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Linking forage choice behavior of goats with the metabolome of contrasting silages

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ABSTRACT

The interrelationships between silage preference of ruminants and compounds that may affect forage choice are not yet fully enlightened. Analysis of the forage metabolome in addition to conventional chemical analysis and preference trials can provide new insights. Six silage treatments each of alfalfa (AL) and red clover (RC), with different dry matter concentrations (222–391 g/kg), silage additives, and intended addition of soil, were produced in quadruplicate to obtain a range of qualities. After 120 d of ensiling, silages were sampled for chemical analysis, vacuum-packed, and refrigerated for subsequent preference trials with goats. Within 21 d, each possible combination of 2 silages and an AL hay that served as control (n = 21) was presented to goats (Saanen-type wethers, n = 8, body weight 105 ± 2.7 kg) for 3 h for ad libitum intake. Comparisons among means for 3-h dry matter intake (DMI) for forages offered in choice situations were made using variance analysis, including terms for treatment and animal and the Waller-Duncan k-ratio *t*-test to separate means. The most preferred and avoided treatments of AL and RC silage amounted to 863, 858, 226, and 282 g DMI/3 h, respectively. To further explore relations between silage composition and preference, a metabolome analysis of the most preferred and most avoided AL and RC treatments were conducted. Metabolites (all low molecular weight molecules) were analyzed by a non-targeted metabolite profiling in the range of 50–1,700 Da. Metabolites showing the most distinct difference between preferred and avoided silages were identified by partial least squares discriminant analysis. In the 2 selected treatments of each plant species (those that were most different in forage preference), more than 6,400 compounds were detected and 2,010 were identified. Between preferred and avoided treatments, 934 of the detected compounds differed in RC and 1,860 in AL, of which 475 were altered in both plant species (251 were reduced and 186 were increased; only 38 behaved contrarily, meaning that they were increased in one substrate and decreased in the other). The database provides a useful foundation for the approach of explaining silage preference by ruminants.
Key words: goat, metabolome, preference, silage

INTRODUCTION

At the end of the 1990s, a trend was observed over the preceding 30 years that indicated that the proportion of forage conserved as silage had increased, whereas the proportion of haymaking had declined (Wilkinson et al., 1996). Since then, this development has continued (Martin et al., 2017). A comprehensive survey in the United States revealed that corn and alfalfa silages accounted for more than one-third of the dietary DM of dairy cows (Thoma et al., 2013). For many dairy feeding systems, silages provide the forage base because they supply energy, protein, and digestible fiber to ruminant diets (Martin et al., 2017). However, ensiled forage offered to ruminants has often resulted in a lower voluntary DMI compared with fresh (Donaldson and Edwards, 1976) or dried feed (Thiago et al., 1992). Various attempts have been made to establish relationships between silage composition and feed intake (e.g., Huhtanen et al., 2002, 2007) as well as feed preference, including volatile fatty acids, alcohols, acetone, NH_3-N, ethyl esters (Gerlach et al., 2013, 2019; Brüning et al., 2018), and biogenic amines (Scherer et al., 2019). These studies are valuable for the exploration of the relationships between the variety of nutrients and sensory properties of silages and forage choice of ruminants. However, much is still unknown, and the specific mechanism or single compounds affecting intake or preference in many cases have not been elucidated (Grant and Ferraretto, 2018). Only recently, Guo et al. (2018) examined the metabolome of ensiled alfalfa (*Medicago sativa* L.) and detected 280 substances, of which 120 were identified. However, the focus of Guo et al. (2018) was on microbial community dynamics involved in en-

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siled alfalfa inoculated with lactic acid bacteria and not on the relationship between feed composition and feed choice or intake. Other than in silage research, metabolomics has been used for several years in food science research programs, such as Metabolomics for Plants, Health and OutReach, and it has the potential to address problem-solving approaches to global nutrition (Hall, 2007). In addition, the metabolome of a variety of foods and beverages such as vegetables and tea has already been characterized extensively (Ulrich et al., 2015; Xu et al., 2015).

This study aimed to make a snapshot of the metabolic status of 4 different silages with a known forage choice data to identify metabolites that may be related to a reluctant or improved silage preference. To achieve the most holistic view of relations between fermentation conditions, resulting silage composition, and feed choice behavior of goats, we, therefore, conducted a combination of comprehensive chemical analysis including analyses of fermentation acids and biogenic amines, CP fractionation, and forage preference trials (Scherer et al., 2019) with metabolomics. It is hypothesized that metabolomics can help to carve out links between forage and animals that are not detectable with the conventional chemical characterization of feedstuffs.

MATERIALS AND METHODS

Silage Preparation and Sampling and Feed Choice Trial

Silages were prepared from pure stands of alfalfa (**AL**, *Medicago sativa* L., first cut) and red clover (**RC**, *Trifolium pratense* L., first cut). Alfalfa was cultivated at the Educational and Research Center Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany). Red clover was cultivated at the Educational and Research Center Hofgut Neumühle, Münchweiler an der Alsenz, Germany. Both species were harvested at the late vegetative stage, and 6 silage treatments were prepared for each forage species. The treatments included untreated control silages with 2 different DM levels (low, 274 and 232, and high, 380 and 301 g/kg for AL and RC, respectively), silage additives [biological additive based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; 3.0 × 1,011 colony-forming units/g; **BIO**); chemical silage additive (2.5 L/t) based on sodium nitrite and hexamine (hexamethylenetetramine; **CHE1**); and chemical silage additive (4 L/t) based on 75% formic acid buffered with sodium hydroxide to pH 2.5 (**CHE2**)], and intended addition of soil to simulate a soil-contaminated substrate (**SOIL**, the addition of 7,600 g soil/t; for details see Scherer et al., 2019). The BIO and CHE1 treatments were prepared from the high DM levels and the CHE2 and SOIL treatments were prepared from the low DM levels. Refined sugar (sucrose; Diamant Zucker, Pfeifer & Langen, Cologne, Germany) was added to AL and RC at ensiling to ensure adequate substrate availability. For this reason, 125 g/kg DM and 75 g/kg DM sucrose were added to AL and RC, respectively. The forage was processed in a clean dry place immediately after unloading from the loader wagon. For this purpose, the appropriate quantities were spread on a tarpaulin (3 × 4 m) and treated accordingly. Afterward, each treatment was ensiled in quadruplicate in plastic barrels with a lid (120 L). Each barrel was sampled after 120 d of fermentation for analyses and then homogenized, vacuum-packed in 2 to 3 kg portions, and stored in a cooled chamber until feeding.

For a detailed description of the preparation of silages, the general and chemical analyses of fermentation variables, and the experimental procedure, see Scherer et al. (2019). Briefly, after predrying at 60°C, the DM of the silages was estimated by oven-drying a triplicate subsample overnight at 105°C. A correction of DM (DM_{cor}) for the loss of volatiles during drying was conducted using the following equation (Weißbach and Strubelt, 2008): $DM_{cor} = DM + (1.05 - 0.059 \times pH) \times$ total VFA (C2 to C6) + 0.08 × lactic acid + 0.77 × 1,2-propanediol + 0.87 × 2,3-butanediol + 1.00 × total of other alcohols (C2 to C4). All concentrations are expressed as g/kg. Samples for all other analyses were freeze-dried in duplicate (P18K-E-6; Dieter Piatkowski, Munich, Germany) and then ground using 3- and finally 1-mm screens.

