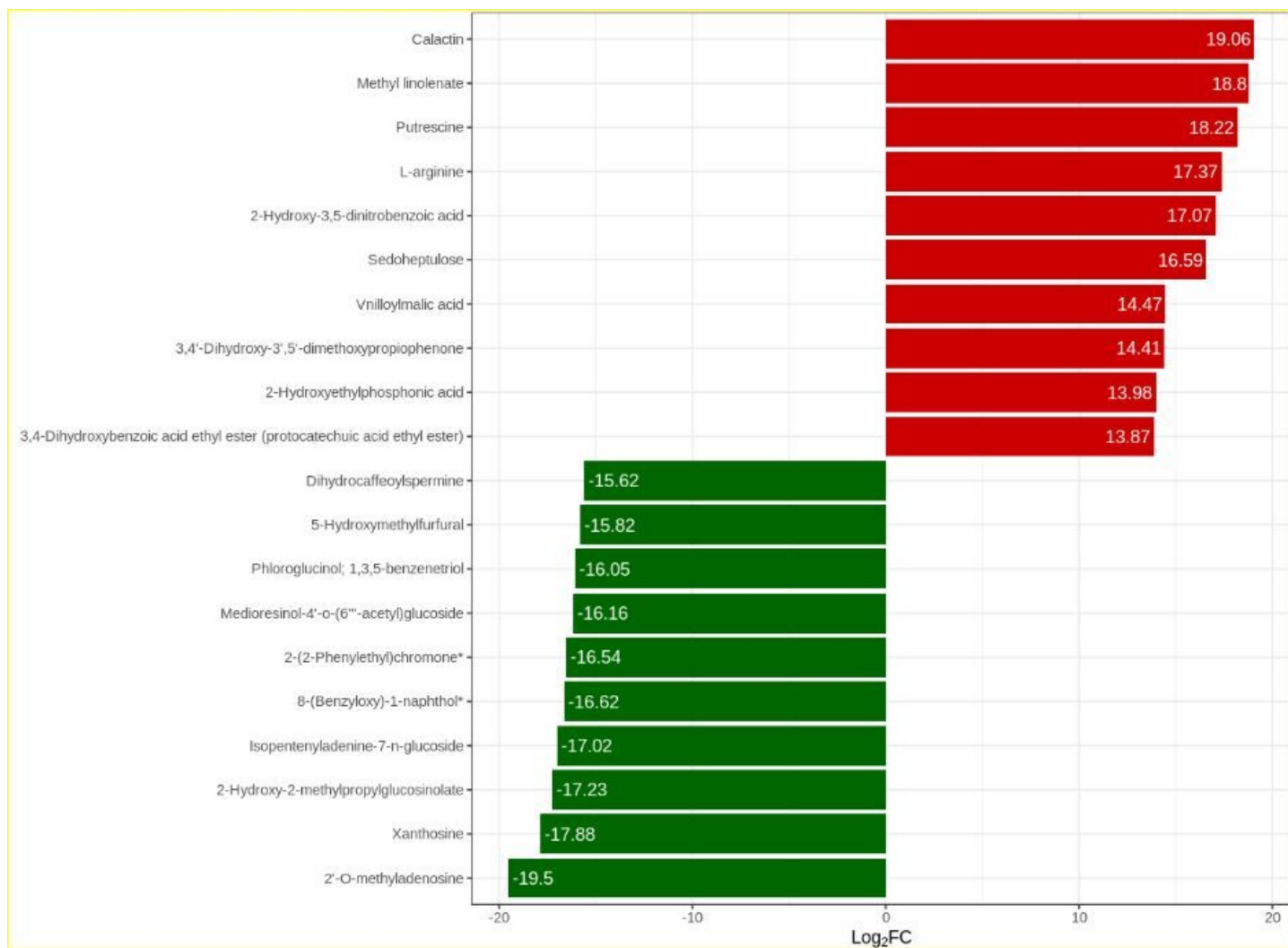


Figure 5

Bar chart of top dynamic differential metabolites

Spearman Correlation Plot of Sample2_vs_Sample1



Figure 6

Heat map of the Spearman rank's correlation of different metabolites. The colors represent the correlation coefficient with the scale seen on the right (The darker the red, the stronger the positive correlation; the darker the green the stronger the negative correlation). The figure shows the top 50 differential metabolites based on VIP.

