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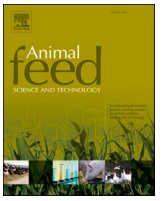

Survey on the occurrence of silage volatile organic compounds in the Po Valley - Italy

Samantha Sigolo [a,1], Francesco Fancello [b,2], Francesca Ghilardelli [a,3], Martina Mosconi [a], Aldo Prandini [a,4], Francesco Masoero [a], Xianjun Yuan [c,5], Antonio Gallo [a,*,6]

[a] Department of Animal Science, Food and Nutrition - DIANA (DIANA), Università Cattolica Sacro Cuore, via Emilia Parmense 84, 29122 Piacenza, Italy

[b] Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy

[c] Institute of Ensiling and Processing of Grass, College of Agro-grassland Science, Nanjing Agricultural University, No. 1 Weigang, Nanjing, Jiangsu Province 210095, PR China

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ABSTRACT

The volatile organic compounds (VOC) are the fermentation products and used to assess the quality of ensiled forages. The study aimed to monitor the VOC concentrations in silage and haylage by GC/FID system and to reveal the relationships among these different end-fermentation products by carrying out a multivariate analysis. Forty-six compounds were quantified by GC/FID system in a single chromatographic run. The average repeatability and reproducibility coefficients of variation obtained on standard solutions, composed by 0.1 mL/L of each VOC (solution A) or volatile fatty acids (VFA, solution B), were 0.028 and 0.017 (absolute value), respectively. The average repeatability and reproducibility coefficients of variation obtained on silage samples were 0.026 and 0.027, respectively. Mean values of recovery obtained on the standard solutions at different concentration levels of compounds were in the range 0.95–1.04 (absolute values), and no effect of analyte concentration was observed on recovery. When multivariate analysis was carried out on whole database, 7 factors were retained and they explained 0.50 of the total

Abbreviations: BCAA, branched chain amino acids; DM, dry matter; F, factor; FID, flame ionization detector; GC, gas-chromatography; HPLC, high liquid performance chromatography; HS, headspace; IS, internal standard solution of pivalic acid at 15 g/L of distilled water; KdcA, keto acid decarboxylase; Kivd, α-ketoisovalerate decarboxylase; LAB, lactic acid bacteria; LB, *Lentilactobacillus buchneri*; LOD, limit of detection; LOQ, limit of quantification; MS, mass-spectrometry; PCA, principal component analysis; rCV, coefficient of variation of repeatability; RCV, coefficient of variation of reproducibility; RF, factor of instrumental response; rSD, standard deviation of the repeatability; RSD, standard deviation of the reproducibility; solution A, mixture standard solution of aldehydes, ketones, alcohols, and esters at 0.1 mL/L each compound in distilled water; solution B, mixture standard solution of organic acids at 0.1 mL/L each compound; SPME, solid-phase microextraction; VFA, volatile fatty acids; VOC, volatile organic compounds; Zvar, standardized data vector.

* Corresponding author.
E-mail address: antonio.gallo@unicatt.it (A. Gallo).
[1] **ORCID:** https://orcid.org/0000–0001-9081–0565
[2] **ORCID:** https://orcid.org/0000-0002-0023-7492
[3] **ORCID:** https://orcid.org/0000–0001-8631–1544
[4] **ORCID:** https://orcid.org/0000–0002-8650–8766
[5] **ORCID:** https://orcid.org/0000–0001-9053–1937
[6] **ORCID:** http://orcid.org/0000–0002-4700–4450

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variability. The factors were interpreted for their biological meanings, based on specific VOC loading on each extracted latent structure. Alfalfa haylage and ryegrass silage were characterized by higher contents of valeric and butyric acids, methyl and ethyl butyrate, 2,3-butaneldiol, 1-butanol, and 2-butanol compared with the other fermented products. Except for mixture crop silage, all the fermented products containing legume crops had higher levels of 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenyl ethanol, and methanol and lower contents of ethanol, ethyl lactate, and ethyl acetate compared with the other fermented products. Silage containing grass crops had higher levels of acetic and lactic acids, and 1,2-propanediol respect to the other investigated products. The most part of the investigated fermented products were characterized by the presence of 2-methyl propionaldehyde and 2-methyl butyraldehyde. The VOC profile determination of ensiled products provides useful information for an overall evaluation of the fermentative quality of silages and haylage.