Proximate analyses of the silage samples and of an AL hay that served as a control in both preference trials were performed according to VDLUFA (2012), and method numbers were given. Ash and crude lipids (**CL**) were analyzed using methods 8.1 and 5.1. Crude protein was determined by Dumas combustion (4.1.2, Elemental Analyzer rapid micro N cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). The concentrations of NDF [6.5.1; assayed with heat-stable amylase (aNDF)], ADF (6.5.2), and ADL (6.5.3) were analyzed using the Fiber Analyzer Ankom A2000 (Ankom Technology, Macedon, NY). The NDF and ADF values are expressed exclusive of residual ash (aNDFom, ADFom). In accordance with point 8.8 of method 6.5.2, the analysis of ADFom was performed sequentially for pectin-containing AL and RC samples.

The Hohenheim gas test (method 25.1; VDLUFA, 2012) was conducted to measure the 24-h in vitro gas production (**GP**) and used to estimate the concentration of ME of the AL and RC silages using the following equation (GfE, 2017): ME (MJ/kg organic matter) = 12.49 − 0.0114 × ADFom + 0.00425 × CP + 0.0269 ×

CL + 0.01683 × GP; ME (MJ/kg DM) = ME (MJ/kg organic matter) × [1,000 − ash]/1,000. Ash, CP, CL, and ADFom are in g/kg DM, and GP is in mL/200 mg DM.

Frozen subsamples (50.0 g) of silages were used for the determination of lactic acid, acetic acid, pH, ammonia-N, and water-soluble carbohydrates. Cold-water extracts were prepared by blending the frozen samples with a mixture of 300 mL of distilled water and 1 mL of toluol, kept in a refrigerator overnight, and afterward, filtered with a folded filter paper (MN 615, Macherey-Nagel, Düren, Germany). The pH in the extract was determined potentiometrically using a calibrated pH electrode. The extract was filtered through a Minisart syringe filter (pore size 0.45 μm; Sartorius, Göttingen, Germany) according to Weiß and Kaiser (1995). Volatile fatty acids and alcohols were determined by gas chromatography (flame ionization detector, Shimadzu Deutschland, Duisburg, Germany), as described by Weiß (2001). The analysis of propanol, methanol, 1-butanol, and 2-butanol was performed following Weiß and Sommer (2012). The lower detection limit for VFA and alcohol was 0.01%, and for esters, it was 0.001%. The NH_3-N concentration was analyzed colorimetrically based on the Berthelot reaction, using a continuous flow analyzer (Skalar Analytical, Breda, Netherlands). The concentration of water-soluble carbohydrates was determined with the anthrone method according to von Lengerken and Zimmermann (1991).

For each forage species, one preference trial with Saanen-type wethers (German Improved White Goat breed, n = 8, body weight 105 ± 2.7 kg) was conducted to evaluate the feed choice behavior following the procedure of Burns et al. (2001). Animal care and experimental procedures were conducted according to the German Guidelines and Regulations on Animal Care (Deutsches Tierschutzgesetz). Two wethers shared an indoor pen of approximately 2 × 3 m bedded with straw. Every morning, before offering the silages, the animals were tethered for the duration of experimental feeding, and it was ensured that they could drink and lie down. Crucial for collecting reliable preference data is the adaptation period before the experimental phase (Kyriazakis et al., 1990), where single meals of each AL and RC silage treatment and AL hay were offered once in randomized order to allow the animals to associate the forage with the smell, taste, and postingestive metabolic response. The AL hay was used as a standard in both runs so that quantitative comparisons between both runs were possible.

Within the 21 d of the following experimental period, each possible combination of 2 silages and the AL hay (n = 21) was presented for 3 h for ad libitum intake to goats in randomized order. Each forage of the pair was offered in a separate plastic box (400 × 340 × 250 mm), and the forage pairs were presented side by side and were randomly allocated each day to prevent a habit reflex. Goats had free access to both feeding boxes so that free choice between the 2 forages could be guaranteed. The boxes were weighed 30 min after starting to feed (initial forage choice) and after 3 h. To ensure ad libitum feed intake, the respective forage was refilled as soon as less than 300 g remained in the box. Each day, the experimental meal was offered for 3 h, starting at 0730 h.

To have the optimum comparison of feed composition with regard to preferred and avoided feed compounds, the most and least preferred silage treatment of each preference trial was selected for metabolome analysis. In both forage species, it was the untreated, high DM control (**CON**) and the soil-treated, low DM forage (SOIL). The abbreviations for the treatments, which are used in the following, are composed of the abbreviation for the forage species (AL or RC), the DM concentration (38, 30, 27, or 23%), and CON or SOIL (i.e., AL38CON, RC30CON, AL27SOIL, and RC23SOIL). The chemical composition and preference (expressed as g DMI/3 h for forages offered in choice situations) of the AL and RC silages selected for metabolome analysis are shown in Table 1.

Sample Preparation and Processing

Freeze-dried samples of the 4 silage treatments (n = 4 per treatment) selected for metabolome analyses were homogenized, and 10 mg filled in 2-mL micro test tubes (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) and forwarded to an external laboratory (Metabolomic Discoveries GmbH, Potsdam, Germany). One milliliter methanol (−20°C) including internal standards was added to 10 mg of freeze-dried silage and immediately vortexed for 15 s. For metabolite extraction, the tubes were incubated at 70°C, with continuous shaking at 1,000 rpm for 15 min. Then 480 μL of cold H_2O was added. Samples were vortexed for 15 s and centrifuged at 13,500 rpm for 15 min. An aliquot of 200 μL was taken and stored at −80°C for further analyses.

Nontargeted Metabolite Profiling

For the metabolome analysis, the nontargeted profiling approach was chosen. Nontargeted metabolite profiling comprised analyses by gas chromatography-mass spectrometry (**GC-MS**) and liquid chromatography-quadrupole-time of flight-mass spectrometry (**LC-**

Table 1. Chemical composition[1] and preference (expressed as g of DMI for forages offered in choice situations) of alfalfa (AL) and red clover (RC) silages (g/kg of DM unless stated; n = 4)

	Treatment[3]			
Variable[2]	AL38CON	AL27SOIL	RC30CON	RC23SOIL
DM (g/kg)	374	276	301	234
Ash	106	144	103	130
CP	171	178	159	162
Crude lipids	25	30	28	32
NDF	393	439	335	365
ADF	326	324	241	248
ADL	79	85	42	49
ME (MJ/kg DM)	11.0	11.4	12.1	12.0
pH	4.57	4.55	4.36	4.03
Lactic acid	59	66	65	95
Acetic acid	19	62	24	46
Ammonia-N (g/kg N)	112	147	72	67
Water-soluble carbohydrates	61	11	127	31
DMI (g/30 min)	376	68	434	87
DMI (g/3 h)	748	226	858	283

[1]For more details see Scherer et al. (2019).

[2]Neutral detergent fiber was analyzed with heat-stable amylase and expressed exclusive of residual ash; ADF was expressed exclusive of residual ash.

[3]Treatments: CON = untreated control, SOIL = addition of 7,600 g of soil/t; 38, 27, 30, and 23 = DM concentration (%) of treatment. Treatments were chosen as the most preferred (AL38CON and RC30CON) and the most avoided (AL27SOIL and RC23SOIL) silages from 2 preference trials with a total of 12 silage treatments (Scherer et al., 2019).

QTOF/MS). Metabolites can be analyzed in the range of 50–1,700 Da, with an accuracy of up to 1 to 2 ppm and a resolution of mass/Δmass = 40,000. Metabolites measured in the LC were annotated according to their accurate mass, subsequent sum formula prediction, and retention time. In some cases, several metabolite annotations were possible. Only the metabolite with the highest probability score or defined retention time was considered. Metabolites that were not annotated in the LC-MS and GC-MS analyses were not included in the subsequent data analysis.