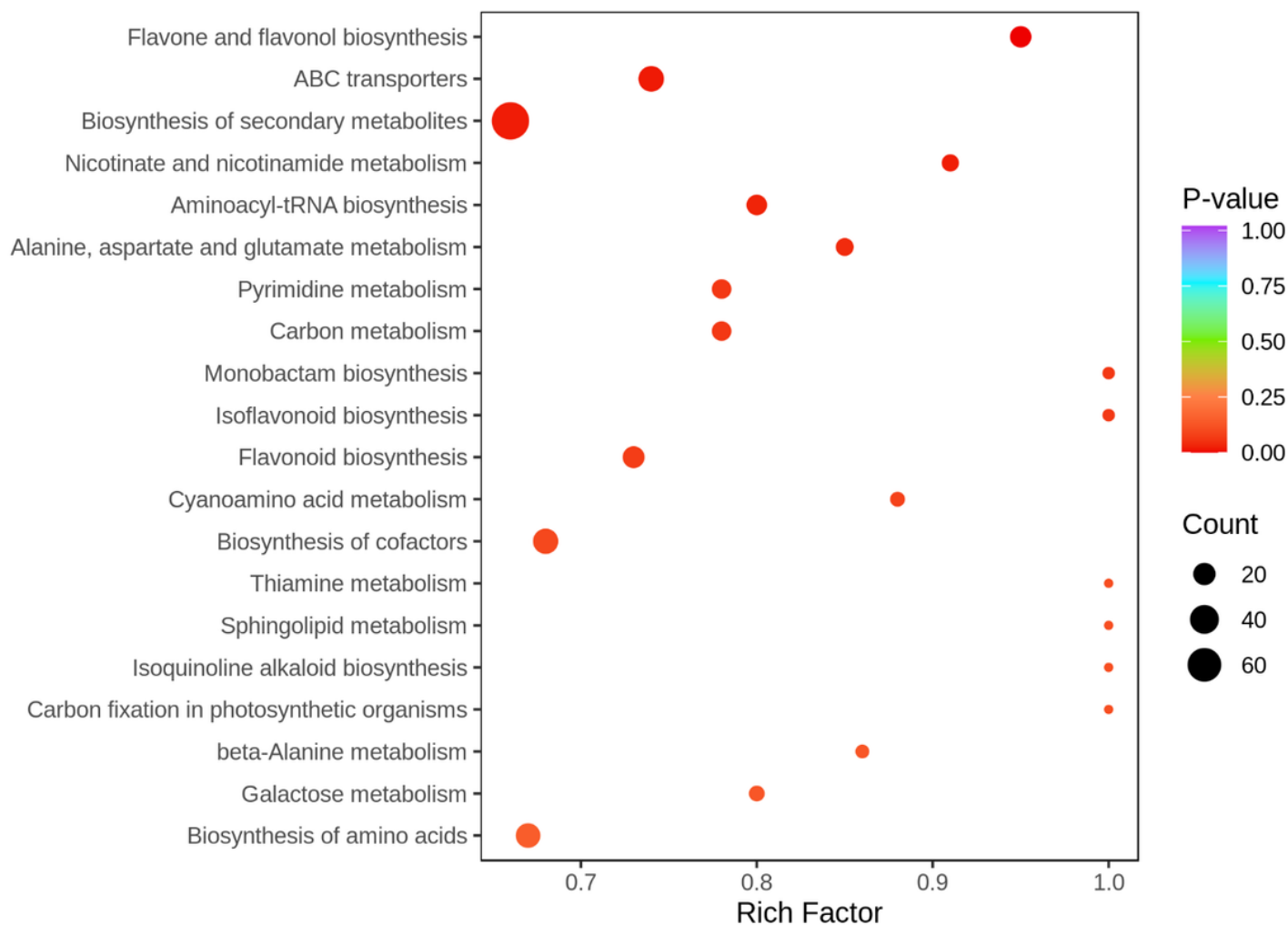


Figure 7

KEGG enrichment diagram of differential metabolites. The X-axis represents the rich factor and the Y-axis represents the pathway. The color of points reflects the *P*-value. The closer the *P*-value to 0, the more significant the enrichment is. The size of the dots in the figure represents the number of significantly different metabolites enriched in the corresponding pathway.