1. Introduction

Silage is a fermented, high-moisture stored feed that can be fed to ruminants or used as a biofuel feedstock for anaerobic digesters (Kalač, 2011). The anaerobiosis is the most important condition should be assured during ensiling (Kung et al., 2018). Under anaerobic condition, the growth of lactic acid bacteria is supported to detriment of other microorganisms, which compete for the available nutrients, especially water-soluble carbohydrates. Consequently, the productions of either lactic acid or, to a lesser extent, acetic acid increase the acidity of the ensiled forage preventing the growth of unfavorable microorganism and the occurrence of undesirable fermentations (McDonald et al., 1991).

The most common parameters used in the evaluation of silage fermentation quality were pH, the concentrations of main organic acids, few alcohols, ammonia nitrogen as well as counts of specific microbial populations (Kung et al., 2018). However, ensilage is a very complex process and a great number of volatile organic compounds (VOC) could be generated as a result of the specific fermentation processes. In particular, Hafner et al. (2013) reviewed the majority of papers published from 1974 to 2012 used VOC in the evaluation of fermentation processes occurring during ensiling and these authors discussed as the main VOC detected in silage could be ascribed to four main categories, being alcohols, aldehydes, esters and ketones (Chemlová et al., 2009; Daniel et al., 2013; Weiss, 2017).

Thus, there has been a growing interest for the development of analytical methods based on gas chromatography (GC) for the VOC profile determination of silage (Weiss and Sommer, 2012; Weiss et al., 2016; Gomes et al., 2019) and their use in developing fermentative quality indexes to assess the overall quality of ensiled forage (Gallo et al., 2016b; Andrighetto et al., 2018; Tharangani et al., 2021).

The aim of our work was to monitor the production of VOC in different silage and haylage categories and to verify relationships among these different end-fermentation products by adopting multivariate statistical techniques, such as principal component analysis (PCA). For this purpose, a sensitive, accurate and fast method using GC/FID equipment has been proposed and evaluated to determine in a single chromatographic run a large number (48 in total) of VOC.

2. Material and methods

2.1. Sampling, preparation, and chemical analyses

A total of 111 fermented products (i.e., 10 corn silage, 7 sorghum silage, 10 small grain silage, 8 alfalfa silage, 8 ryegrass silage, 10 mixture crop silage, 11 alfalfa haylage, 8 legume haylage, 20 grass haylage, and 19 mixture crop haylage) were collected in different dairy farms located in the Po Valley (Italy) on 2020 and 2021 harvest seasons. These fermented products were sampled at least 10 weeks after ensiling from 53 horizontal bunker silos and 58 haylages. Within each silage category, the sample analysed in current survey were randomly selected and no selection criteria were adopted.

The sampling of horizontal bunker silos was carried out from four randomly selected points of silage core of the feed-out face, collecting about 2 kg on a wet weight basis. All the sampled horizontal bunker silos were characterized by appropriate coverage with gravel bags and optimal use of plastic film to cover silage. In particular, all the sampled horizontal bunker silos had a proper sealing system obtaining using plastic films on the longitudinal side, lateral side and as a bunker cover. Specifically, thin oxygen barrier films were used in combination with black or black-on-white plastic films and strong protective nets against crows. Regarding haylage, the sample was carried out using a forage corer, sampling from two randomly selected points of the bale. The bales, obtained with appropriate plastic film and with a proper degree of compression, had been opened within approximately 12 h prior to sampling. All the samples did not show visible signs of mold contamination. All fermented products obtained from the leguminous, ryegrass, grass and mixture crops (in this case in the haylage) were obtained by subjecting the crop to pre-wilting in the field.

