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RESEARCH ARTICLE

European oak metabolites shape digestion and fitness of the herbivore *Tortrix viridana*

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Abstract

1. Plants harbour a wide range of leaf-feeding insects whose survival and fitness are influenced by both energy-rich molecules and phytochemicals in the host foliage. Yet, how leaf host chemical diversity and insect microbiota—key factors in ecological and physiological processes—impact insect nutrition and fitness are still poorly understood.
2. To study the effects of leaf metabolic composition on insect herbivory resistance and performance, we fed the larvae of the specialist herbivory *Tortrix viridana* with leaves of susceptible and resistant *Quercus robur* trees that are characterized by contrasting metabolomes. We analysed the larval performance and mortality, the metabolomes in plant leaves, and in the insects' saliva and faeces by non-targeted metabolomics. Using chemometrics, mass difference network analysis and metabarcoding, we show the metabolome changes and chemical reactions associated with the different diets as well as their impact on insect fitness and gut microbiota.
3. In the saliva and faeces of larvae, plant secondary metabolites (e.g. flavonoids) persisted more the insect digestion while compounds from primary metabolism were more depleted. In addition, metabolic reactions within the larvae indicated different degradation pathways used on the two plant metabolic types (syn. metabotypes), including sulfation and sulfonation. We show that feeding insects with resistant oak leaves, enriched in secondary metabolites and depleted in primary metabolites, impaired insect performance and mortality. Although the insects' gut microbiota was slightly different upon the contrasting diets, overall, it was fairly stable. Despite the impact of host chemicals on herbivores, larvae were generally highly efficient in nutrient assimilation (feed conversion ratios of 3.3–3.6) and able to minimize plant defences (78% of secondary metabolites were converted, broken down or sequestrated).

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4. The comparison of the oak metabotypes showed how the foliar composition of resistant oaks affected insect fitness by influencing their digestion. Herbivores feeding on resistant oaks were less efficient due to their lower ability to metabolize and detoxify higher levels of host phytochemicals, whereas those on susceptible oaks were more efficient as they could degrade the host metabolome. This study highlights the importance of the oak leaf chemical composition to insect digestion and fitness of a specialized herbivore.

KEYWORDS
European oak, faeces, herbivore resistance, mass difference network analysis, metabolomics, metabotypes, microbiome, *Tortrix viridana*

1 | INTRODUCTION

Nutrition plays a crucial role in shaping patterns in ecology and strongly impacts plant-insect interactions. The performance and development of insect herbivores depend on their host plants' abundance and variability of energy-rich molecules, nutrients and defensive chemicals (Awmack & Leather, 2002; Wetzel et al., 2016). It has also been recognized that microorganisms are an essential part of the ecological and physiological processes based on the insect's nutritional ecology (Douglas, 2009). However, little is known about how leaf chemical diversity and the resulting changes in host plant food quality affect insect development, fitness, and gut microbiota.

Plants host a wide range of herbivorous insects as a result of coevolution. Herbivores essentially rely on plant foliage for nutrition as unique food. While plants have evolved traits to reduce herbivory damage, natural selection favours insect species that can overcome plant defences (Mopper, 1996). This evolutionary race has resulted in complex mechanisms of plant resistance and insects overcoming it (Gatehouse, 2002). Although it is well-understood that coevolution has resulted in an array of plant chemical defence mechanisms against herbivory, evidence of the importance of plant chemical diversity and insect-associate microbial community on plant resistance is still scarce. European oak *Quercus robur* (L.) hosts hundreds of insect species (Kersten et al., 2013; Schroeder & Tiberi, 2014; Yela & Lawton, 1997) and is periodically attacked by the specialized herbivorous insect *Tortrix viridana* L. (Lepidoptera, Tortricidae) a serious oak pest in Europe (Simchuk et al., 1999). Chemical diversity and spatial distribution of *Q. robur* results in insect-susceptible (S-), and insect-resistant (T-) oaks, phenotypically different from their degree of defoliation in periods of outbreaks (Bertić et al., 2021; Ghirardo et al., 2012). Previous research has shown that resistance and susceptibility of oaks attacked by *T. viridana* is related to growth-defence trade-offs in host leaf metabolism (Bertić et al., 2021). The susceptible phenotype allocates more resources in the biosynthesis of leaf metabolites related to growth processes (e.g. carbohydrates), while the resistant phenotype invests more resources in the formation of constitutive defence compounds such as phenolics (Bertić et al., 2021; Ghirardo et al., 2012; Kersten et al., 2013; Schröder et al., 2015); thus, the two oak phenotypes (S- and T-) represent different metabotypes. However, it is not yet known how the variation in nutrient content and quality of *Q. robur* leaves—both key ecological factors for insect community formation (Kelly & Southwood, 1999)—may affect the fitness and mortality of *T. viridana* larvae, and further, what significance the different diet compositions may have for uptake and metabolism in the insect gut and the diversity of the gut microbiota.

To answer these questions, we fed *T. viridana* larvae on leaves of either susceptible (S-) or resistant (T-) oaks and examined the chemical composition and abundance of metabolites in the diet (leaf metabolome), processed during chewing (salivary metabolome) and excreted by the insects (faecal metabolome) and linked them to insect larval performance and mortality. In addition, we determined the adaptation of the insect gut microbiome to the two food sources using metabarcoding approaches of faecal samples to assess bacterial diversity. The correlation of insect-related phenotypes with leaf metabolome profiles defines the plants' metabotype (Bertić et al., 2021), which is of great help in gaining insight in plant-insect interactions (Johnson et al., 2016). We used non-targeted metabolomics as it allowed studying the extraordinary phytochemical diversity of plants while providing a direct and comprehensive view of the biochemical composition of diverse samples from (a) the intact leaf (phytochemicals) through (b) the metabolome of larval saliva (during chewing, i.e. plant and insect chemicals) to (c) the larvae's faecal metabolome (processed phytochemicals by insect in interaction with gut microbiota). Differences in the salivary and faecal metabolome of larvae that developed on T- and S-oak leaves, respectively, could reflect altered metabolism due to malnutrition (Zhang et al., 2019) or insufficient detoxification of growth-inhibiting food ingredients, and moreover, the faecal metabolome can also be used as a functional measure of the gut microbiome performance (Zierer et al., 2018).

Here, we studied (i) whether the phenotype-dependent chemical diversity of European oak leaves affects *T. viridana* larvae performance and development; (ii) how the metabolome of host oak plants with greater resistance to herbivory persists in larval digestive system; (iii) what is the larval food conversion and ability to assimilate carbon and nitrogen nutrients and neutralize plant chemical defences (phenols); and (iv) how the different nutrition affects the gut microbiome of larvae feeding on S- and T- oak leaves.

2 | MATERIALS AND METHODS

2.1 | Plant and insect material

All experiments were performed on grafted trees of *Quercus robur* L., using susceptible (S-) and resistant (T-) tree phenotypes to *Tortrix viridana* infestation (Bertić et al., 2021; Ghirardo et al., 2012; Schröder, 2010). In the years 2003 to 2005 when outbreaks of *T. viridana* were observed in North Rhine-Westphalia (Germany), the susceptibility/resistance of European oaks to herbivory have been monitored phenotypically as defoliation rates, which is 5%–60% for T-oaks and >95% for S-oaks (Bertić et al., 2021). Plants were grafted onto *Q. robur* saplings (Schröder, 2010) from different scion mothers (Bertić et al., 2021), which are genetically different (Kersten et al., 2013). In 2008, 2011 and 2015, we grafted 100, 100 and 50 plants for each of the three T-genotypes and the two S-genotypes, respectively. The T-genotypes are the lines ASB2a, ASB14a, ASB17a and the S-genotpyes are the lines ASB13b and ASB47b from the oak stand "Asbeck" in North Rhine-Westphalia (for details see Ghirardo et al., 2012; Kersten et al., 2013). Grafted plants had been growing since then and until experiments (2021) in the plant garden of the Thünen-Institute of Forest Genetics, Grosshansdorf, Germany. Genetically, the trees belong to the same oak population (Schroeder & Degen, 2008) and the purity of *Q. robur* was ascertained by microsatellite markers (Ghirardo et al., 2012; Scotti-Saintagne et al., 2004).