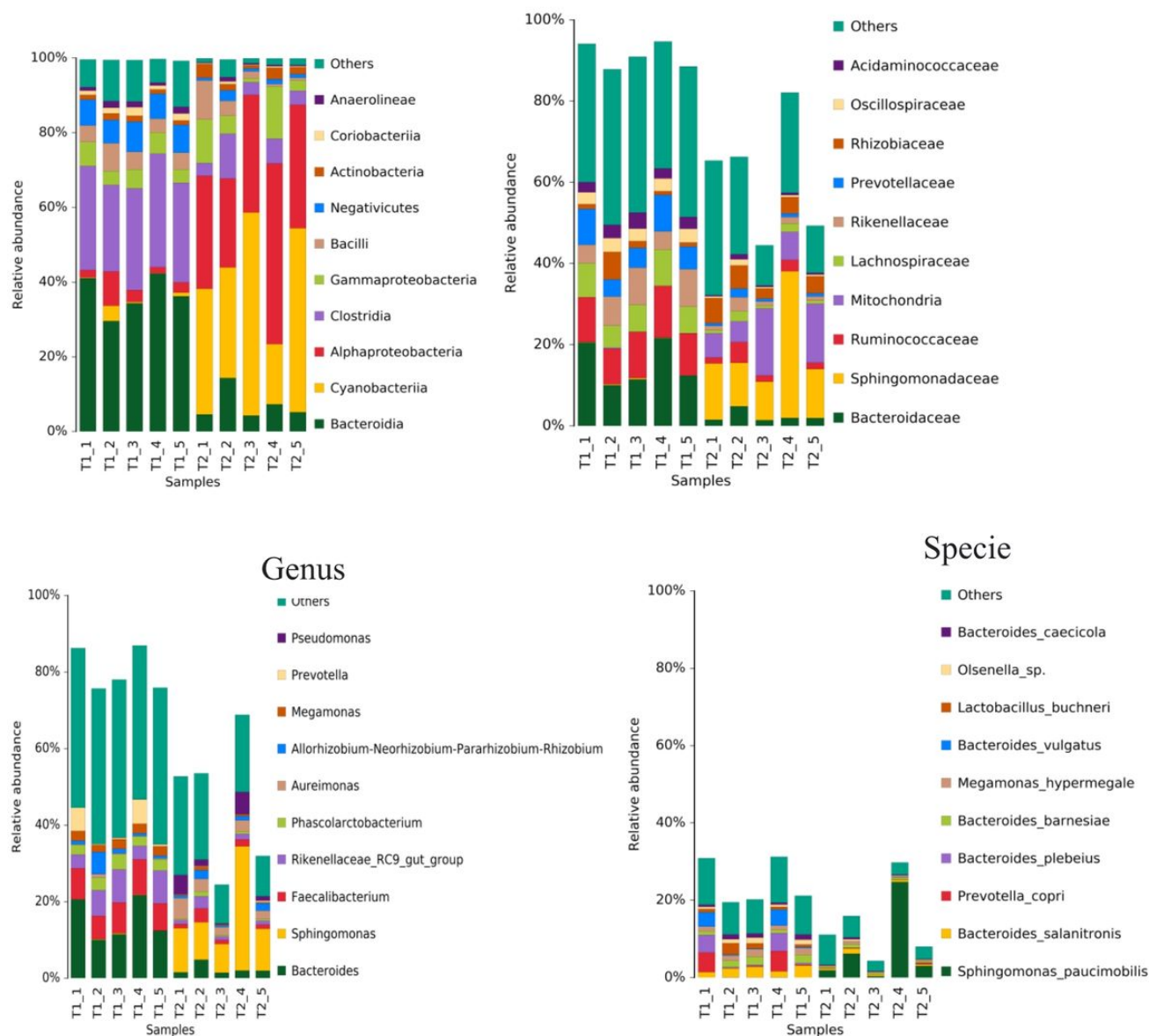


Figure 8

Bacterial composition and structure from ensiled (T1) and non-ensiled (T2) samples.