The VOC were extracted from fresh sample (50 g) using a Stomacher blender (Seward Ltd., West Sussex, UK) for 3 min with 150 mL of distilled water. The blend was filtered through medical non-woven gauze. To remove solid impurities, the filtered blend (10 mL) was centrifuged at 3000 rpm for 15 min and the liquid phase supernatant was micro-filtered on Minisart GF-prefilters syringe filter 0.45 μm pore size (Sartorius, AG, Germany). An aliquot of micro-filtered supernatant liquid phase (1.5 mL) was transferred in vial, added with 10 μL of internal standard solution (pivalic acid at 15 g/L of distilled water; IS) and analyzed by GC/FID for VOC, and by high liquid

performance chromatography (HPLC; Carvalho et al., 2012) after twice dilution with distilled water for lactic acid as described by Gallo et al. (2016a). The pH was measured on the filtered blend with a pH meter (Crison Micro-pH, Barcelona, España). Dry matter was determined according to AOAC (1995) method 945.15 with correction for the loss of volatiles during drying as reported by NorFor (2011). Ammonia nitrogen concentration was determined in accordance with NorFor (2011). Briefly, 20 g of fresh sample were mixed with 150 mL of distilled water and magnesium oxide (10 g/sample). The Kjeldahl method was used to steam distilling this solution and determinate the ammonia nitrogen concentration, expressed on a total nitrogen (TN) basis (AOAC, 1995 method 984.13; Gallo et al., 2018).

2.2. Chemical standards

Supplier and purity grade of the chemical standards used in this study are shown in Table1. Two mixture standard solutions were prepared according to Ortega et al. (2001) and FDA (2015), with adaptations to the aim of present study: 1) a mixture standard solution of aldehydes, ketones, alcohols, esters, glycols, and caproic acid at 0.1 mL/L each compound in distilled water (solution A); and a mixture standard solution of VFA at 0.1 mL/L each compound obtained by diluting 10 times with distilled water WSFA-2 standard mix (Sigma-Aldrich, St. Louis, MO, USA; solution B). To estimate recovery rate at different concentration levels of analytes, standard solutions at 0.02 and 0.002 mL/L each compound were obtained by diluting the standard solutions A and B.

2.3. Gas-chromatography

All the gas-chromatographic analyses were conducted using a 2025 Shimadzu GC (Shimadzu S.r.l., Milano, Italy) equipped with auto-sampler (AOC-20i Shimadzu S.r.l., Milano, Italy), FID detector, and capillary column DB-WAX UI (60 m × 0.250 mm; 0.25 μm; Agilent Technologies S.p.A., Milano, Italy). The GC setting conditions were in accordance with previous works with adjustments (Ortega et al., 2001; Symeou et al., 2007). The injector and detector temperatures were 200 and 230 °C, respectively. Carrier gas was hydrogen and the analyses were conducted at constant flow of 1.5 mL/min. The injection volume was 1 μL and split ratio was 30:1. The temperature program was as follows: 35 °C × 5 min, after 110 °C at 3 °C/min, after 200 °C at 2 °C min (total running time = 75 min). Data acquisition and their processing were conducted using LabSolutions Lite software (version 5.82; Shimadzu S.r.l., Milano, Italy).

The VOC in silage samples were identified by comparison with retention times of the external standards and calculated through peak areas (corrected by instrumental response factors, RF; AOAC, 2000) using peak area of pivalic acid as IS at known concentration (Fussell and McCalley, 1987). The VOC were expressed as mg/kg dry matter (DM).

2.4. Gas-chromatographic method performance

The VOC were determined by gas-chromatographic method except for lactic acid which was analyzed by HPLC. In this study, the assessment of method performance referred only to the gas-chromatographic approach being the HPLC technique widely used for the acid lactic determination (Canale et al., 1984; Carvalho et al., 2012; Gallo et al., 2016a).

Measurements of analytical accuracy were performed on 3 silage samples (1 grass silage and 2 corn silage) and standard solutions A and B for each detected compound. In particular, the VOC extraction was carried out in single for each silage sample on the same day. The GC quantification was conducted on both silage samples and standard solutions 6 times/day for 4 consecutive days. The measurements carried out on the same samples or standard solutions in the same days were analytical repetitions (n = 6, for repeatability or rSD), whereas the measurements carried out in different days were considered experimental replicates (n = 4 for reproducibility or RSD). The rSD, RSD, and their coefficients of variations (i.e, rCV and RCV, respectively) were expressed as absolute values and calculated according to Symeou et al. (2007).