Derivatization and analyses of metabolites by a GC-MS 7890A mass spectrometer (Agilent, Santa Clara, CA) were carried out as described by Lisec et al. (2006). Metabolites were identified in comparison to entries of authentic standards in the database of Metabolomic Discoveries. The LC separation was performed using hydrophilic interaction chromatography with a ZIC-HILIC 3.5 μm, 200 A column (Merck Sequant, Umeå, Sweden), operated by an Agilent 1290 UPLC system (Agilent). The LC mobile phase was (A) 95% acetonitrile, 5% 10m*M* ammonium acetate and (B) 95% 10 m*M* ammonium acetate, 5% acetonitrile, with a linear gradient from 95% A to 72% A in 7 min, to 5% A in 8 min, followed by 3 min wash with 5% A. The flow rate was 400 μl/min, and the injection volume was 1 μl. The MS was performed using a 6540 QTOF/MS detector and an Agilent jet stream electrospray ionization (AJS ESI) source.

Taxonomical Classification of Metabolites

The metabolites that could be assigned to the Metabolomic Discoveries internal database were classified into the chemical taxonomy including superclass, class, subclass, and the direct parent of each metabolite. As a plant-related database was not available, the classification was conducted with version 3.6 to 4.0 of the Human Metabolome Database (**HMDB**; HMDB, 2020), a freely available electronic database providing detailed information about metabolites of the human body. It contains 42,632 metabolite entries including both polar and nonpolar metabolites as well as metabolites that would be regarded as either abundant (>1 u*M*) or relatively rare (<1 n*M*). The HMDB supports extensive text, sequence, chemical structure, and relational query searches and is complemented by 4 additional databases: the DrugBank (drug metabolites, http://www.drugbank.ca), T3DB (Toxin and Toxin Target database, common toxins, and environmental pollutants, http://www.T3db.ca), SMPDB (the Small Molecule Pathway Database, pathway diagrams, and disease pathways, http://www.smpdb.ca), and FooDB (food components and food additives, http://www.foodb.ca).

Calculations and Statistical Data Evaluation

Detailed information on statistical evaluation of the forage preference data is given in Scherer et al. (2019).

The experimental design allowed statistical analysis of the preference trials by multidimensional scaling, as previously described by Burns et al. (2001) and Scherer et al. (2019) as well as by 2-factorial ANOVA after averaging the DMI of each forage offered in choice situations (averaged across each combination, n = 6). The ANOVA included terms for animals and forage. Within the forage treatments, the means were separated using the minimum significant difference from the Waller-Duncan k-ratio *t*-test (k = 100; Burns et al., 2001). Based on results from the ANOVA, the most and least preferred silage treatments (n = 4 with 4 replicates each) from both runs were selected for metabolome analysis.

The multivariate statistical analysis of the silage metabolite profiles was performed using a web tool MetaboAnalyst 4.0 (Chong et al., 2018; for detailed methodology see http://www.metaboanalyst.ca). Briefly, the metabolite data were log-transformed and Pareto scaled to correct for heteroscedasticity (variable variance) and the skewness of the data (van den Berg et al., 2006). A principal component analysis was conducted to illustrate the variances between the 4 different sample groups. The initial evaluation of the effect of single metabolites on feed choice was made calculating the metabolite ratio of the avoided to the preferred silage, i.e., AL27SOIL to AL38CON and RC23SOIL toRC30CON and ANOVA for comparison of those ratios. Compounds that differed (Bonferroni adjusted $P < 0.01$) in both forage species between avoided and preferred silages, in the same manner, were defined as choice-relevant metabolites which might influence feed selection. Metabolites showing ratios > 0 may have a negative influence on forage choice and metabolites showing ratios < 0 may have a positive influence on forage choice.

The variable selection was performed with a score of Variable Importance in Projection (VIP) from a partial least squares discriminant analysis (**PLS-DA**) using the plsr function provided by the R pls package (Mevik and Wehrens, 2007), to identify the differential metabolites between the groups and to rank them according to their importance in discriminating groups. Permutation tests were performed with PLS-DA models to validate the accuracy of the model. The classification and cross-validation were performed using the corresponding wrapper function offered by the caret package (Kuhn, 2008).

Furthermore, correlations (Pearson coefficients) of the choice-relevant metabolites and preference (expressed as g of DMI/3 h for forages offered in choice situations, n = 16) were calculated. All *P*-values from the correlations were corrected for multiple testing using Benjamini and Hochberg (1995) false discovery rate (**FDR**, 1%) adjustment by assuming n number of tests performed on the metabolites, and the *P*-values were sorted from lowest to highest. The following equation was used to calculate the FDR:

$$FDR = \frac{n \times P\left(k\right)}{k},$$

where k = the individual relative test position; P = *P*-values. Significance was declared at $P < 0.05$, and a trend was denoted when $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

Descriptive Analysis

For each forage crop, 6 different treatments were applied before ensiling to obtain a range of silage qualities prepared from the same sward. Although forage treatments generated only a few differences in fermentation acids and CP fractions, feed choice behavior of goats was strongly divergent (Table 1). In both forage species, the high DM CON silage was highly preferred with 748 g of DM/3 h (AL38CON) and 858 g of DM/3 h (RC30CON). Wilting of forages to impair growth of undesired microorganisms in combination with providing sufficient fermentable substrate for lactic acid fermentation led to silages with high acceptance whereas low DM SOIL amounted to the lowest DMI with 226 g of DM/3 h (AL27SOIL) and 283 g of DM/3 h (RC23SOIL). According to our expectations, the intended addition of soil resulted in silages with the lowest preference. Contamination with soil during harvest increases the buffering capacity of the substrate such that its ensilability is reduced. Furthermore, it may increase the number of clostridial spores. Avoided silages contained the highest concentrations of acetic acid, which has been shown to reduce the DMI of ruminants before (Eisner et al., 2006). However, on the other hand, silages with the lowest acetic acid concentrations were not the most preferred treatments. Despite extensive chemical analysis (excluding the metabolomics approach), it was impossible to clearly assign differences in preference to the specific groups of compounds (Scherer et al., 2019). The contrasting forage choice behavior may, therefore, have had other causes.

Metabolome analysis of the 4 selected silage treatments revealed 6,403 metabolites in total, of which 1,860 differed between preferred and avoided AL treatments and 934 between the 2 RC treatments. About 1,100 (AL) and 500 metabolites (RC) were more con-

centrated in the avoided compared with the preferred silages, and around 700 (AL) and 400 metabolites (RC) were less concentrated in the avoided compared with the preferred silages. Of the total number of detected metabolites, 4,393 could not be annotated. We characterized the remaining 2,010 metabolites with HMDB, of which 334 metabolites could not be classified either because chemical taxonomic assignment failed or no unique hit in the database was found. Thus, we identified 1,678 metabolites by classifying them with their chemical taxonomy. The classification resulted in 16 superclasses, 68 classes, 162 subclasses, and 462 direct parents. The superclass of organic compounds represented 91% of all metabolites. According to the Encyclopedia Britannica (2009), organic compounds comprise any of a large class of chemical compounds in which one or more atoms of carbon are covalently linked to atoms of other elements, most commonly hydrogen, oxygen, or nitrogen. The highest shares of metabolites belonging to this superclass comprised peptides (40.2% oligopeptides and 8.4% dipeptides) and amino acids (8.6%). Table 2 shows relative shares of superclasses and classes each based on the entity of annotated metabolites (n = 1,678).

Of the annotated metabolites (n = 1,678), 68% contained nitrogen (Table 3). It was either pure nitrogen or in combination with one or 2 hydrogen atoms and one or 2 oxygen atoms. Partially, N, NH, and NH_2 played a role as functional groups of biogenic amines. Of the annotated metabolites, 20% contained sulfur. For absolute and relative shares of N and S containing metabolites in the entity of annotated metabolites, see Table 3. Moreover, relative shares of N and S containing metabolites are shown in Table 3. A total of 1,044 metabolites contained aromatic compounds. Among them, 448 metabolites had one, 296 had 2, and 300 had 3 or more aromatic rings.