Differences in leaf metabolomics, volatilomics, and transcriptomics in S- and T-oaks have previously been reported in detail (Ghirardo et al., 2012; Kersten et al., 2013; Orgel et al., 2021; Schröder, 2010; Schröder et al., 2015). *Tortrix viridana* is a specialist insect whose larvae prefer to feed on the species *Q. robur* (Du Merle, 1999; Hunter et al., 1997). The moth of *T. viridana* is particularly attracted to the chemical clues of the S-phenotype of oaks, whose leaves emit a different blend of volatiles and have a different phenolic composition than T-oaks (Ghirardo et al., 2012; Kersten et al., 2013). All metabolomics analyses were performed from feeding experiments using the 4th instar of the larvae on 50 of the grafted trees as in previous studies (Ghirardo et al., 2012; Kersten et al., 2013). In 2007, breeding of the larvae of *T. viridana* was established at the Thünen Institute of Forest genetics using caterpillars from 15 oak stands in North Rhine-Westphalia. In 2008, larvae from Finland were added to the breeding campaign. Since then, as far as possible every year some new larvae from other stands in North Rhine-Westphalia or Schleswig-Holstein were added to the breed to refresh the gene pool. Every spring under natural conditions hibernated eggs were transferred into room temperature and after hatching of the 1st instars, the larvae were put on opening buds of the ramets of the T- and S-oak trees. The larvae stay on the trees until pupation. The pupae were sampled and individually reared to adults in petri dishes. One female and one male were then put in boxes with paper and sugar water for mating and laying eggs. To avoid inbreeding, the females and males for crossings were chosen from different populations. The crossings of the females and males were documented over the years. Thereby, larvae are available every year for different experiments.

2.2 | Performance of *Tortrix viridana* feeding on the two European oak phenotypes

A total of 307 *T. viridana* larvae were used in the feeding trial. Before the experiment, all larvae were hatched, lived and fed on leaves of same oak tree phenotype as during the experiment. Freshly hatched 1st instar larvae were placed on just opening tree buds. Using a fine brush, 150 larvae were placed on T-oaks and 157 larvae on S-oaks. Each larva was given an individual number and was isolated on the trees in one "crispac" bag per larva. The 'crispac' are thin microperforated bags, allowing air to circulate and preventing larvae from escaping. The number of bags per tree with one larva each varied due to the number of available buds in the correct conditions (the 1st instar needs "just opening" buds) when the larvae hatched. Larvae developed on trees under controlled climate chamber conditions (12–13°C and 10 h/14 h day/night regime) until pupation. From the 3rd instar onwards, the weight of the larvae was measured every 4 days. Feeding was assessed by measuring the consumed leaf area on a scale paper and assuming leaf symmetry. The freshly eaten leaves were placed on the scale paper, and the boxes of the "removed surface" were counted. From the counted boxes, the feeding area was calculated. In addition to the weight and feeding measurements, the mortality and developmental stage of the larvae were recorded on a daily base. Due to variation in the length of the developmental stages, individual larvae were weighed 1–3 times per stadium. Fifth instar larvae were only weighed twice to avoid disturbance in their preparations for pupation. Feed conversion ratio (FCR) was calculated for each individual larva as the ratio between feed consumed (in kg) to obtain 1 kg of animal body weight increase (Oonincx et al., 2015).

2.3 | Sampling of leaves, faeces and saliva

In the experiment, the larvae were hatched and grown on the same tree phenotype (either on S- or T-) they were used to for years leading to an adapted gut microbiome. Larvae were starved for 24 h and then placed on the leaves for 48 h feeding. We used one tree per clone, that is, three trees of T- and two trees of S-oaks with five larvae per tree in the 4th instar. All branches with larvae were wrapped with crispac bags. For trials, we used larvae of different crosses enclosed in separated crispac bags for a total of 12 bags (Table S1 and Figure S1). After 2 days of feeding, we sampled the faeces of the larvae and the leaves on which the insects were feeding (10 S- and 15 T- leaves) by flash-freezing in liquid-N_2. To later get metabolomic information at the onset of digestion, we also collected the saliva of the larvae by using autoclaved cut filter paper (1 × 0.5 cm, Macherey-Nagel, Düren, Germany). The edge of the paper was put on the mouth of the larvae and stirred to soak the saliva. Filter papers were always handled with ethanol-sterilized tweezers. Control samples consisted of autoclaved filter papers. All samples of plant leaves, insect saliva and faeces were immediately stored at −80°C until metabolomic analyses.

2.4 | Metabolomic analysis

All materials (i.e. leaves, saliva, faeces) were freeze-dried at −50°C under the vacuum condition of 0.040 mbar (Alpha 1–4 LDplus, Christ). To prepare for extraction, leaves were homogenized to fine powder using mortar and pestle, whereas faeces and saliva were used as they were. Extracts were made of 20 mg (dry weight, DW) of fine leaf powder, 1 filter paper for the saliva and 9 mg DW of faeces (corresponding to 10 little "balls"). Filter papers without saliva but handled and stored exactly as the samples were used as blanks for background correction (in triplicates). The internal standard (IS) mixture (0.028 μmol ml^{-1} of magnolol, rosmarinic acid, 3,4-dihydromandelic acid and 3′,4′-dihydroxyacetophenone) was added to extraction solvent mixture, methanol:2-propanol:water (1:1:1, v/v/v). For detailed extraction protocol, see the work of Bertić et al., 2021.

We used the same non-targeted approach as before (Bertić et al., 2021), based on ultra-performance liquid chromatography (UPLC) ultra-high resolution (UHR) tandem quadrupole/time-of-flight (QqToF) mass spectrometry (MS) (UPLC: Ultimate 3000RS UPLC from Thermo Fisher, Bremen, Germany; MS: Bruker Impact II (QqToF) and an Apollo II electrospray ionization (ESI) source from Bruker Daltonic). All the samples were measured separately with reversed-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) columns, and MS operating in both positive (+) and negative (−) ESI modes. Compound elution was achieved using solvent A (H_20 in formic acid 99.9% [v/v]) and solvent B (acetonitrile in formic acid 99.9% [v/v]). In RPLC, the gradient was: 0–1 min, 95% A (isocratic); 1–15 min, 95%–70% A, 15–17 min, 70%–20% A, 17–20 min 20% A (isocratic), 20–22 min 20%–0.5% A, 22–27 min 0.5% A (isocratic), 27–29 min 0.5%–95% A, and 29–31 min 95% A (isocratic). In HILIC, the gradient was: 0–1 min 5% A (isocratic), 1–16 min 5%–30% A, 16–18 min 30%–80% A, 18–19 min 80% A (isocratic), 19–20 min 80%–95% A and 20–21 min 95%–4.5% A. Solvent flow (0.4 mL min^{-1}) and column temperature (40°C) were kept constant. The injection volume was 5 μL. Mass calibration was performed with the calibration mixture of 50 mL of water, 50 mL 2-propanol, 1 mL NaOH and 200 μL of formic acid. The MS was operated in both positive (+) and negative (−) ionization modes at nebulizer pressure of 2.0 bar, dry gas flow of 8.0 L min^{-1}, dry gas temperature of 200°C, capillary voltage of 4500 V for (+) and 3500 V for (−); endplate offset, 500 V; mass range, 20–2000 m/z. The chemicals (LCMS hyper grade) methanol, water, formic acid, NaOH were purchased from Merck (Darmstadt, Germany), 2-propanol and acetonitrile from Honeywell (Puchheim, Germany). See Supplementary material in Bertić et al., 2021 for more details.

Exported raw data table consisted of 89 columns (73 samples) and 18,966 rows (18,953 mass features; Table S2). A mass feature was considered absent (zero) within a sample type group either when the average group peak area was less than the threshold (1000 area units), or the mass feature was not detected (zero) within the group in more than 70% of the cases. The zeros were then replaced with random numbers from the range of 75%–125% of half value of the detection limit (LoD = 1000 area units). Metabolite annotation was achieved by level 1 using pure standards (Reisdorph et al., 2020; Sumner et al., 2007) and by level 2 using MS/MS library spectra matching (see methods in Bertić et al., 2021). The rest of mass features are tentatively annotated with molecular formula and chemical class (level 3) using the smart formula annotation algorithm of the Metaboscape software (4.0, Bruker Daltonics, Bremen, Germany) predicting the formula according to molecular mass and considering natural ratios of elements in biomolecules (Kind & Fiehn, 2007) and multidimensional stoichiometric compound classification (MSCC) algorithm (Rivas-Ubach et al., 2018). Prior to any statistical analysis, data were processed in the same way as described in Bertić et al., 2021.

2.5 | Conversion of European oak leaf metabolome following insect intake

We assessed "metabolite conversion" of leaf metabolites after passing through the insect digestion system as indication of insect intake (catabolism and sequestration), by relating the compound abundances occurring in the leaves and those occurring in the insect faeces. The rates were calculated from the same data table (Table S3) after normalization to the values of ellagic acid and glucogallin, two compounds that were least affected by insect ingestion, as explained hereafter. In our search for stable compounds, little or hardly digestible, we focused on the class of secondary metabolites, as these are chemicals that are likely to be more resistant to passing through the insect digestive system compared to the other classes of compounds analysed (i.e. carbohydrates, lipids, proteins and nucleotides). We assumed that the ratios of stable compounds passing through larval digestion remain the same even though their absolute values change from leaves to faeces, which have different densities. Therefore, we calculated the ratios between the average areas of all secondary metabolites in the list of leaves (L_R, "L ratio", calculated separately for S and T metabotypes). We repeated the same procedure for the faeces ("F_R") for S- and T- metabotype, separately. The ratios for S- and T- metabotype were calculated independently to ensure that the final compounds used for the normalization were stable in both metabotypes. After that, we calculated the ratio of ratios, that is, $L_R/F_{R,}$ for each possible compound comparison. In the last step, we calculated the logarithms for each compound ratios, that is, ($\log2[L_R/F_R]$), to scale all the values and to easier identify candidates (compounds with the value closest to zero). From the list of secondary metabolites that could be adequately annotated, we selected the 30 major metabolites for which the average ratio was closest to zero (Table S3). Compounds with final average ratio values close to zero were considered the most stable to pass through the insect digestion system. We used ellagic acid and glucogallin because their $\log2(L_R/F_R)$ averages were −0.17 and −0.29, and additionally, they could be annotated (level 2) and were highly abundant in all measured samples (Table S3). The normalization procedure to ellagic

acid and glucogallin was required to compare the relative compound abundances between leaves, saliva and faeces, which have different biomass densities.