Only the top 10 taxa of abundance are displayed on the bar chart. The remaining proportions to get 100% on each bar were unclassified taxa.

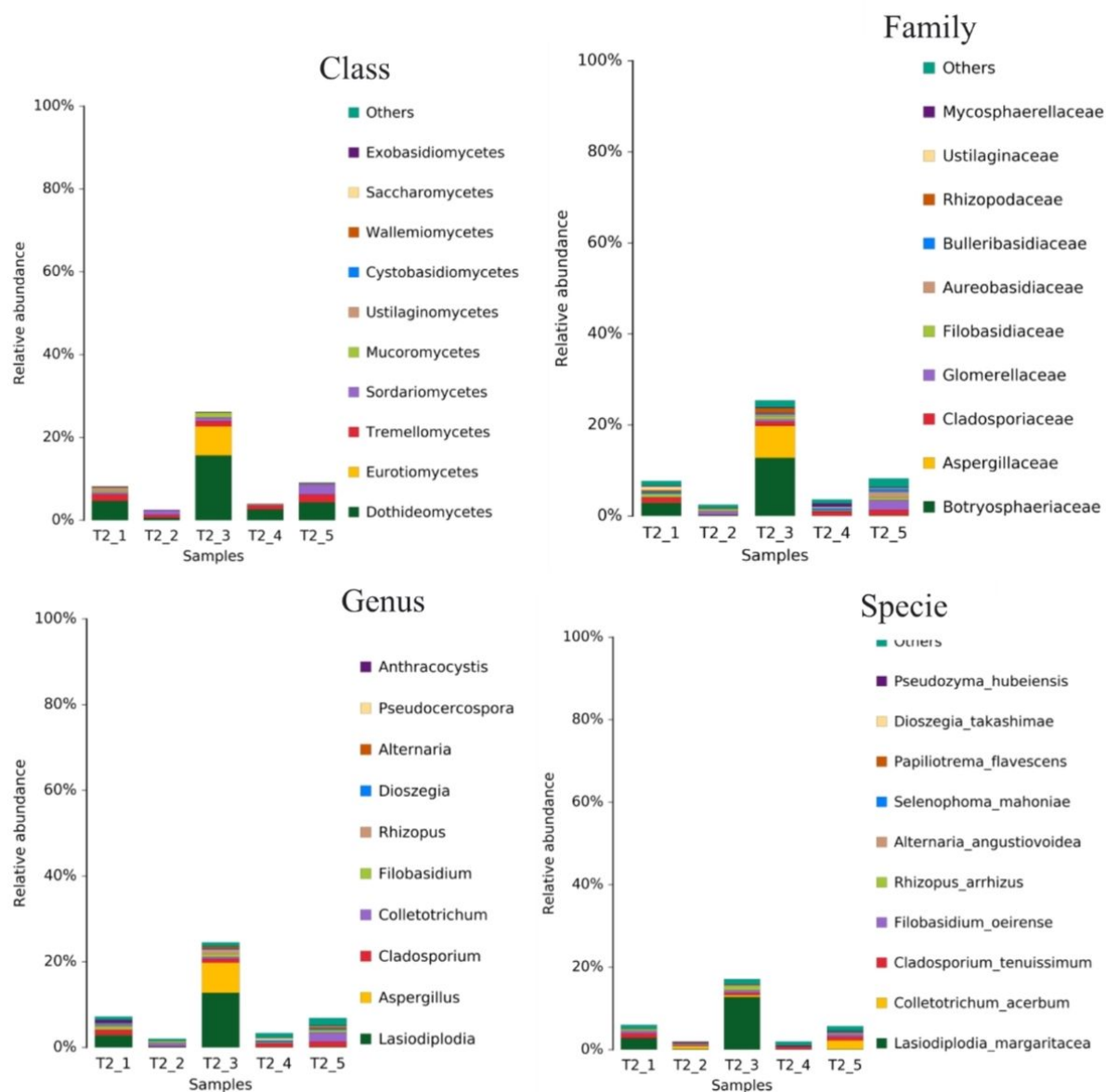


Figure 9

Fungi, mold composition and structure from non-ensiled (T2) samples.