Despite not yet being able to annotate all metabolites, variances can be calculated via principal component analysis to depict variances in the sample sets. Our results revealed that principal component (**PC**) 1 clearly separated AL and RC (the substrate used for ensiling), explaining 40.7% of all variances; PC 2 separated the different silage treatments (AL38CON/RC30CON and AL27SOIL/RC23SOIL), explaining 22.9% of all variances in the data set.

Effect of Single Metabolites on Forage Choice

About 600 (AL) and 350 metabolites (RC), respectively, differed between the avoided and the preferred silages. In both plant species, approximately 200 metabolites were more concentrated, and 408 (AL) and 166 metabolites (RC), respectively, were concentrated less in the avoided compared with the preferred silages. For both forage species, the same treatments (Scherer et al., 2019) were selected for metabolome analysis such that the metabolite ratios between both AL and RC silages could be compared. Statements about the possible relationships between metabolites and forage choice might thereby have higher validity than findings within 1 forage species. Compounds that differed in both forage species ($P < 0.01$) in the same manner were defined as choice-relevant metabolites. This definition applied to 160 of the 1,678 annotated metabolites. Very little information is available on metabolites in feedstuffs for ruminants and their effects on feed choice. Only major volatile organic compounds in silages have been studied in some detail, but for most of the numerous other products, the knowledge on their formation and mode of action in ruminants is limited (Gerlach et al., 2018). Of those metabolites that could be annotated and whose ratio of avoided to preferred silage was increased (AL27SOIL/AL38CON and RC23SOIL/RC30CON, respectively, >0), 66 metabolites overlapped in the AL and RC silages. Of those whose ratio of avoided to preferred silage was decreased (AL27SOIL/AL38CON and RC23SOIL/RC30CON, respectively, <0), 94 annotated metabolites overlapped. All metabolites that were changed significantly (Bonferroni adjusted $P < 0.05$) in both RC (RC23SOIL/RC30CON) and AL (AL27SOIL/AL38CON; ratio of each calculated comparison as $\log_2$-value >0 and <0, respectively) inclusive related superclasses, classes, subclasses, and direct parents are shown in Tables 4 and 5. Metabolites showing ratios >0 may have a negative influence on forage choice (Table 4), and metabolites showing ratios <0 may have a positive influence on forage choice (Table 5). Pearson correlation coefficients and *P*-values corrected for FDR between metabolites of choice-relevant compounds and the preference (expressed as g DMI/3 h for forages offered in choice situations) of goats of the RC and AL silage (n = 16) are shown in Tables 4 and 5. The majority of compounds that differed between preferred and avoided silage manifests a significant relation to preference, also after correction for FDR.

It has to be kept in mind that intake data from preference trials are not to be equated with DMI from production trials. Typically, differences in feed intake are much stronger when cows are having the possibility to choose between 2 or more feedstuffs (Keady and Murphy, 1998). Besides studying the metabolome of contrasting silages one objective of the study was to identify silage characteristics related to preference or avoidance. Because feeding behavior is more sensitive to feed characteristics in choice situations (Baumont, 1996), the design of the preference trial was chosen and judged as being appropriate for reaching these aims.

Table 2. Relative shares of superclasses and classes each based on the entity of annotated metabolites (n = 1,678) detected in alfalfa and red clover silages (n = 4 with 4 replicates each)

Superclass	Share (% of all superclasses)	Class	Share (% of all classes)
Organic compounds	17.14	Organic acids and derivatives	4.28
		Benzenoids	4.28
		Organoheterocyclic compounds	15.51
		Naphthofurans	0.53
		Organic oxygen compounds	1.60
		Phenylpropanoids and polyketides	9.10
		Nucleosides, nucleotides, and analogs	5.35
		Alkaloids and derivatives	1.60
		Lignans, neolignans and related compounds	2.14
		Lipids and lipid-like molecules	2.68
		Organosulfur compounds	3.21
		Hydrocarbons	1.07
Organic acids and derivatives	8.57	Carboxylic acids and derivatives	1.07
		Organic carbonic acids and derivatives	0.53
		Vinylogous acids	0.53
		Thiocarboxylic acids and derivatives	0.53
		Keto acids and derivatives	0.53
		Hydroxy acids and derivatives	0.53
Organic oxygen compounds	1.43	Organooxygen compounds	0.53
Organooxygen compounds	4.29	Carbohydrates and carbohydrate conjugates	1.07
		Alcohols and polyols	0.53
		Carbonyl compounds	2.67
Organoheterocyclic compounds	22.86	Pyridines and derivatives	1.07
		Quinolines and derivatives	0.53
		Dihydrofurans	0.53
		Pyrans	0.53
		Indoles and derivatives	1.07
		Piperidines	0.53
		Heteroaromatic compounds	0.53
		Benzopyrans	1.07
		Diazines	1.07
		Imidazopyrimidines	0.53
		Pteridines and derivatives	0.53
		Pyrrolidines	0.53
		Trithianes	0.53
		Benzoxazines	0.53
		Oxanes	0.53
		Benzothiazoles	1.07
Benzenoids	2.86	Naphthalenes	1.07
		Benzene and substituted derivatives	3.74
Organic nitrogen compounds	4.29	Organic nitrogen compounds	1.07
		Organonitrogen compounds	1.07
		Amines	0.53
Alkaloids and derivatives	5.71	—	0.53
		Aporphines	0.53
		Tropane alkaloids	0.53
		Harmala alkaloids	0.53
Nucleosides, nucleotides, and analogs	1.43	Purine nucleosides	0.53
Phenylpropanoids and polyketides	14.29	Flavonoids	0.53
		Phenylpropanoic acids	0.53
		Isoflavonoids	0.53
		Cinnamic acids and derivatives	1.60
		Tannins	0.53
		2-arylbenzofuran flavonoids	0.53
		Stilbenes	0.53
		Linear 1,3-diarylpropanoids	0.53
		Macrolides and analogs	0.53
		Neoflavonoids	0.53
Lignans, neolignans and related compounds	1.43	Furanoid lignans	0.53
Inorganic compounds	1.43	Homogeneous nonmetal compounds	0.53
Lipids and lipid-like molecules	5.71	Gycerolipids	1.07
		Prenol lipids	2.67
		Fatty acyls	3.21
		Lineolic acids and derivatives	0.53
Organosulfur compounds	4.29	Organic disulfides	0.53
		Thioethers	1.60
		Isothiocyanates	0.53
Hydrocarbons	1.43	Polycyclic hydrocarbons	0.53
Organophosphorus compounds	1.43	Organic phosphoric acids and derivatives	0.53

Table 3. The number and relative shares of N and S containing metabolites in the entity of annotated metabolites (n = 1,678) in alfalfa and red clover silages[1] (n = 4 with 4 replicates each) and relative shares of N and S containing metabolites each based on the totality of N (n = 1,139) and S (n = 338) containing metabolites

Element/ compound	Number of all identified metabolites (n = 1,678)	Relative share (%) of all annotated metabolites (n = 1,678)[2]	Relative share (%) of N- (n = 1,139) and S- (n = 338) containing metabolites[2]
N	326	19.4	28.6
NH	660	39.4	57.9
NH_2	881	52.5	77.3
NO	2	0.1	0.2
NO_2	1	0.1	0.1
S	167	10.0	49.4
SH	183	10.9	54.1
SO	3	0.2	0.9
SO_3	4	0.2	1.2
SO_4	15	0.9	4.4

[1]Silages were chosen as the most preferred (AL38CON and RC30CON) and the most avoided (AL27SOIL and RC23SOIL) silages from 2 preference trials with a total of 12 silage treatments (Scherer et al., 2019). AL = alfalfa silage; RC = red clover silage; CON = untreated control; SOIL = addition of 7,600 g of soil/t; 38, 27, 30, and 23 = DM concentration (%) of treatment.