2.6 | Analysis of leaf composition

We additionally analysed by traditional colorimetric assays the levels of the major classes of leaf compounds important for insect nutrition. In addition to sugars and phenolics analyses (confirmatory to our non-targeted analysis), we measured the soluble proteins, cell walls and lignin, which could not be detected by LCMS-based metabolomics. Detailed descriptions of analyses can be found in the Supplementary methods S1–S4. In short, total soluble sugar content was determined with adjusted standard "Dubois" colorimetric assay (Dubois et al., 1956); soluble proteins according to Bradford's assay (Marshall & Williams, 1993); total phenolics using the modified "Folin–Ciocalteu" assay (Folin & Denis, 1912). Estimation of protein-free cell wall content was determined by the gravimetric analysis of the protein-free cell wall fraction during the analysis of lignin, which content was measured by the acetyl bromide method after small modifications (see Supplementary methods S1–S4 & Moreira-Vilar et al., 2014). Cell wall and lignin contents are shown as % of cell wall and lignin per dry weight, respectively.

2.7 | Microbiome analysis

Microbiome analysis was performed using a molecular barcoding approach to assess bacterial diversity. In short DNA was extracted from faecal samples using Nucleospin Soil Kit (Macherey Nagel), followed by sequencing the V3–V4 hypervariable region of the 16 S rRNA gene (primers 335F and 769R [Dorn-In et al., 2015]) on a MiSeq Illumina instrument. After quality filtering, sequences were analysed on the European Galaxy web platform (Afgan et al., 2018) via DADA2 pipeline (Callahan et al., 2016). Subsequent statistics and plots were performed in R. A detailed description of methods and data analysis is given in the supplementary methods S5.

2.8 | Mass difference enrichment analysis

Mass difference enrichment analysis (MDEA) was performed as described in (Moritz et al., 2017), using an in-house algorithm and by targeting the mass differences (MDs) between compounds uniquely identified. We used compounds putatively identified in faeces (B) and their connection to the metabolites putatively identified in leaf samples (A) following the general conversion reaction $A + MD \leftrightarrow B$. Enrichment of mass differences was calculated using in-house algorithm and was expressed as *Z* score as described elsewhere (Clancy et al., 2018; Moritz et al., 2017). All mass differences with Z score higher than two (which equal to *p*-values < 0.05) were considered significantly changed from source (leaf material) to target (faecal material).

Overall, MDEA examines all pairs of mass features for mass differences for known biochemical transformations. The pairs of MS features connected by a biochemical transformation formed the source (substrate) of lower mass and a target node (product) of higher mass. The MDEA tests whether a specific type of transformation (e.g. sulfation and sulfonation) occurs with the mass characteristics of interest. With this approach on leaf (source) and faecal (target) metabolomes, we were able to study the reactions that occur during insect's digestion.

2.9 | Statistical analysis

For all the metabolomic analyses, we used in total 25 oaks, composed of 15 insect-resistant (T-) and 10 insect-susceptible (S-) independent plants. Insect materials (saliva and faeces) collected from the same tree was treated as one replicate. Therefore, the number of biological replicates for metabolomic analysis of leaves, saliva and faeces was $n = 15$ for T-metabotypes and $n = 10$ for S-metabotypes collected on respectively number of different trees.

The multivariate statistics orthogonal partial least squares—discriminant analysis (OPLS-DA) was performed using SIMCA-P v.13.0.3.0 (Umetrics) as described in the work of Bertić et al., 2021. The significance of OPLS-DA was tested by cross-validated ANOVA (Eriksson et al., 2008) and given at $p < 0.05$. Hierarchical clustering based on Euclidian distance, statistical analyses for feeding experiment including mortality rates, metabolites subsets were made with R software (R Core Team D, 2021) using package HEATMAP (Gu et al., 2016), GGPLOT2 (Wickham, 2011), and VENNDIAGRAM (Chen & Boutros, 2011), respectively.

To test for statistical significances of the insect microbiome, Kruskal-Wallis test and PERMANOVA (adonis function) was used. For identification of biomarker taxa, two generalized linear models accounting for over-dispersion and zero inflation (R packages MASS and PSCL) as well as LEfSe (Linear discriminant analysis Effect Size), differential expression analysis (DeSeq2 and EdgeR, both based on log-fold changes) and random forest decision trees were used with the R package MICOBIOMEMARKER(Cao, 2020). Multiple test corrections were performed by *p*-value adjustment via the FDR method. All the microbiome analyses were performed in R.

3 | RESULTS

3.1 | Leaf composition of resistant oak metabotypes impacts insect performance and mortality

We studied the effects of dietary components of S- and T-oaks on the feeding behaviour, growth performance and mortality of *T. viridana* larvae (Figure 1). For this, we performed laboratory feeding

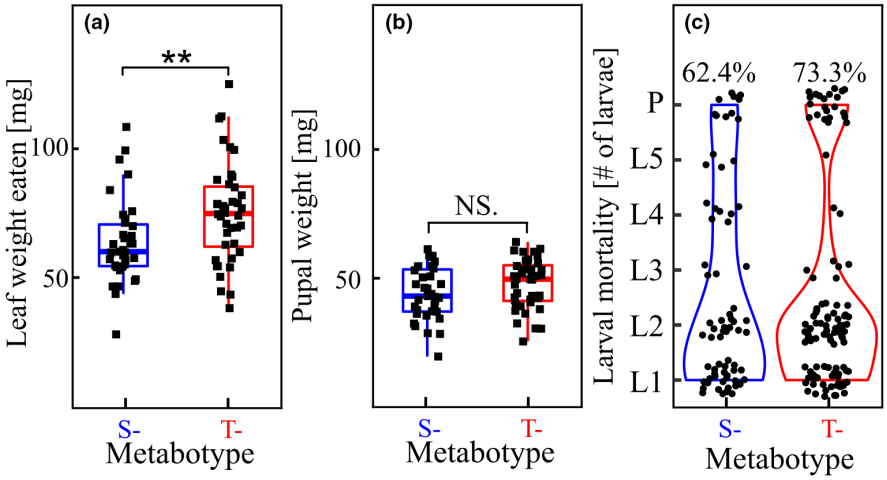


FIGURE 1 Herbivore performance of *T. viridana* larvae reared on oak leaves differently resistant to *T. viridana* defoliation. (a) Leaf weight consumed by larvae after 24 h of feeding, and (b) final larval pupal weight to reach the same pupal stage. The colours indicate susceptible (S-, in blue) and resistant (T-, in red) oak metabotypes to the defoliation of *T. viridana* (Bertić et al., 2021). The *T. viridana* larvae were reared on either S- or T-leaves under controlled environmental conditions. (c) Distribution of mortality through all developmental stages (from instar L1 to L5 and P, pupa) of *T. viridana* fed on S- and T-leaves, showing that total mortality of *T. viridana* is higher upon T-diet. Percentages are the mean values of mortality. In T-leaves, 40 out of 150 larvae reached adulthood, compared to 59 out of 157 in S-leaves. **, $p < 0.01$; (two sample *t*-test).

experiments where either S- or T-leaves were available to the larvae. We followed the larval development from the first instar (L1) to the pupal stage and analysed the metabolome in leaves, saliva and faeces (Figures 1 and 2) in relation to the amount of leaf ingested by the larvae (Figure 1a), the larval body weight gained (Figure 1b) and mortality (Figure 1c).