The recovery test was based on blank method in agreement with Naidis and Turpeinen (2009). In particular, the recovery of three replicates was calculated both on standard solutions at different concentration levels of analytes (i.e., 0.1, 0.02, and 0.002 mL/L; instrumental recovery) and on dry corn silage (blank) spiked with the standard solutions added to a constant weight to volume ratio of 1 g to 9 mL. Dry corn silage was used as blank to reduce VOC levels in the original sample that could naturally contain very high concentrations of some VOC, such as ethanol and acetic acid, and therefore to avoid excessive analyte concentrations in GC/FID analysis. The relative recovery for each analyte was estimated according to Symeou et al. (2007). The limit of detection (LOD) and limit of quantification (LOQ) were evaluated by using signal-to-noise approach (Armbruster and Pry, 2008). The LOD was estimated at a signal-to-noise ratio of 3:1, whereas LOQ was three times the LOD (Armbruster and Pry, 2008).

2.5. Statistical analysis

The main effect of concentrations (i.e., 0.1, 0.02, and 0.002 mL/L) on recovery was studied in agreement to a completely randomized design by using GLM procedure of SAS, and means were post-hoc compared by using orthogonal contrasts to test linear response. The significance of linear trend was at a $P \leq 0.05$.

A descriptive statistic (i.e., mean, standard deviation) was carried out on different silage and haylage categories. Then, variables were analyzed using a multivariate approach with the PROC FACTOR of SAS (version 9.2). In a factorial model, the value of the i^{th} observation of X_i can be decomposed as follows:

$$X_i = \Sigma_j b_{ij} \times F_j + e_i \text{ (for } j = 1,m) \quad (1)$$

Table 1
Retention times and factors of instrumental response (RF) of the investigated volatile organic compounds and volatile fatty acids. Repeatability (r, n = 6 for each day) and reproducibility (R, n = 4 days) data (absolute values) for each analyte determined on the standard solutions at 0.1 mL/L each compound. Mean recovery values of three replicates (absolute values) for each analyte obtained on the standard solutions at different compound concentrations (0.1, 0.02, and 0.002 mL/L each compound) and effect of analyte concentration on recovery. Purity of chemical standards (%).