[2]The relative share of elements and compounds of the respective element (i.e., N, NH, NH_2, NO, NO_2, S, SH, SO, SO_3, and SO_4) do not give 100% because several compounds containing N and S, respectively, can be part of a metabolite.

The metabolites we defined as choice-relevant compounds may have contributed to sensory characteristics of the silages or the postingestive feedback of the goats. Volatile compounds could have had the greatest effect on preference and avoidance of silage because they are probably the most flavorful compounds in silage and the first that is sensed (by smell or taste) when silage is offered to animals. Among other substances, they are composed of monoterpenes, sesquiterpenes, alcohols (monoterpene and sesquiterpene alcohols), ketones, phenols, aldehydes, coumarins, esters, and oxides (Parker, 2015), which were all found in the samples.

However, it has to be considered that sample preparation for metabolome analysis required freeze-drying and grinding of the silages, which can have caused the loss of volatile compounds. Their contribution to the overall profile might, therefore, be underestimated. Working on fresh, unground silage as an alternative would make it challenging to obtain a homogeneous representative sample, therefore, we decided to use freeze-dried, ground material, accepting that some volatiles might get lost.

The 66 metabolites being more concentrated in the avoided compared with the preferred silages could potentially have a negative effect on forage choice; most of them were negatively correlated (FDR-corrected P-values <0.01) to preference expressed as short-time DMI for forages offered in choice situation. These metabolites consisted of oligopeptides (33), dipeptides (3), amino acids (6), lipids (fatty acyls, glycerolipids, and glycerophospholipids; 12), indolacetaldehyde, 1-pentenyl glucosinolate, styrene, methylfurane, xanthine, diadenosine tetraphosphate, L-carnitine, hydrocinnamic acid, ethyl 1-(ethylthio)ethyl disulfide, sphingosine, D-threitol, and erythritol.

Almost all sugars are decreased in the avoided compared with the preferred silages. The levels of glucose and fructose, 2 main sugars in plants, especially are very low in SOIL silages. Ruminants generally prefer feedstuffs with a sweet taste and avoid bitter substances (Provenza, 1995; Forbes, 2007). In goats, we have shown that the addition of sugar beets (containing considerable amounts of sucrose, fructose, glucose, and glycerol) to the ration strongly increased preference (Gerlach et al., 2017). Compounds that might positively contribute to sensory characteristics of silages were higher in the preferred silages: citric acid, malic acid (acidic, fruity), and glycerol with a sweet taste (HMDB, 2020), as well as volatile compounds such as ethyl butyrate that is known for a fruity and sweet odor, at least from a human perspective (Högnadóttir and Rouseff, 2003).

Contrary to suggestions of Weiß et al. (2016), ethyl esters showed no negative influence on forage choice, and ethyl butyrate even had a positive effect. Esters are known to be odorants, which is why they probably have an effect on the flavor of silage (Mo et al., 2001). According to Figueiredo et al. (2007), esters are the most abundant class of volatile compounds in RC silages, with ethyl esters being the predominant subclass of all esters. Ethyl acetate and ethyl lactate artificially added to forages did not affect the forage choice of goats (Gerlach et al., 2019) such that authors concluded that it is unlikely that ethyl esters as a single substance affect preference behavior of ruminants. Based on the current investigations, ethyl esters in combination with other compounds might also not be responsible for reduced silage acceptance.

Table 4. Metabolites that were changed significantly (global adjusted $P < 0.05$) in both red clover silage (RC23SOIL/RC30CON) and alfalfa silage (AL27SOIL/AL38CON; ratio >0) and related superclasses, classes, subclasses, and direct parents, and correlations[1]

Metabolite	Superclass	Class	Subclass	Direct parent	Ratio >0.0		r (DMI, g/3 h)	FDR 1% corrected *P*-value
					RC23SOIL/RC30CON	AL27SOIL/AL38CON		
Isoleucine	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids	0.32	0.52	−0.46†	0.20
L-2-Amino-5-ydroxypentanoic acid					1.13	0.55	−0.70	0.02
L-Lysopine					1.86	2.44	−0.81	<0.01
Methionine					0.64	0.85	−0.60	0.07
N-(4-Hydroxycinnamoyl) tyrosine					0.80	1.37	−0.15	0.75
N-Acetyl-L-methionine					0.73	2.25	−0.66	0.04
N-Carbamoyl-2-amino-2-(4-hydroxyphenyl)acetic acid					0.78	1.32	−0.74	0.01
Phenylalanine					0.60	0.56	−0.53	0.12
Ala-Asn-Gly				Oligopeptides	4.30	3.60	−0.92	<0.01
Ala-Lys-Ala-Gln					0.80	1.12	−0.66	0.04
Ala-Lys-Ala-Gly					2.49	3.60	−0.97	<0.01
Ala-Lys-Asn-Gln					4.00	5.83	−0.98	<0.01
Ala-Lys-Thr-His					3.11	2.73	−0.90	<0.01
Ala-Met-Phe-Arg					0.78	1.07	−0.89	<0.01
Ala-Val-Val-Ala					3.65	0.69	−0.58	0.08
Arg-Glu-Asn-Ile					3.02	3.57	−0.92	<0.01
Asn-Asn-Asp-Gly					1.81	2.20	−0.66	0.03
Asn-Ile-Ile-Pro					3.06	2.52	−0.98	<0.01
Asn-Phe-Gln-His					3.12	3.15	−0.78	<0.01
Asn-Trp-Cys-Cys					2.96	3.76	−0.80	0.03
Asp-Asp-Asp-Tyr					6.68	5.92	−0.74	0.01
Asp-Cys-Gln-Tyr					1.50	1.93	−0.87	<0.01
Asp-Met-Cys-His					1.78	1.69	−0.85	<0.01
Asp-Trp-Asp-Gly					0.49	1.08	−0.64	0.05
Gln-Met-Trp-Trp					2.59	2.34	−0.90	<0.01
Glu-Lys-Asp-Arg	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Oligopeptides	4.26	6.34	−0.89	<0.01
His-Trp-Trp-His					2.11	1.30	−0.96	<0.01
Ile-Asp-Val					2.17	1.56	−0.44	0.22
Ile-Gly-Val					1.84	1.26	−0.71	0.02
Ile-His-Ile					1.27	0.94	−0.95	<0.01
Ile-Ile-Lys					2.02	1.36	−0.73	0.02
Ile-Lys-Val					1.63	1.24	−0.67	0.04
Ile-Met-Pro					0.51	1.37	−0.61	0.06
Ile-Pro-Val					2.72	1.48	−0.63	0.05
Leu-Gly-Pro					1.47	1.16	−0.39	0.30
Val-Val-His					1.68	1.11	−0.97	<0.01
Glycylprolylhydroxyproline				Dipeptides	1.88	1.19	−0.82	<0.01
Leucyl-histidine					0.27	0.78	−0.58	0.08
(2R,3R,4R)-2-Amino-4-hydroxy-3-methylpentanoic acid				Isoleucine and derivatives	6.20	5.51	−0.90	<0.01
Propyl propionate				Carboxylic acid esters	2.87	5.41	−0.76	<0.01

Continued

Table 4 (Continued). Metabolites that were changed significantly (global adjusted $P < 0.05$) in both red clover silage (RC23SOIL/RC30CON) and alfalfa silage (AL27SOIL/AL38CON; ratio >0) and related superclasses, classes, subclasses, and direct parents, and correlations[1]