Larval feeding was higher on T-leaves, as the larvae required more foliage material to reach the same developmental stage (two sample *t*-test, $p < 0.01$) or achieve a similar pupal weight (two sample *t*-test, $p > 0.05$; Figure 1a,b, Supporting Information Figure S3). The insect mortality on T-leaves was ~10% higher compared to larvae feeding on S-leaves (Figure 1c). In terms of feed conversion ratio (FCR), which is the ratio of feed consumed (in kg) to obtain 1 kg of insect body weight gain (Oonincx et al., 2015), the FCR was 3.34 for S-larvae and 3.62 for T-larvae. Therefore, we studied how T- and S-leaf metabolomes varied in interaction with the insect, by comparing the metabolome of leaves with those of the insect saliva and faeces and upon T- or S-feeding (Figure 2a). We observed significant differences in the metabolic composition of leaves, saliva, and faeces collected from diets exclusively based on leaves of the S- or T-metabotypes (Figure 2b,c; $p < 0.001$, cross-validated ANOVA). Unsurprisingly, a large part of the total metabolic variance (~58%, depicted in the first 2 components, Figure 2b) explained the extremely diverse sample types (leaves vs saliva vs faeces) compared to the differences of T- and S-within leaf, saliva, and faeces (1.46% of total variance, disentangle by the 3rd component of OPLS-DA, Figure 2c). The 191 significant discriminant mass features found within the groups 'leaves', 'saliva' and 'faeces' that were up- or down-regulated in the comparison T/S (Variable Importance in Projection (VIP) > 2, Log2(T/S) > 3, and Log2(T/S) < −3, $p < 0.05$) consist mainly of plant secondary metabolites, protein-related metabolites, lipids and few carbohydrates (Figure 3a). Also, each sample material is characterized by an unique number of mass features (80 in leaves, 130 in saliva, 73 in faeces, see Venn diagram in Figure 3a), indicating metabolic degradation and transformation of leaf metabolites, or metabolites originating from larvae. We therefore analysed those mass features appearing only in either saliva or faeces but that were not detected in the leaf metabolomes that significantly differed in their relative abundance between T/S (Figure 3b). We refer to them as "unique compounds", which include new conjugates and breakdown products. Besides general metabolic changes, hierarchical clustering analyses of those unique compounds mainly showed an increased level of (modified) plant secondary metabolites in the saliva of larvae fed with T-leaves, which vanished in the faecal metabolome concomitant to an increase of unknown metabolites. Further changes were observed for protein-related metabolites and lipids (Figure 3b). Taken together, the data indicate a different nutritional quality of T-oak leaves, which are digested differently by the larvae and have a negative impact on insect performance.

3.2 | Increased depletion of the S-leaf metabolome following larval ingestion

We sought to study why the larvae require less leaf material of S-oaks than T-oaks to gain the same pupal weight. An option is that components of S-leaves are somehow easier to catabolize. To this end, we first compared the differences of metabolite abundances in faeces versus leaves within the same metabotype and calculated the "percent of metabolite conversion", that is, the decrease of compounds after passing-through the larvae (see materials), as a proxy of insect uptake/sequestration. We observed a greater decrease of

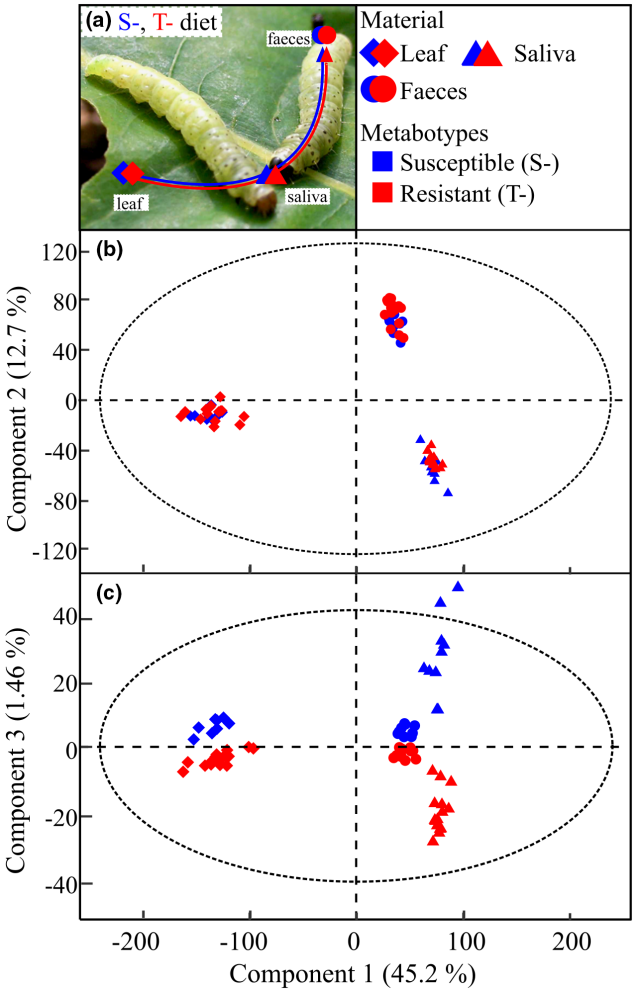


FIGURE 2 (a) Picture of the feeding experiment depicting the symbols used for leaf (diamonds), saliva (triangles) and faeces (circles) samples. The colours indicate the two susceptible (S-, in blue) and resistant (T-, in red) oak metabotypes to the defoliation of *T. viridana*. The *T. viridana* larvae were fed on either S- or T-leaves for 24 h. (b) Scores of first vs second and (c) first vs third component of orthogonal partial least square regression discriminant analysis (OPLS-DA) of S- and T-metabotypes from metabolomics extracts of leaf, saliva, and faeces samples. OPLS-DA model fitness $R^2X(cum) = 0.662$, $R^2Y(cum) = 0.584$, $Q^2Y(cum) = 0.459$ using three predictive components. RMSEE/RMSEcv (sample material): 0.27/0.27 (leaf), 0.25/0.26 (saliva), 0.28/0.26 (faeces). CV-ANOVA, $p = 8.019 \times 10^{-17}$. *, $p < 0.05$ (two-sample *t*-test).

carbohydrates, lipids, nucleotides, secondary metabolites, amino acids and protein related compounds when larvae ate S-leaves in respect to T-leaves (Figure 4a–c). Overall, secondary metabolites were decreasing the least (average metabolite conversion of 78%) while carbohydrates and protein-like compounds the most (85%) (Figure 4c). We then analysed the cell wall, lignin and total protein contents in the oak leaves by traditional colorimetric assays, to gain further general indicators of leaf diet quality that otherwise cannot be deduced from our metabolomics dataset. Total soluble proteins, cell wall components and lignin were more abundant in T- than in S-leaves (two-sample *t*-test, $p < 0.05$, Figure 4d). Complementary colorimetric analyses of the main leaf components showed that the total soluble carbohydrate level was higher in S- than in T-leaves, whereas total phenolic content was similar in both types of oak leaf metabotypes (Figure 4d).

3.3 | Metabolic reactions within the larvae

We focused the analysis on the "unique compounds" in the faecal metabolome (i.e. compounds not found in leaves) to shade light on chemical transformations of leaf metabolites after passing-through the insect digestive system. The unique compounds found in faecal samples originating either from T- or S-leaf diets were significantly different in their metabolite compositions ($p < 0.01$, CV-ANOVA, Figure 5a,b). We then applied the mass difference enrichment analysis (MDEA) approach, to study the reactions occurring within the larvae that results in the unique compounds of the faecal metabolome. We found a strong enrichment of reactions related to the insertion of sulphur and loss of nitrogen (Figure 5c). The sulfation and sulfonation reactions led to the formations of several sulfated flavonoids (e.g. sulfates of quercetin, quercetin 3-glucoside, kaempferol, isorhamnetin, isoorientin and hispidulin) which appeared in the list of annotated mass features detected in the faecal metabolome (Figure 5d, Table S4). Notably, sulfated flavonoids were differently occurring in S-faeces compared to T-faeces (Figure 5d). Among others, the analysis showed the presence of typical catabolic products such as hippurate (from the catabolism of polyphenols), and xanthourenic acid, quinolinic acid, kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid (from the catabolism of tryptophan) in the larval faeces (Table S4).

3.4 | Insect gut microbiota differs with European oak metabotypes

As diet is one of the key factors structuring gut microbiota composition, we studied the bacterial communities in the gut of *T. viridana* larvae that exclusively fed on either T- or S-oak leaves. The different food quality, S- vs T-leaves, had no effect on α-diversity, indicating no differences in the overall diversity of gut bacterial community as seen from the analysis of amplicon sequence variant (ASV) richness, Shannon diversity index, Pielou's evenness (Figure S4). ß-Diversity analysis based non-metric multidimensional scaling (NMDS) (Figure 6a) showed also no major clustering between the microbiome in the faeces originating from S- and T- diet but strong individual variabilities independent from the type of diet (Figure 6a). A detailed analysis of individual taxa revealed overall, 385 genera composing 21 phyla. Most of the annotated reads (>90%) belonging to three major phyla: Proteobacteria (60%), Actinobacteria (20%) and Firmicutes (12%) (Figure 6b). Among the genera, several members of Proteobacteria (Pseudomonas, Massilia, 1174-901-12, Acidiphilium), Friedmanniella and Staphylococcus dominate the insect faecal microbial community (Figure S5). Interestingly, ASVs