VOC (0.1 mL/L)	Retention time (min)	RF[1]	rSD	rCV	RSD	RCV	Recovery[2]	Supplier	Purity (min %)
Aldehydes									
Acetaldehyde	3.686	1.61	0.018	0.018	0.018	0.018	1.04	Sigma-Aldrich	99.5
Propionaldehyde	4.521	2.34	0.040	0.040	0.016	0.017	1.04	Sigma-Aldrich	97.0
2-Methyl propionaldehyde	4.845	2.15	0.029	0.029	0.018	0.018	1.04	Sigma-Aldrich	99.0
Butyraldehyde	6.096	2.33	0.029	0.029	0.018	0.018	1.04	Sigma-Aldrich	99.0
2-Methyl butyraldehyde	7.077	1.37	0.035	0.035	0.019	0.019	1.04	Sigma-Aldrich	95.0
3-Methyl butyraldehyde	7.222	1.26	0.025	0.025	0.018	0.018	1.04	Sigma-Aldrich	97.0
Valeraldehyde	9.365	1.22	0.024	0.024	0.018	0.018	1.04	Sigma-Aldrich	97.0
Hexanal	13.835	1.44	0.023	0.023	0.018	0.018	1.04	Sigma-Aldrich	98.0
Ketones									
Acetone	4.883	1.65	0.086	0.086	0.016	0.017	1.04	Merck	99.8
2-Butanone	6.725	1.17	0.038	0.038	0.018	0.018	1.04	Sigma-Aldrich	99.0
Alcohols									
Methanol	6.767	2.71	0.021	0.021	0.018	0.018	1.04	VWR	99.9
Ethanol	8.051	1.57	0.032	0.032	0.017	0.018	1.04	Sigma-Aldrich	99.0
1- Propanol	12.210	1.19	0.037	0.037	0.017	0.019	1.04	Carlo Erba	99.7
2-Propanol	7.873	1.53	0.038	0.038	0.018	0.018	1.04	Carlo Erba	99.9
2-Propen-1-ol (Allyl alcohol)	16.146	1.02	0.019	0.019	0.018	0.018	1.04	Merck	99.5
1-Butanol	17.530	0.84	0.016	0.016	0.018	0.019	1.04	Sigma-Aldrich	99.5
2-Butanol	11.636	1.20	0.037	0.037	0.016	0.018	1.04	Carlo Erba	99.0
2-Methyl-1-propanol (Isobutanol)	15.145	1.24	0.033	0.033	0.018	0.018	1.04	Carlo Erba	99.0
2-Methyl-1-butanol	20.314	0.92	0.012	0.013	0.018	0.017	1.04	Sigma-Aldrich	99.0
3-Methyl-1butanol	20.386	0.88	0.016	0.016	0.018	0.018	1.04	Carlo Erba	97.0
1-Pentanol	22.369	0.96	0.016	0.017	0.017	0.016	1.04	Carlo Erba	99.0
2-Pentanol	16.471	1.04	0.016	0.016	0.018	0.018	1.04	Sigma-Aldrich	98.0
1-Hexanol	27.094	0.89	0.017	0.017	0.018	0.017	1.04	Sigma-Aldrich	99.0
2-Phenyl ethanol	53.668	0.57	0.017	0.017	0.018	0.018	1.04	Sigma-Aldrich	99.0
Esters									
Methyl acetate	5.129	2.36	0.030	0.030	0.017	0.018	1.04	Merck	99.8
Ethyl acetate	6.427	1.78	0.033	0.033	0.018	0.019	1.04	Merck	99.8
Propyl acetate	9.311	1.44	0.134	0.133	0.019	0.018	1.04	Sigma-Aldrich	98.0
Butyl acetate	13.524	1.19	0.024	0.024	0.018	0.019	1.04	Sigma-Aldrich	99.0
Ethyl propionate	8.677	1.42	0.029	0.029	0.017	0.018	1.04	Sigma-Aldrich	99.0
Methyl butyrate	9.709	1.38	0.017	0.017	0.018	0.018	1.04	Sigma-Aldrich	99.0
Ethyl butyrate	11.896	1.26	0.031	0.030	0.018	0.018	1.04	Sigma-Aldrich	99.0
Butyl butyrate	20.526	1.36	0.016	0.016	0.018	0.019	1.04	Sigma-Aldrich	99.0
Methyl valerate	14.098	1.23	0.037	0.037	0.018	0.018	1.04	Sigma-Aldrich	99.0
Ethyl valerate	16.323	1.20	0.031	0.030	0.019	0.019	1.04	Sigma-Aldrich	98.0
Methyl hexanoate	18.874	1.19	0.031	0.030	0.019	0.018	1.04	Sigma-Aldrich	99.0
Ethyl hexanoate	21.188	1.19	0.015	0.014	0.019	0.018	1.04	Sigma-Aldrich	99.0
Ethyl lactate	26.529	1.50	0.016	0.016	0.018	0.018	1.04	Sigma-Aldrich	98.0
Glycols									
1,2-Propanediol	38.192	0.95	0.025	0.024	0.019	0.018	1.05	Carlo Erba	99.5
2,3-Butanediol[3]	34.451/37.585	0.90	0.051	0.050	0.018	0.018	1.04	Sigma-Aldrich	97.0
Volatile fatty acids									
Acetic acid	31.271	2.40	0.019	0.019	0.022	0.022	1.05	Sigma-Aldrich	WSFA-2
Propionic acid	35.306	1.43	0.018	0.018	0.016	0.015	1.00	Sigma-Aldrich	WSFA-2
Iso-butyric acid	36.748	1.11	0.014	0.014	0.006	0.006	0.99	Sigma-Aldrich	WSFA-2
Butyric acid	39.642	1.11	0.011	0.011	0.015	0.014	0.98	Sigma-Aldrich	WSFA-2
Iso-valeric acid	47.705	0.95	0.011	0.011	0.010	0.010	0.95	Sigma-Aldrich	WSFA-2
Valeric acid	45.068	0.96	0.013	0.013	0.013	0.012	0.98	Sigma-Aldrich	WSFA-2
Caproic acid	50.433	0.97	0.026	0.026	0.018	0.018	1.04	Sigma-Aldrich	99–100
Coelutions									
Isoamyl acetate + propyl butyrate	15.720	1.00	0.032	0.015	0.038	0.018	-	Emplura + Sigma-Aldrich	both 99.0
Internal standard									
Pivalic acid	37.247							Sigma-Aldrich	100