Metabolite	Superclass	Class	Subclass	Direct parent	Ratio >0.0		r (DMI, g/3 h)	FDR 1% corrected P-value
					RC23SOIL/RC30CON	AL27SOIL/AL38CON		
N-Acetylhistamine				*N*-acetyl-2-arylethylamines	1.91	5.69	−0.64	0.05
Styrene		Benzenoids	Benzene and substituted derivatives	Styrenes	3.72	4.55	−0.76	<0.01
2-Methylfuran		Organoheterocyclic compounds	Heteroaromatic compounds	Heteroaromatic compounds	1.77	8.78	−0.62	0.06
Indoleacetaldehyde			Indoles and derivatives	3-Alkylindoles	4.24	5.51	−0.92	<0.01
Xanthine			Imidazo-pyrimidines	Xanthines	0.55	1.56	−0.69	0.03
1-Pentenyl glucosinolate		Organic oxygen compounds	Organooxygen compounds	Glucosinolates	1.73	2.59	−0.82	<0.01
Erythritol/Threitol				Sugar alcohols	2.18	2.76	−0.94	<0.01
Diadenosine tetraphosphate		Nucleosides, nucleotides, and analogs	(5′–>5′)-dinucleotides	(5′–>5′)-dinucleotides	2.46	3.18	−0.89	<0.01
Ethyl 1-(ethylthio)ethyldisulfide		Organosulfur compounds	Organic disulfides	Dialkyldisulfides	2.14	2.28	−0.87	<0.01
3-Dehydroxycarnitine		Lipids and lipid-like molecules	Fatty acyls	Straight chain fatty acids	3.08	1.99	−0.58	0.08
5-Hexyltetrahydro-2-furanoctanoic acid				Prostaglandins and related compounds	1.26	1.59	−0.57	0.09
Avenoleic acid				Lineolic acids and derivatives	1.49	1.14	−0.69	0.03
DG(18:3(6Z,9Z,12Z)/15:0/0:0)			Glycerolipids	1,2-Diacylglycerols	1.47	2.24	−0.64	0.05
DG(18:4(6Z,9Z,12Z,15Z)/15:0/0:0)	Organic compounds	Lipids and lipid-like molecules	Glycerolipids	1,2-Diacylglycerols	1.80	2.12	−0.67	0.04
DG(20:2n6/0:0/22:6n3)				1,3-Diacylglycerols	1.58	1.41	−0.88	<0.01
MG(15:0/0:0/0:0)				1-Monoacyl-glycerols	1.00	1.60	−0.68	0.03
PE(18:3(6Z,9Z,12Z)/20:0)				Phosphatidyl-glycerols	1.32	0.39	−0.79	<0.01
PE(22:5(7Z,10Z,13Z,16Z,19Z)/18:0)					1.15	0.52	−0.72	0.02
PG(16:1(9Z)/16:0)					1.28	0.67	−0.86	<0.01
PG(18:3(6Z,9Z,12Z)/16:0)					2.14	0.62	−0.86	<0.01
Phosphatidylglycerol-32:0					1.55	0.69	−0.88	<0.01
L-Carnitine	Organic nitrogen compounds	Organonitrogen compounds	Quaternary ammonium salts	Carnitines	4.92	5.07	−0.92	<0.01
Sphingosine		Organic nitrogen compounds	Organonitrogen compounds	1,2-Aminoalcohols	0.61	0.52	−0.65	0.04
Hydrocinnamic acid	Phenylpropanoids and polyketides	Phenylpropanoic acids	—	Phenylpropanoic acids	0.64	0.85	−0.60	0.07

[1]Correlations = Pearson correlation coefficients; r and P-values corrected for false discovery rates (FDR) between metabolites and preference (expressed as g of DMI/3 h for forages offered in choice situations) of RC and AL silage of goats (n = 16); CON = untreated control, SOIL = addition of 7,600 g of soil/t, 38, 27, 30, and 23 = DM concentration (%) of treatment. Treatments were chosen as the most preferred (AL38CON and RC30CON) and the most avoided (AL27SOIL and RC23SOIL) silages from 2 preference trials with a total of 12 silage treatments (Scherer et al., 2019).

*$P < 0.10$, $P < 0.05$, $P < 0.01$, $P < 0.001$.

Table 5. Metabolites that were changed significantly (global adjusted $P < 0.05$) in both red clover silage (RC23SOIL/RC30CON) and alfalfa silage (AL27SOIL/AL38CON) (ratio <0) and related superclasses, classes, subclasses, and direct parents (data are based on differential analysis) and correlations[1]

Metabolite	Superclass	Class	Subclass	Direct parent	Ratio <0.0		r (DMI, g/3 h)	FDR 1% corrected *P*-value
					RC23SOIL/RC30CON	AL27SOIL/AL38CON		
(R)C(S)S-Alliin	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids	−1.83	−4.55	0.84	<0.01
2,5-Diaminopentanoate					−0.82	−1.50	0.56	0.09
4-Methylene-L-glutamine					−1.27	−2.33	0.62	0.06
Argininosuccinic acid					−1.26	−2.48	0.72	0.02
Citrulline					−1.28	−2.32	0.61	0.06
Tyrosine					−1.87	−2.67	0.62	0.06
Ala-Ala-Gly-Pro				Oligopeptides	−1.61	−3.93	0.55	0.10
Ala-Asp-Asp-Gln					−1.47	−2.25	0.80	<0.01
Ala-Leu-Asn-Asp					−2.58	−6.26	0.95	<0.01
Ala-Thr-Asp-Tyr					−0.71	−1.19	0.34	0.38
Ala-Tyr-Arg					−2.67	−2.75	0.97	<0.01
Ala-Val-Ser-His					−3.50	−2.53	0.87	<0.01
Asn-Thr-Thr-Asp					−1.62	−6.09	0.94	<0.01
Asp-Gln-Gln-Gln					−1.75	−1.65	0.84	<0.01
Asp-Glu-Glu-Trp					−1.15	−3.42	0.93	<0.01
Asp-Thr-Ser-Tyr					−1.17	−0.85	0.35	0.37
Cys-Cys-Gly					−3.51	−5.13	0.59	0.08
Cys-Lys-Gly-Gly					−1.45	−2.89	0.79	0.04
Lys-Ala-Cys					−3.02	−4.18	0.81	0.03
Met-Phe-Val					−3.39	−5.57	0.73	0.02
Met-Phe-Val-Pro					−3.09	−3.63	0.97	<0.01
Val-Gly-His					−3.66	−3.16	0.88	<0.01
Asn-Asp-Asp-Pro					−1.10	−4.32	0.60	0.07
Glu-Ala-Asp					−3.27	−4.03	0.64	0.05
Gly-Pro-His					−1.21	−3.97	0.90	<0.01
Ile-Met-Phe-Pro					−4.18	−4.04	0.96	<0.01
Ser-Trp-Gly-Ser					−2.60	−3.97	0.86	<0.01
Thr-Cys-His	Organic compounds	Organic acids and derivatives	Carboxylic acids and derivatives	Oligopeptides	−2.11	−5.66	0.66	0.04
Alanyl-histidine				Dipeptides	−2.53	−3.87	0.90	<0.01
Arginyl-proline					−3.69	−5.77	0.88	<0.01
Glutamyl-gamma-glutamate					−2.88	−2.97	0.73	0.02
Glutamyl-tyrosine					−3.29	−3.39	0.88	<0.01
L-gamma-glutamyl-L-leucine					−0.50	−1.72	0.79	<0.01
L-phenylalanyl-L-proline					−0.64	−3.59	0.77	<0.01
Lysyl-Glycine					−1.41	−5.61	0.76	<0.01
Pyro- L-glutaminyl-L-glutamine					−2.82	−7.25	0.91	<0.01
Serinyl-valine					−0.65	−2.29	0.84	0.01
Trp-Tyr					−3.21	−5.32	0.80	<0.01
Tryptophyl-arginine					−3.49	−1.52	0.73	0.02
Valyl-aspartate					−1.05	−2.41	0.88	<0.01
Mukoline				Histidine and derivatives	−1.02	−1.89	0.76	<0.01
N-(1-Deoxy-1-fructosyl) histidine					−2.37	−5.09	0.61	0.07