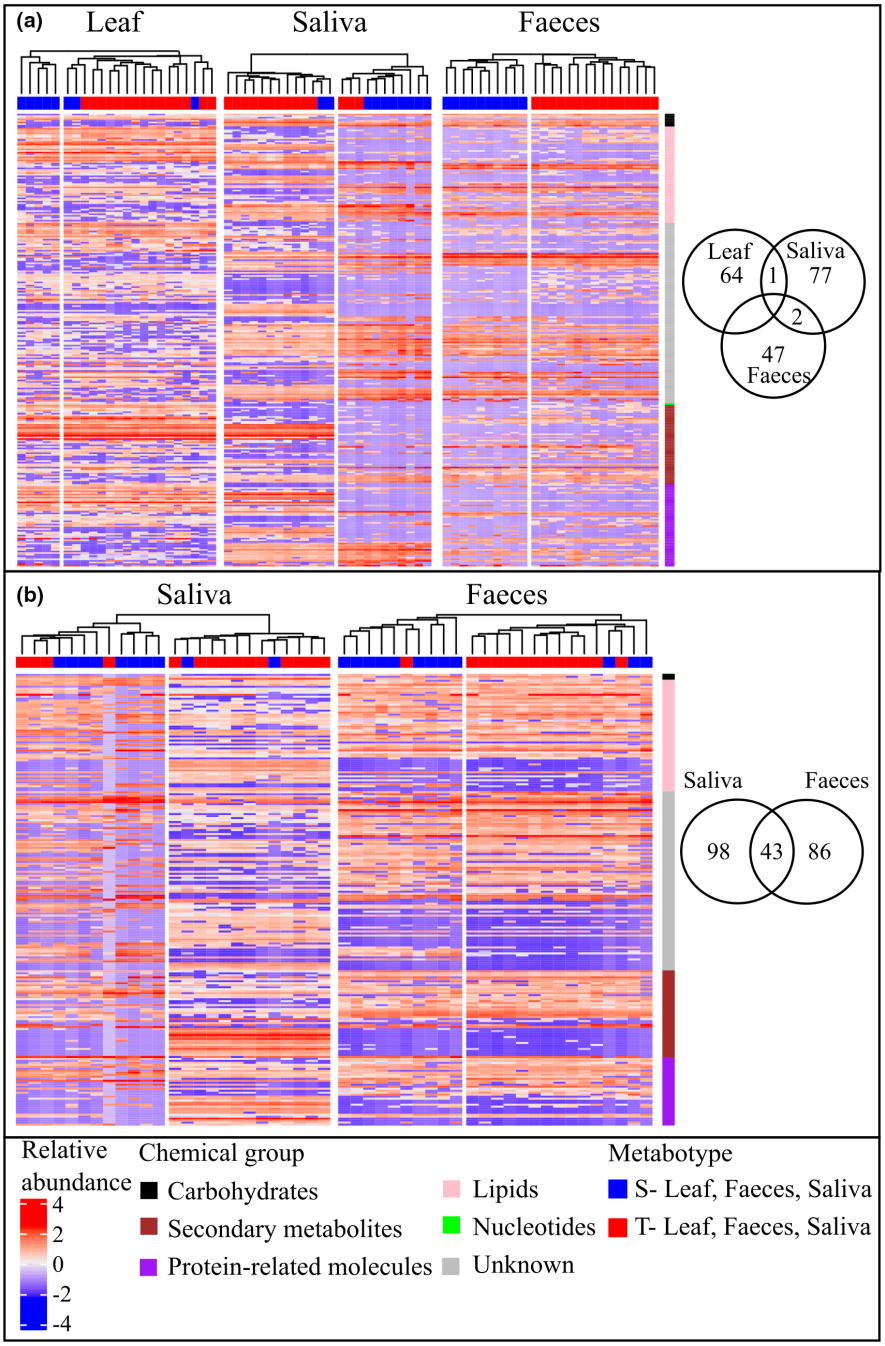


FIGURE 3 Hierarchical clustered heatmap of the significant phenotype-specific metabolome-related mass features. (a) Mass features with VIP > 2, Log2(T/S Log2(T/S) >3, and) <−3 of leaf, saliva and faecal metabolomes cluster the two susceptible (S-) and resistant (T-) oak metabotypes in each of three sample material. Each sample material is characterized by unique number of mass features showed in the Venn diagram. (b) Mass features appearing only in either saliva or faeces (unique compounds) showing different metabolic patterns of two oak phenotypes (VIP > 2, Log2(faeces/leaf), Log2(saliva/leaf) >2) (see methods for mass feature exclusion conditions). The Venn diagram shows the number of mass features which are in common, or saliva- and faeces-specific.

assigned to the phylum Proteobacteria were relatively more abundant in faeces of larvae fed uniquely with T-leaves, whereas ASVs associated to Firmicutes were relatively low (Figure 6b). To classify discriminant groups of bacteria species based on metabarcoding in more detail, we performed linear discriminant analysis (LDA) via LEfSe, and calculated Random Forest trees on both species and ASV level (Figure 6c,d). Five taxa and ASVs, respectively, were influenced by the diet, four increased upon S-diet (*Nakamurella* (ASV17), *Clostridium* sensu stricto 1 (ASV19), *Buchnera aphidicola* (ASV31) and *Staphylococcus warneri* (ASV33)) and one upon T-diet (*Modestobacter multiseptatus* [ASV28]) (Figure 6c,d). All identified taxa/ASVs showed high LDA scores ranging from 4.2 to 5.0 (adj. $p < 0.05$).

4 | DISCUSSION

Using comprehensive analyses, starting from leaf metabolic composition of two *Q. robur* phenotypes, we traced down the catabolism of these food sources into the excreted faeces and deciphered chemical reactions associated with the digestion of the contrasting diets of T- an S-oak metabotypes. Globally, plant secondary metabolites were less depleted by the larvae, while metabolites from the plant's primary metabolism were more taken up and metabolized after ingestion. We identified differences in the catabolism of the different European oak food sources, which could be associated to varying performance and mortality of the larvae. However, the gut microbiota of the larvae grown up on the two diets turned out to be quite

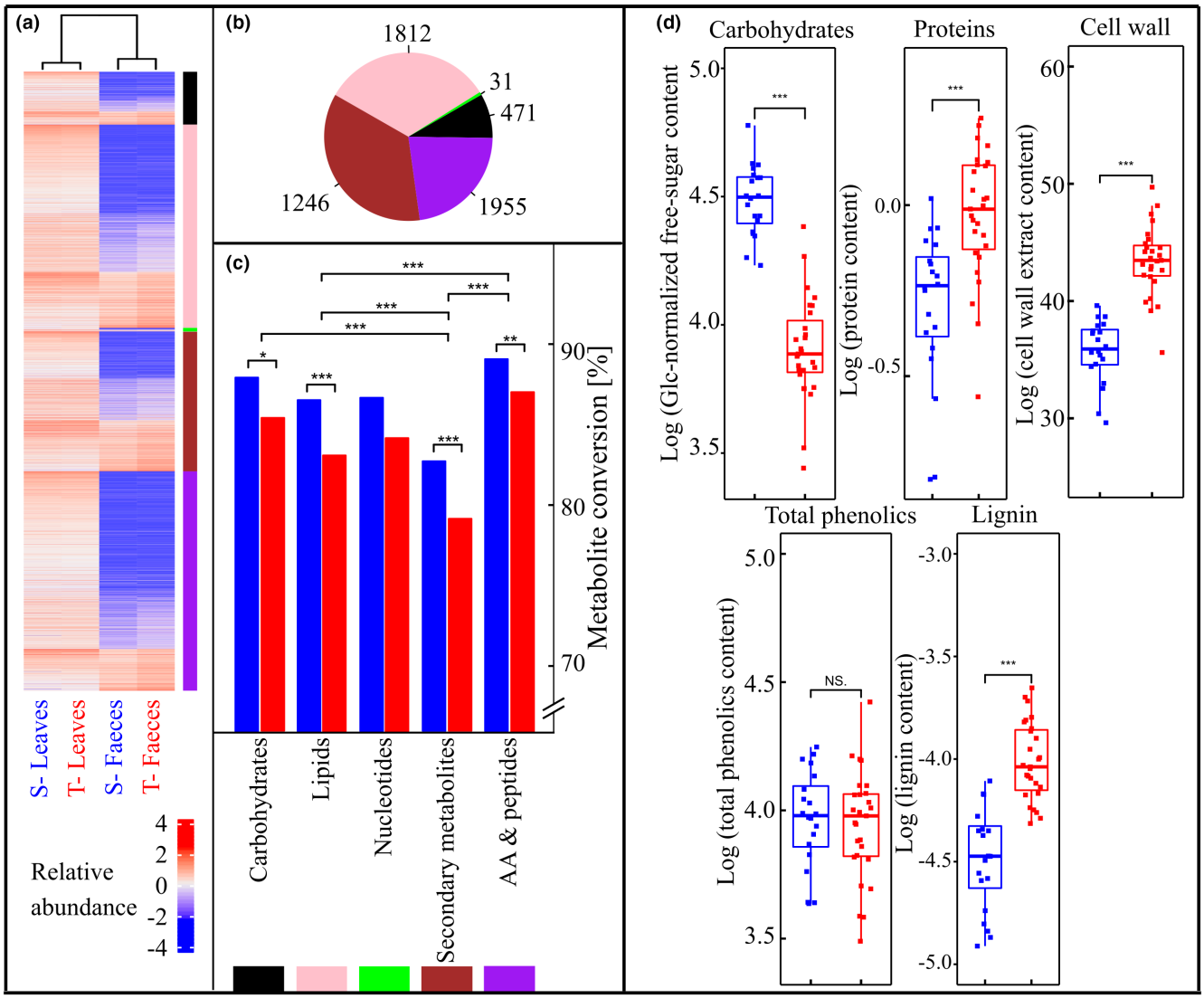
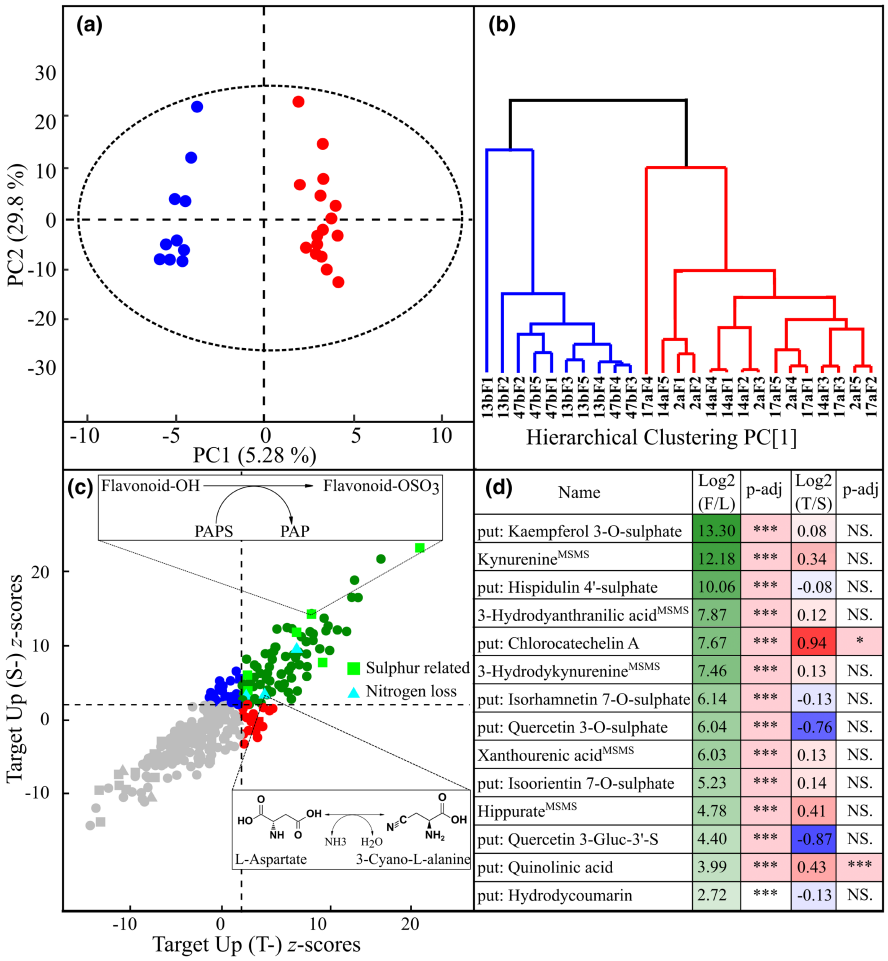