Only the top 10 taxa of abundance are displayed on the bar chart. The remaining proportions to get 100% on each bar were unclassified taxa.

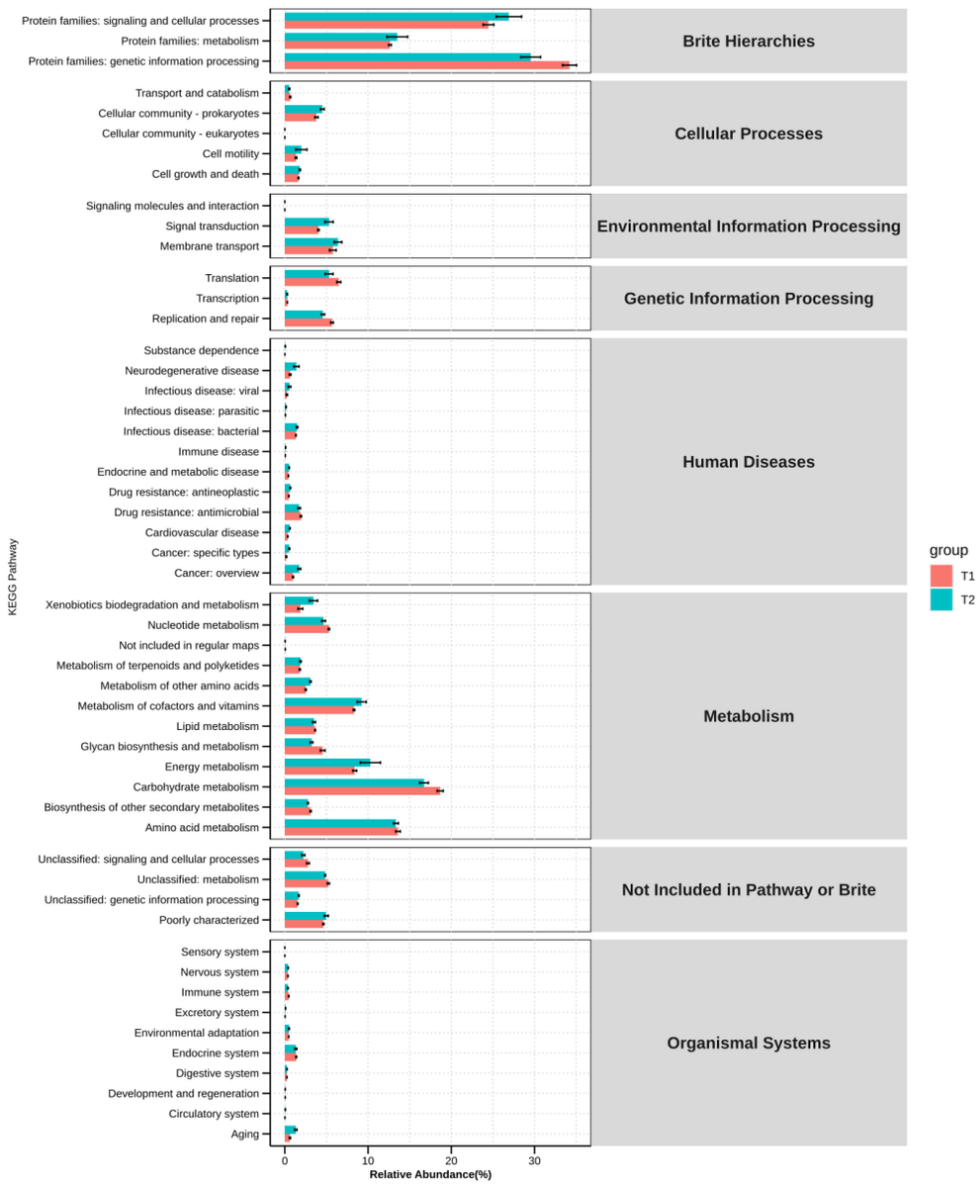


Figure 11

PICRUSt2 relative abundance of microbial KEGG pathways