rCV, coefficient of variation of repeatability; RCV, coefficient of variation of reproducibility; rSD, standard deviation of repeatability; RSD: standard deviation of reproducibility.

Carlo Erba Reagents S.r.l., Milano, Italy; Merck, Darmstadt, Germany; Sigma-Aldrich, St. Louis, MO, USA; VWR International S.r.l., Milano Italy.

WSFA-2, mix of acetic, propionic, butyric, isobutyric, valeric, isovaleric acids was 0.1% each analyte in deionized water.

[1] Factors of instrumental response for each chemical compound determined respect to pivalic acid on average of five injections.

[2] No effect of each analyte concentration was observed on recovery.

[3] Mixture of racemic and meso forms of 2,3-butanediol which splits in two peaks when injected into GC/FID equipped with capillary column DB-WAX UI. The parameters of instrumental method performance (i.e., rCV, RCV, and recovery) were determined on peak at 37.585 min, peak at 34.451 min being below the limit of detection at the highest evaluated concentration of solution standard (i.e., 0.1 mL/L).

Table 2
Repeatability (r, n = 6 for each day) and reproducibility (R, n = 4 days) data (absolute values) for each detected analyte obtained from grass and corn silage. Mean recovery values of three replicates (absolute values) for each analyte obtained on dry corn silage spiked with standard solutions at different compound concentration (0.1, 0.02, and 0.002 mL/L each compound) and effect of analyte concentration on recovery.