FIGURE 4 (a) Relative abundances of metabolite-related mass features in leaves and faeces showing different metabolite profiles dependent on S- and T-diet and after intake and passage through *T. viridana* body. Mass features were normalized by ellagic acid and glucogallin content and selected by means of highest values of VIP, Log2(T/S) ratios and lowest *p*-values to discriminate two phenotypes (OPLS-DA, see methods). (b) Pie chart with the number of the most relevant phenotype specific metabolites of main chemical groups. (c) Percentages of decreases of carbohydrates, lipids, nucleotides, secondary metabolites, amino acids and protein related in faecal compared to foliar metabolome upon S- and T- diet. Metabolites belonging to different chemical groups were classified according to multidimensional stoichiometric compound classification (MSCC) approach and are depicted with different colours in (a), (b) and (c) (see legend in c). (d) Content of the main oak foliar components in S- and T- phenotypes: total soluble sugars, total soluble proteins, total phenolic content, cell wall content and lignin content. ***$p < 0.001$; **$p < 0.01$; $p < 0.05$ (two sample *t*-test).

stable. Despite these differences, the larvae were extremely efficient at nutrient uptake and able to minimize the anti-feeding properties of many phytochemicals.

4.1 | Leaf quality as a key factor in larval performance

The present metabolome analysis of the saliva and faeces of larvae showed that secondary metabolites (mainly flavonoids) persist more the insect digestion system and that their higher abundance in the leaves of resistant European oaks results in a lower insect performance and higher mortality. Quantitative levels and chemical diversity of plant metabolites are known as key ecological factors for insect performance (Wetzel & Whitehead, 2020). The variability in host plant nutrient composition can suppress herbivore populations (Wetzel et al., 2016). Compared to the susceptible metabotype, T-oaks do not contain other foliar chemicals (i.e. same chemical richness) but differ in the relative concentrations (i.e. different chemical evenness) of primary (carbohydrates and amino-acid derivatives, higher in S-metabotypes) and secondary metabolites (some specific flavonoids, higher in T-metabotypes) as



Name	Log2 (F/L)	p-adj	Log2 (T/S)	p-adj
put: Kaempferol 3-O-sulphate	13.30	***	0.08	NS.
Kynurenine^{MSMS}	12.18	***	0.34	NS.
put: Hispidulin 4'-sulphate	10.06	***	-0.08	NS.
3-Hydrodyanthranilic acid^{MSMS}	7.87	***	0.12	NS.
put: Chlorocatechelin A	7.67	***	0.94	*
3-Hydrodykynurenine^{MSMS}	7.46	***	0.13	NS.
put: Isorhamnetin 7-O-sulphate	6.14	***	-0.13	NS.
put: Quercetin 3-O-sulphate	6.04	***	-0.76	NS.
Xanthourenic acid^{MSMS}	6.03	***	0.13	NS.
put: Isoorientin 7-O-sulphate	5.23	***	0.14	NS.
Hippurate^{MSMS}	4.78	***	0.41	NS.
put: Quercetin 3-Gluc-3'-S	4.40	***	-0.87	NS.
put: Quinolinic acid	3.99	***	0.43	***
put: Hydrodycoumarin	2.72	***	-0.13	NS.

FIGURE 5 (a) Orthogonal partial least square regression—discriminant analysis (OPLS-DA) scores and (b) hierarchical clustering based on Ward distance of discriminant mass features uniquely found in faecal samples of susceptible (S-) and resistant (T-) leaves diet. Fingerprints of unique metabolites in the insect faeces are separating the S- and T- metabotypes. OPLS-DA model fitness $R^2X(cum) = 0.428$, $R^2Y(cum) = 0.977$, $Q^2Y(cum) = 0.595$ using three predictive components ($p < 0.01$, CV-ANOVA). (c) Z-scores of upregulated mass differences (MD) at the faecal level. Blue dots represent *Z* scores of MDs significant only for faeces originating from S- diet, red dots are *z*-scores of MDs significant for faeces originating from T- diet, in green are *z*-scores of MDs significant for whole faecal sample material, where light green rectangular are reactions in which sulphur atom was added to the final product (compound found only in faeces), and in cyan triangles are reactions in which nitrogen loss was observed in the final product, and grey dots represent non-significant *z*-scores. (d) Metabolites found only in faecal samples with Log2(faeces/leaf), and Log2(T-faeces/S-faeces) ratios and corresponding adj. *p*-values based on Benjamini-Hochberg correction. Abbreviations: F, faeces; L, leaves; MS/MS, annotation based on spectra matches; put, putative annotation; and NS, not significant.

a result due to the growth-defence trade-off (Bertić et al., 2021). As shown here with the oak-specialist *T. viridana*, foliage consumption to reach the pupal stage was higher in larvae feeding on resistant oaks, while survival was about 10% lower than larvae feeding on S-oaks. This might be due to the relatively lower nutrient levels (affecting insect health), increased phytochemicals or both. We observed a specific formation of diet-dependent saliva and faecal metabolomes, indicating that the plant-specific dietary components of the resistant metabotype resulted in a diverse metabolic catabolization of host food intake. Notably, we detected a higher conversion of specific phytochemicals (mainly flavonoids) related to resistant metabotypes in faecal metabolomes, which means that *T. viridana* needs to neutralize the defensive compounds of resistant oaks more than those of S-oaks. On the other hand, the compounds of susceptible metabotypes, enriched in carbohydrates and reduced in secondary metabolites, appear to be more efficiently taken-up and may represent for the insects a more energy-rich resource of food that could be more efficiently converted into insect body mass, as seen by the lower FCR. The lower performance of insects reared on T-leaves may also have been influenced by the increased cell wall and lignin content, which affects the bioavailability and digestibility of nutrients (Calderón-Cortés et al., 2012; Neutelings, 2011). Although insects might be able to degrade the components of the cell walls including cellulose, hemicellulose, pectin and lignin by producing cellulases, hemicellulases, pectinases and ligninases (Calderón-Cortés et al., 2012; Scott et al., 2010), their catabolism in the gut is often helped by symbiotic microorganisms (Scott et al., 2010). We did not detect