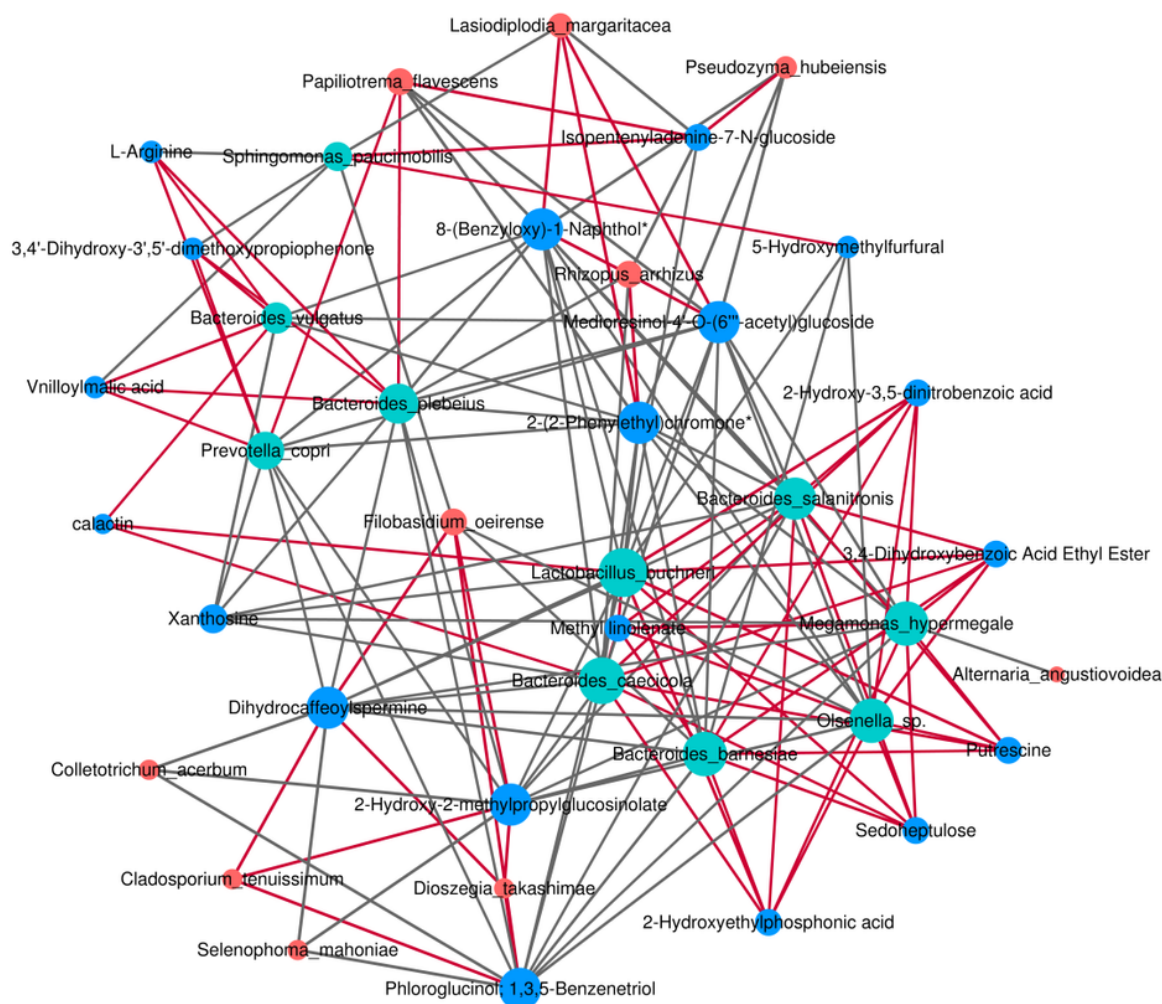


Figure 12

Network correlation analysis between the studied microbiota and the most changing plant metabolites

Supplementary Files

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