Continued

Table 5 (Continued). Metabolites that were changed significantly (global adjusted $P < 0.05$) in both red clover silage (RC23SOIL/RC30CON) and alfalfa silage (AL27SOIL/AL38CON) (ratio <0) and related superclasses, classes, subclasses, and direct parents (data are based on differential analysis) and correlations[1]

					Ratio <0.0			
Metabolite	Superclass	Class	Subclass	Direct parent	RC23SOIL/RC30CON	AL27SOIL/AL38CON	r (DMI, g/3 h)	FDR 1% corrected *P*-value
*N*2-(2-Carboxymethyl-2-hydroxysuccinoyl) arginine				Arginine and derivatives	−1.04	−2.88	0.66	0.03
Ethyl aconitate				Tricarboxylic acids and derivatives	−4.33	−3.86	0.91	<0.01
D-Vacciniin		Benzenoids	Benzene and substituted derivatives	Benzoic acid esters	−1.94	−4.37	0.93	<0.01
Methylgingerol				Dimethoxybenzenes	−1.58	−1.64	0.58	0.08
Propyl gallate				Galloyl esters	−3.09	−2.99	0.93	<0.01
Vanillin acetate			Phenol esters	Phenol esters	−1.24	−3.95	0.50	0.15
2-Carboxy-4-dodecanolide		Organoheterocyclic compounds	Lactones	Gamma butyrolactones	−3.84	−7.11	0.89	<0.01
3-(2-Furanyl)-2-propenal			Heteroaromatic compounds	Heteroaromatic compounds	−2.13	−3.00	0.96	<0.01
3′,4′-Dihydrodiol			Azolidines	Phenylhydantoins	−1.43	−3.04	0.76	<0.01
7-Aminomethyl-7-carbaguanine			Pyrrolopyrimidines	Pyrrolo[2,3-d] pyrimidines	−0.85	−1.36	0.90	<0.01
Rutaecarpine			Indoles and derivatives	Beta carbolines	−1.17	−1.42	0.56	0.10
Deoxyeritadenine			Imidazopyrimidines	6-Aminopurines	−1.96	−4.50	0.66	0.03
Mannose		Organic oxygen compounds	Organooxygen compounds	Hexoses	−1.50	−5.02	0.86	<0.01
5-Methylthioribose				Pentose phosphates	−1.05	−4.93	0.45	0.21
De-O-methylsimmondsin	Organic compounds	Organic oxygen compounds	Organooxygen compounds	O-glycosyl compounds	−3.07	−5.58	0.96	<0.01
D-glycero-L-galacto-octulose				C-glycosyl compounds	−1.57	−3.83	0.77	<0.01
Dhurrin				Phenolic glycosides	−1.21	−4.67	0.75	0.01
Galactose				Hexoses	−3.82	−3.43	0.77	<0.01
Glucose					−3.29	−8.07	0.75	<0.01
Tyramine glucuronide				Phenolic glycosides	−3.52	−2.65	0.73	0.02
Xylose				Pentoses	−4.56	−5.31	0.69	0.03
11-Methylgerberinol		Phenylpropanoids and polyketides	Coumarins and derivatives	4-Hydroxy-coumarins	−0.71	−2.93	0.73	0.02
Gerberinol					−4.53	−2.21	0.73	0.01
3,4′,5,6,8-Pentamethoxyflavone			Flavonoids	8-O-methylated flavonoids	−1.31	−5.57	0.81	<0.01
Glicoisoflavanone				3′-prenylated Flavanones	−3.95	−3.50	0.73	0.01
Glycyrrhizaisoflavone B			Isoflavonoids	Pyranoisoflavonoids	−1.63	−4.28	0.95	<0.01
Isopimpinellin			Coumarins and derivatives	8-Methoxypsoralens	−1.65	−3.53	0.72	0.02
Deoxyadenosine		Nucleosides, Nucleotides, and analogs	Purine nucleosides	Purine 2′-deoxyribonucleosides	−0.92	−2.18	0.61	0.06
Deoxyinosine					−1.97	−3.48	0.93	<0.01
Uridine 5′-monophosphate			Pyrimidine nucleotides	Pyrimidine ribonucleoside monophosphates	−1.53	−4.22	0.94	<0.01

Continued

Table 5 (Continued). Metabolites that were changed significantly (global adjusted $P < 0.05$) in both red clover silage (RC23SOIL/RC30CON) and alfalfa silage (AL27SOIL/AL38CON) (ratio <0) and related superclasses, classes, subclasses, and direct parents (data are based on differential analysis) and correlations[1]

Metabolite	Superclass	Class	Subclass	Direct parent	Ratio <0.0		r (DMI, g/3 h)	FDR 1% corrected *P*-value
					RC23SOIL/RC30CON	AL27SOIL/AL38CON		
Dityrosine		Lignans, neolignans and related compounds	—	Lignans, neolignans and related compounds	−2.93	−5.40	0.71	0.02
(9R,10S,12Z)-9,10-Dihydroxy-8-oxo-12-octadecenoic acid		Lipids and lipid-like molecules	Fatty acyls	Lineolic acids and derivatives	−1.11	−0.52	0.71	0.02
15(S)-Hydroxyeicosa-trienoic acid				Hydroxyeicosatrienoic acids	−1.17	−2.79	0.79	<0.01
3,5,7-Octatriyn-1-ol				Fatty alcohols	−1.82	−2.47	0.96	<0.01
3-Methylglutaconic acid				Methyl-branched fatty acids	−1.83	−3.93	0.76	<0.01
5-O-p-Coumaroylnigrumin				Fatty acyl glycosides of mono- and disaccharides	−2.03	−3.66	0.57	0.09
Ethyl butyrate				Fatty acid esters	−1.70	−2.07	0.94	<0.01
Melibiitol				Fatty acyl glycosides of mono- and disaccharides	−1.24	−2.55	0.96	<0.01
S-(3-Methylbutanoyl)-dihydrolipoamide-E				Fatty acyl thioesters	−1.60	−4.60	0.74	0.01
Vaccenic acid	Organic compounds	Lipids and lipid-like molecules	Fatty acyls	Long-chain fatty acids	−2.02	−2.70	0.64	0.05
Panaquinquecol 1				Long-chain fatty alcohols	−1.65	−0.60	0.58	0.08
Auxin b			Prenol lipids	Monocyclic monoterpenoids	−1.70	−0.61	0.33	0.41
Gibberellin A113				C19-gibberellin 6-carboxylic acids	−3.07	−3.64	0.83	<0.01
Citric acid		Carboxylic acids and derivatives	Tricarboxylic acids and derivatives	Tricarboxylic acids and derivatives	−4.08	−7.62	0.78	<0.01
Malic acid		Hydroxy acids and derivatives	Beta hydroxy acids and derivatives	Beta hydroxy acids and derivatives	−2.60	−5.05	0.77	<0.01
Glucosamine	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Hexoses	−3.51	−1.85	0.44	0.23
Glycerol				Sugar alcohols	−4.08	−3.95	0.99	<0.01
3-O-Feruloylquinic acid	Organooxygen compounds	Alcohols and polyols	Cyclic alcohols and derivatives	Quinic acids and derivatives	−1.21	−3.04	0.95	<0.01
Escitalopram	Benzenoids	Benzene and substituted derivatives	Phenylbutylamines	Phenylbutylamines	−1.63	−5.48	0.79	<0.01
Spermidine	Organic nitrogen compounds	Organic nitrogen compounds	Organonitrogen compounds	Dialkylamines	−1.27	−4.21	0.58	0.08
N-Methylcalystegine C1	Alkaloids and derivatives	Tropane alkaloids	—	Tropane alkaloids	−1.61	−3.78	0.72	0.02
Gravolenic acid	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives	Coumaric acids and derivatives	−3.16	−5.97	0.95	<0.01