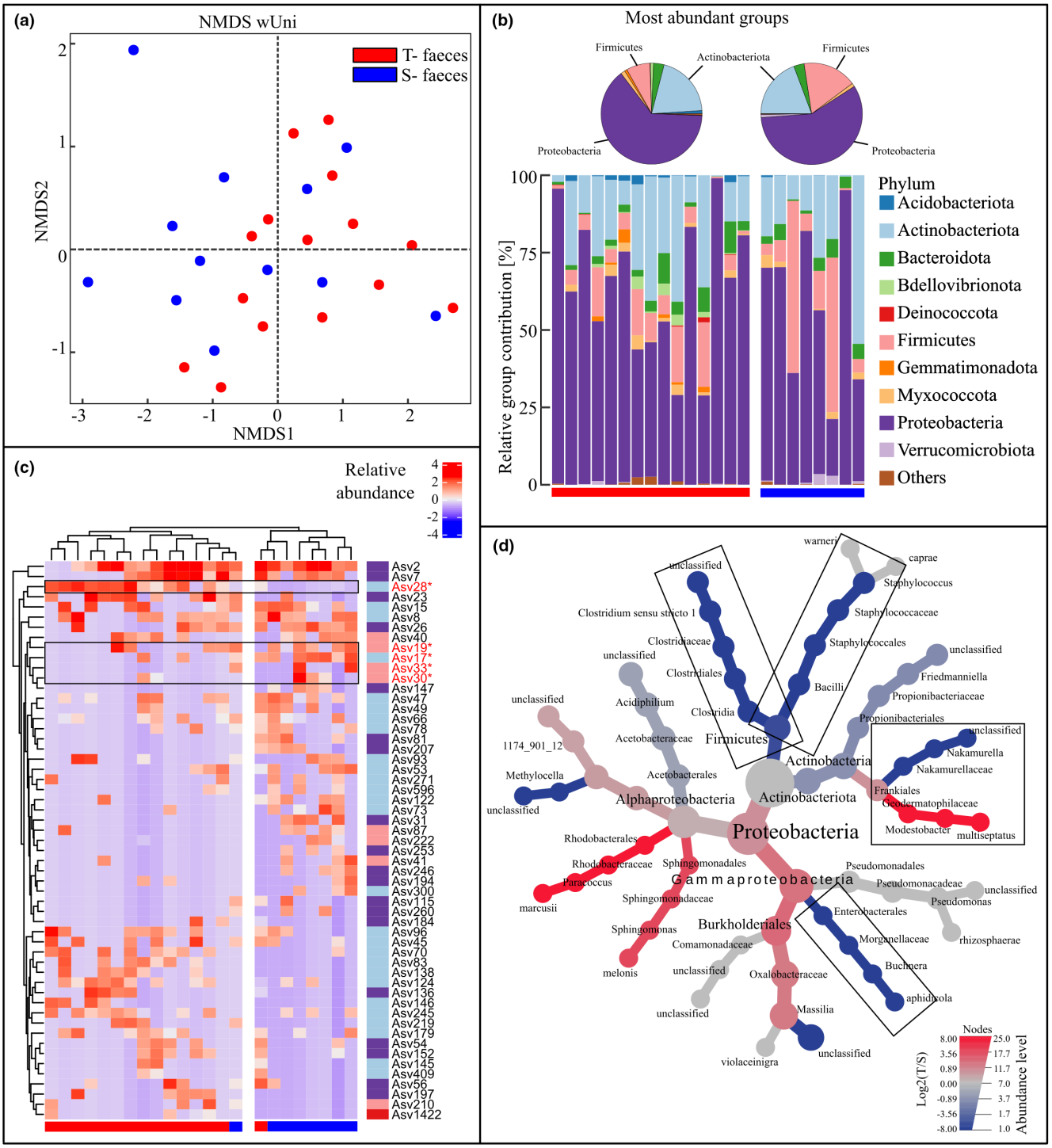


FIGURE 6 Microbiome analysis of larval faeces feeding either susceptible (S-, in blue) or resistant (T-, in red) oak leaves. (a) Two-dimensional non-metric multidimensional scaling (NMDS) plot based on weighted UniFrac distance (wUni) of bacterial communities in faecal samples. (b) Top-10 most abundant bacterial genera present in faecal samples. (c) Clustered heatmap of metabotype-specific amplicon sequence variants (ASV) for faeces originating from S- and T- leaf diets. Fingerprints of ASV are separating the metabotypes that compose the resistant and susceptible oak phenotypes. In red, significant ASV detected by linear discriminant analysis (LDA) via LEfSe and Random Forest trees. Values are relative abundances logarithmically transformed, and Pareto scaled with centering, clustered using Euclidean distance. (d) Phylogenic tree with marked significant bacterial taxa responsible for S- and T-faeces discrimination. The colours and their intensity refer to Log2(T/S) ratios. All identified significant taxa an ASV had LDA scores between 4.2 and 5 at significance level of adj. $p < 0.05$.

major differences in the composition of the faecal microbiome, despite feeding the *T. viridana*'s annual offspring exclusively on one of the two metabotypes for several years and using insects from the same population, that is, genetically close-related and having similar enzymatic degradation potentials. We interpret the higher cell wall and lignin contents in T-leaves as another indication of the low leaf quality that in addition to the lower carbohydrates affected insect performance. Performance of *T. viridana* generally increase with nitrogen and carbohydrate content of the host plant, as demonstrated by rearing the herbivores on different oak species (Yazdanfar et al., 2015). We observed a higher abundance of total soluble protein in T-oaks. This result might appear contradictory. However, besides being a source of nitrogen, whose total content does not differ between the T- and S-oaks (Bertić et al., 2021), protein composition is crucial for insect performance, for example, the presence of protease inhibitors (PIs) (Broadway & Duffey, 1988). Our previous studies showed that the transcript levels of PIs are strongly upregulated (logFC >6) in T-oaks (Kersten et al., 2013) suggesting that protein quality, rather than quantity, is important in the feed performance of *T. viridana*. However, the involvement of PIs in the development of resistance of *Q. robur* to *T. viridana* require future dedicated studies.

4.2 | *Tortrix viridana* efficiency in leaf metabolome utilization

Our data showed that *T. viridana* larvae feeding on *Q. robur* have a high conversion efficiency. Overall, insects are very efficient at converting ingested food into body mass (van Huis & Oonincx, 2017), and mealworms and cockroaches are the most efficient among different insect species, with feed conversion ratios (FCR) of 2.7–4.8 (Oonincx et al., 2015). A low FCR indicates that some insects can overcome phytochemicals or even metabolize them (Mello & Silva-Filho, 2002). In our work, we were able to study the different classes of metabolites beyond the FCR values. *T. viridana* larvae were also very efficient (FCRs of 3.34 and 3.62 for S- and T- larvae respectively) in consuming different classes of chemicals: from carbohydrates to the wide range of secondary metabolites, the metabolite conversion was 77%–87%. Herbivores that specialize on particular plant species possess specific enzyme systems that allow them to metabolize the host's phytochemicals (Pentzold et al., 2014). A considerable percentage (62.20%) of all phytochemical compounds were not found in the faecal metabolome, suggesting that the specialist insect *T. viridana* is able to metabolize, transform and/or sequestrate a large proportion of phytochemicals (Erb & Robert, 2016; Nishida, 2002). Whereas, catabolization of phytochemicals might contribute to the high efficiency in converting food (i.e. low FCR number), the transformation seen (e.g. sulfation and sulfonation reactions) and possibly sequestration may represent an evolved strategy of the specialized *T. viridana* to cope with the high richness and abundance of phytochemicals present in *Q. robur* leaves.

4.3 | Larval digestion ability reflects the chemical compositions of different diets

We showed that *T. viridana* larvae are partially able to remove host phytochemicals, as seen by the strong decrease of phenolics in the faeces compared to the leaf metabolome. Also, we observed metabolic reactions within the larvae that indicate degradation pathways used differentially on the two plant metabotypes. Overall, oak leaves are well-known to be rich in phenolics (Damestoy et al., 2019). In defending against herbivores, some plant phenolics (e.g. 2-hydroxycoumaric acid, quercetin, kaempferol) can act as growth inhibitors or direct toxins (Kumar et al., 2020) or have beneficial effects acting as antioxidants (Johnson & Felton, 2001). Resistant and susceptible European oaks have similar total phenolic content but a different composition: T-oaks are characterized by higher levels of quercetin 3-glycoside, whereas S-oaks by kaempferol 3-glycoside (Ghirardo et al., 2012). Insects on the other side, have evolved mechanisms to evade, neutralize, or even use the very high content of plant phenolics for their own defence against predators (War et al., 2019). One of these mechanisms is the transformation to a more polar compound by sulfation (Ferreres et al., 2008; Salminen et al., 2004); a common detoxification process that also occurs in mammalian livers (Raftogianis et al., 1999). The higher polarity of sulfated flavonoid derivatives increases water solubility and decreases adsorption in the insect's fat body, so they can be more easily excreted (Coughtrie et al., 1998). We found it very intriguing to detect mass differences related to sulfation reactions, in addition to the reactions (e.g. oxidations, hydroxylation, decarboxylation, transamination) and intermediates (e.g. formation of galactinol, acetyl-CoA, alanine, uracil, urea, ketones, amino acids, amines) typically occurring in the catabolism of sugars, lipids, nucleic acids and proteins. Other relevant reactions implied in the detoxification of secondary metabolites are oxidations mediated by oxidases such as the cytochrome P-450 enzymes (Karban & Agrawal, 2002) and metabolization of cyanogens (Engler et al., 2000). Linamarin is a plant cyanogenic glucoside that releases the toxic hydrogen cyanide during larvae digestion. The MDEA dataset shows the degradation of linamarin and the detoxification of the hydrogen cyanide by reaction with aspartate to form the cyanoalanine (Ohlen et al., 2016). Cyanogenic glucosides have not been reported in oak yet, although they presence is widely distributed in the plant kingdom (Zagrobelny et al., 2004).

Although our MDEA data shed some light on a few intriguing putative catabolic processes, mechanistic studies of larval detoxification and catabolism are needed to elucidate the complex processes involved in the insect metabolism of phytochemicals.

4.4 | Contrasting metabotypic diets influence larval gut microbiota

We observed some changes in the insects' gut microbiota upon the contrasting diets, although the microbiome was overall relatively

stable. The major phyla detected in our study are consistent to those described by Yun et al. (2014) and Colman et al. (2012), showing that Proteobacteria (57%–62%), Firmicutes (21%–22%) and Actinobacteria (5%) (seen in Yun et al., 2014) are the major constituents of insect gut microbiota. We detected a very similar proportion of Proteobacteria (60%), but lower relative abundance for Firmicutes (12%) and higher relative abundance for (20%) Actinobacteria. It is intriguing to note that the specialist insect *T. viridana* has a higher proportion of Actinobacteria. Actinobacteria are involved in defensive symbioses with several insect taxa (Kaltenpoth, 2009) and engage a nutritional mutualism with their host (Salem et al., 2013). The lower proportion of Firmicutes is somehow unexpected, as the extreme alkalinity of lepidopteran larvae gut supports the growth of specialized alkaline-tolerant symbiotic bacteria such as Firmicutes and Clostridium (Engel & Moran, 2013). Since *T. viridana* larvae are essentially monophagous and we fed the larvae using specifically *Q. robur* metabotypes, food diversity is very limited. Yun et al. (2014) observed that the bacterial diversity is higher in polyphagous than in monophagous insects when comparing the gut microbiome of 218 insect species. Microbial gut colonization strongly depends on physiochemical conditions. Midguts of lepidopteran larvae are adapted to tannin-rich diets showing an exceptionally high pH of 11 to 12 (Appel & Martin, 1990; Harrison & Shew, 2001). These alkaline conditions are thought to decrease the binding affinity of dietary proteins with ingested tannins, thereby improving nutrient availability. At the same time, the abundance of acidophilic and neutrophilic bacteria decreases (Engel & Moran, 2013) leading to a reduction in the diversity of the gut microbiome.

Another explanation for the relative resilience of the gut microbiome of insects reared either on S- or T-leaves might originate from metabotype-specific microorganisms that colonize oak leaves as epi- and endophytes (phylloplane). Studies suggest that the presence/absence of certain microorganisms is determined by metabolic activities of the host plant. For instance, the volatile isoprene can be metabolized by epiphytes on leaves (Crombie et al., 2018). While European oak is a strong isoprene-emitting plant species (Lehning et al., 1999), we previously found significant differences in the emissions of isoprenoids from T- and S-oaks (Ghirardo et al., 2012). To clarify this, the leaf microbiomes of different oak metabotypes need to be characterized in the future.

Herbivores may undergo gut acclimation in response to changes of nutrition (Wetzel and Thaler, 2016). We observed moderate differences of the *T. viridana* gut microbiota (analysed from the faeces) as consequence of the T/S diet. In comparison, the gut microbiota of *Lymantria dispar* caterpillars, a generalist species also feeding on oaks, was observed more sensitive to the diet (Broderick et al., 2004). This is in line with the larger diversity of phytochemicals that a generalist insect can experience compared to a specialist. However, the gut microbiota stability is rather high in insects (Colman et al., 2012; Yun et al., 2014) compared to, for example, mammals (Engel & Moran, 2013; Ley et al., 2008).

Nevertheless, we could identify individual bacterial taxa that occur in different amount depending on S- and T-diets. The abundances of *Clostridium* sensu *strictu* 1, *Staphylococcus warneri*, *Nakamurella* and *Buchnera aphidicola* were found lower upon T-diet. The genera *Clostridium* and *Staphylococcus* are typical gut bacteria of insects and involved in cellulose degradation (Siddiqui et al., 2022). Therefore, the higher cell wall of T-leaves might have increased their abundances. Also, *Clostridium* aids more efficient biomass usage (Chen et al., 2016). *Buchnera aphidicola* is known as endosymbiont in aphids to which it supply vitamins and essential amino acids (Akman Gündüz & Douglas, 2009; Birkle et al., 2002; Nakabachi & Ishikawa, 1999), although its occurrence in *T. viridana* is currently unknown and the possible presence of aphids in the leaf surface was not investigated. The biological function of *Nakamurella* (higher upon S-diet) and *Modestobacter multiseptatus* (higher upon T-diet) is unknown. However, the differences of the microbiota might, besides other factors, contribute to the lower fitness and higher mortality of larvae reared on T-diet. Future studies are therefore required, which may address also other microbiota of the gut including fungi and yeasts which may respond to various forms of diet differently.

5 | CONCLUSIONS

In oak forests, outbreaks of the specialist herbivory *T. viridana* can lead to complete defoliation of susceptible European oaks (Bertić et al., 2021; Ghirardo et al., 2012). Chemical defence of resistant oaks is avoided by the adult female moth which recognize the chemical volatile cues of S-oaks (Ghirardo et al., 2012). Here, the actual holistic analysis of oak leaf metabolomes and their gradual changes, from leaf—to saliva—to faeces, during the ingestion process of *T. viridana* larvae demonstrates the central importance of food chemical composition in the development and mortality of herbivorous insect larvae. The study provided new insights into the herbivore-plant interaction, from the insect's metabolic transformations (e.g. sulphur insertion) to the effects of variation in secondary metabolites (flavonoids) on herbivory resistance. Comparison of the contrasting metabotypes of *Quercus robur* leaves with respect to the resistance/susceptibility to *Tortrix viridana* showed how plant chemical composition and unevenness can influence the larval digestive system and affect the insect's fitness and microbiota. This underlines the importance of intraspecific plant chemical diversity on insect pressures.

AUTHOR CONTRIBUTIONS

Andrea Ghirardo, Matthias Fladung, Hilke Schroeder and Jörg-Peter Schnitzler designed the study. Marko Bertić conducted metabolomics and all statistical analysis. Franziska Orgel and Hilke Schroeder performed feeding experiments and collected the sample material. Silvia Gschwendtner and Michael Schloter conducted microbiome analysis. Franco Moritz and Philippe Schmitt-Kopplin computed the MDEA. Andrea Ghirardo, Marko Bertić, Franziska Orgel, Hilke Schroeder and Jörg-Peter Schnitzler wrote the manuscript. All authors read, corrected and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study can be found here: https://osf.io/u38e7/ and at https://www.ncbi.nlm.nih.gov/ (accession number PRJNA817404).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Representative picture of a feeding experiment with larvae set on oak leaves and enclosed with a crispac bag.

Figure S2. Refraction curves reflecting the saturation that has occurred at subsample level.

Figure S3. Leaf area eaten by larvae at all developmental stages in relation to larval weight gained during larval development.

Figure S4. Microbe a-diversity estimation: ASV richness, Shannon diversity index and Pielou's evenness indices.

Figure S5. Top-twenty most abundant bacterial taxa between faeces originating from two diets.

Methods S1. Colorimetric soluble sugar assay.

Methods S2. Soluble protein assay.

Methods S3. Total phenolic assay.

Methods S4. Determination of lignin.

Methods S5. Microbiome analysis.

Table S1. Experimental conditions of the larval 48 h feeding experiment.

Table S2. Metabolomic data matrix of leaf, saliva and faeces samples from feeding experiment (enclosed).

Table S3. Calculated ratios between metabolites stable through larval digestion system (enclosed).

Table S4. Unique metabolite in faecal samples (enclosed)

Table S5. Mass difference enrichment analysis between masses of source (leaf) and target (faeces) (enclosed).

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