[1]Correlation = Pearson correlation coefficients; r and *P*-values corrected for false discovery rates (FDR) between metabolites and preference (expressed as g of DMI/3 h for forages offered in choice situations) of RC and AL silages of goats (n = 16); CON = untreated control, SOIL = addition of 7,600 g of soil/t, 38, 27, 30, and 23 = DM concentration (%) of treatment. Treatments were chosen as the most preferred (AL38CON and RC30CON) and the most avoided (AL27SOIL and RC23SOIL) silages from 2 preference trials with a total of 12 silage treatments (Scherer et al., 2019).

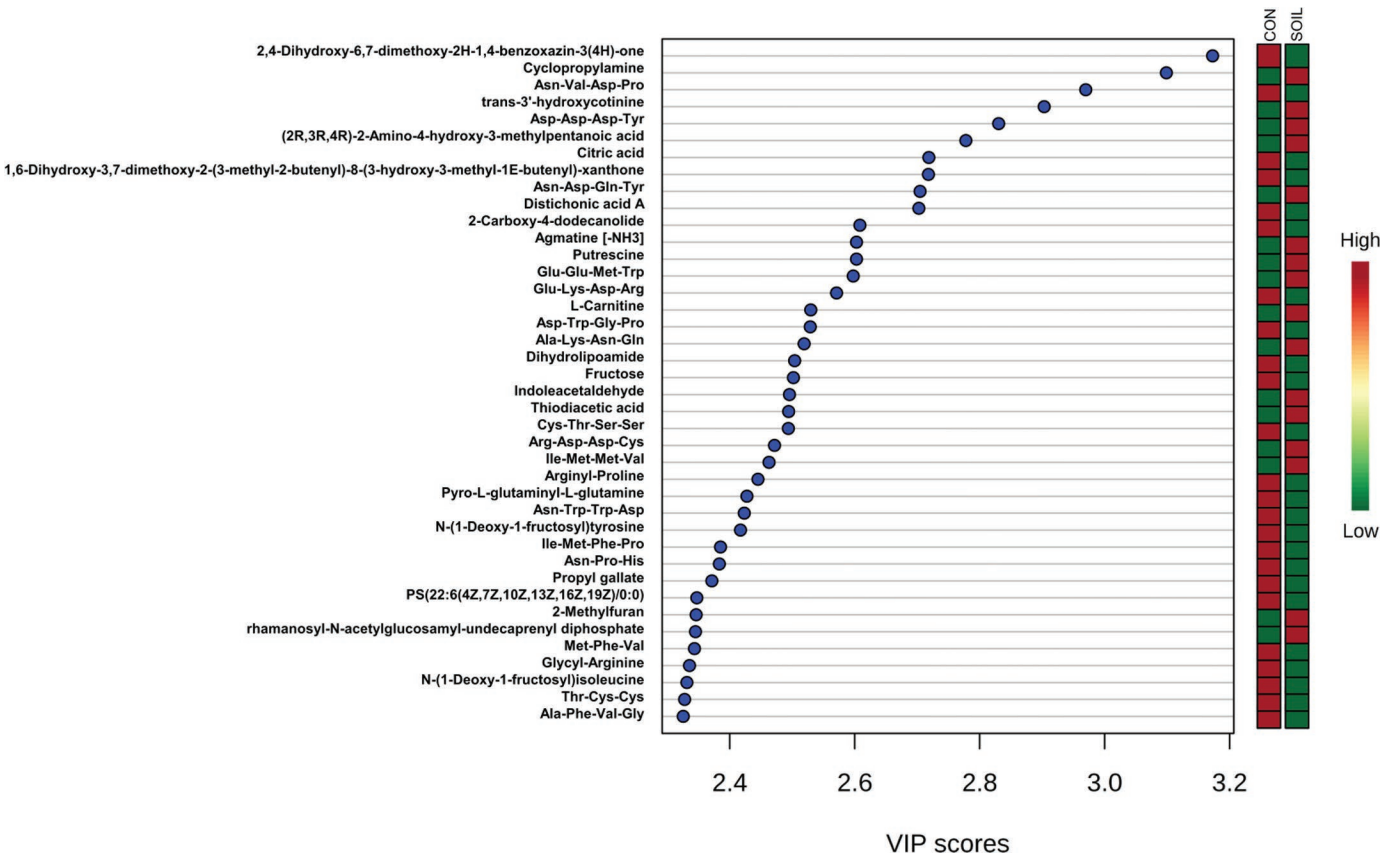

Figure 1. Important metabolites ranked by variable importance in projection (VIP) between the group based on the partial least squares discriminant analysis model. There are 29 metabolites that show a VIP score of > 2.4. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in preferred (CON) versus avoided (SOIL) silages.

The 40 most important metabolites differing between CON and SOIL silages identified by PLS-DA are shown in Figure 1. A big share of them (15) belongs to oligopeptides, but there is no clear grouping of whether they are increased or decreased in avoided silages. Putrescine, the biogenic amine arising from decarboxylation of arginine, was higher concentrated in avoided silages. As reviewed by Scherer et al. (2015), the effect of ensiling conditions on its formation as well as its effect on DMI by ruminants has not yet been fully clarified. Putrescine administered in high concentrations of 100 g/d to dairy cows influenced both milk yield and forage intake, and it was discussed that it might be a contributory factor, alone or combined with other amines, for the development of ketonemia (Lingaas and Tveit, 1992). Our data support the assumption that increased concentrations of putrescine in silages might also reduce forage preference. Carnitine is a quaternary ammonium salt, increased in avoided silages, accounts for a bitter taste, and acts as a taste modifying molecule (Behrens and Meyerhof, 2015). Also, thiodiacetic acid, known for its unpleasant odor (HMDB, 2020), and cyclopropylamine, described to have an ammoniacal to fish-like odor, were more concentrated in the avoided silages, but to the best of our knowledge, there has been no research on their effect on DMI or preference by ruminants yet. This list may provide approaches for further investigations on compounds in silages potentially influencing the forage choice or metabolism of ruminants. The effect of single substances identified in the present work on forage choice or DMI by ruminants should, therefore, be studied in future projects.

With the present study, we have demonstrated with a mainly descriptive approach how broad the chemical spectrum of the metabolites in silage immediately after opening the silos is and which classes of metabolites dominate. We consider this to be essential for a well-founded overview of silage composition and the launch of a deeper exploration of feed-preference-relationships. However, although databases have been constructed (Johnson and Gonzalez, 2012), such as the HMDB (Wishart et al., 2013), identifying metabolites still represents a limiting step of metabolomics workflow; hence there is a need for progress that is currently going on.

CONCLUSIONS

Preferred and avoided silages made from red clover and alfalfa differed in a huge number of metabolites identified by a nontargeted metabolomics approach. Some of the identified compounds offer the potential for being a preference-influencing compound through smell or taste, whereas others might affect the metabolism of ruminants. Therefore, the whole data set offers an important new approach to better understand the forage choice behavior of ruminants.

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