VOC (mg/kg DM)	Grass silage	Corn silage 1	Corn silage 2	rSD	rCV	RSD	RCV	Recovery
Aldehydes								
Acetaldehyde	15.71	38.47	24.68	0.011	0.040	0.013	0.045	0.71
Propionaldehyde	66.73	24.68	n.d.	0.008	0.026	0.006	0.017	0.68
2-Methyl propionaldehyde	n.d.	n.d.	n.d.	-	-	-	-	0.87
Butyraldehyde	n.d.	n.d.	n.d.	-	-	-	-	0.57
2-Methyl butyraldehyde	39.45	34.63	20.52	0.013	0.041	0.014	0.043	0.58*
3-Methyl butyraldehyde	30.11	31.02	25.46	0.008	0.028	0.007	0.024	0.56*
Valeraldehyde	n.d.	n.d.	n.d.	-	-	-	-	0.62
Hexanal	n.d.	n.d.	n.d.	-	-	-	-	0.66
Ketones								
Acetone	130.90	30.56	33.90	0.014	0.025	0.007	0.018	0.60
2-Butanone	n.d.	n.d.	n.d.	-	-	-	-	0.80
Alcohols								
Methanol (g/kg DM)	0.57	0.50	0.48	0.001	0.017	0.001	0.010	0.64*
Ethanol (g/kg DM)	7.57	6.19	7.08	0.001	0.010	0.001	0.012	0.78
1- Propanol (g/kg DM)	8.34	3.20	6.17	0.001	0.009	0.001	0.010	0.76
2-Propanol (g/kg DM)	4.43	0.04	0.44	< 0.001	0.024	0.001	0.024	0.77
2-Propen-1-ol (Allyl alcohol)	n.d.	14.57	n.d.	0.002	0.051	0.005	0.094	0.77
1-Butanol	48.51	9.03	n.d.	0.006	0.047	0.005	0.033	0.75
2-Butanol	83.42	46.01	290.06	0.032	0.029	0.041	0.035	0.76
2-Methyl-1-propanol (Isobutanol)	n.d.	n.d.	n.d.	-	-	-	-	0.86
2-Methyl-1-butanol	16.30	n.d.	n.d.	0.002	0.044	0.001	0.024	0.71
3-Methyl-1butanol	23.28	9.17	7.54	0.003	0.025	0.002	0.013	0.58
1-Pentanol	8.99	n.d.	n.d.	0.001	0.025	< 0.001	0.013	0.70
2-Pentanol	n.d.	n.d.	n.d.	-	-	-	-	0.71
1-Hexanol	n.d.	n.d.	n.d.	-	-	-	-	0.58
2-Phenyl ethanol	12.35	n.d.	n.d.	0.001	0.034	0.001	0.028	0.70
Esters								
Methyl acetate	75.20	n.d.	22.47	0.009	0.029	0.004	0.015	0.63
Ethyl acetate	128.01	42.98	108.60	0.018	0.021	0.019	0.026	0.60
Propyl acetate	154.47	32.66	108.19	0.030	0.034	0.018	0.019	0.62
Butyl acetate	n.d.	n.d.	n.d.	-	-	-	-	0.76
Ethyl propionate	n.d.	n.d.	n.d.	-	-	-	-	0.57*
Methyl butyrate	n.d.	n.d.	n.d.	-	-	-	-	0.56
Ethyl butyrate	n.d.	n.d.	n.d.	-	-	-	-	0.60
Butyl butyrate	n.d.	n.d.	n.d.	-	-	-	-	0.83
Methyl valerate	n.d.	n.d.	n.d.	-	-	-	-	0.61
Ethyl valerate	n.d.	n.d.	n.d.	-	-	-	-	0.55
Methyl hexanoate	n.d.	n.d.	n.d.	-	-	-	-	0.88
Ethyl hexanoate	n.d.	n.d.	n.d.	-	-	-	-	0.97
Ethyl lactate	40.95	99.38	96.19	0.017	0.022	0.011	0.013	0.80
Glycols (g/kg DM)								
1,2-Propanediol	6.67	3.25	0.70	< 0.001	0.011	< 0.001	0.011	1.04
2,3-Butanediol[1]	0.05	0.16	n.d.	< 0.001	0.031	< 0.001	0.042	1.06
Volatile fatty acids (g/kg DM)								
Acetic acid	38.58	23.89	19.87	0.002	0.008	0.002	0.008	0.94
Propionic acid	2.03	0.72	0.57	< 0.001	0.010	< 0.001	0.006	0.88
Iso-butyric acid	n.d.	n.d.	n.d.	-	-	-	-	0.94 *
Butyric acid	0.45	0.45	0.02	< 0.001	0.013	< 0.001	0.019	0.93 *
Iso-valeric acid	n.d.	n.d.	n.d.	-	-	-	-	0.92 *
Valeric acid	0.05	0.01	n.d.	< 0.001	0.044	< 0.001	0.040	0.95
Caproic acid	0.01	0.03	n.d.	< 0.001	0.046	< 0.001	0.083	1.05
Coelutions (mg/kg DM)								
Isoamyl acetate + propyl butyrate	n.d.	n.d.	n.d.	-	-	-	-	-

n.d., not detected; rCV, coefficient of variation of repeatability; RCV, coefficient of variation of reproducibility; rSD, standard deviation of repeatability; RSD, standard deviation of reproducibility.
[1]Sum of all the isomers of 2,3-butanediol.
* The recovery was influenced by concentration of specific analyte at a $P \leq 0.05$.

Table 3
Loading vectors of silage and haylage chemical parameters (dry matter, ammonia nitrogen, and pH), volatile organic compounds, and volatile free fatty acids on varimax rotated extracted factors (F) as estimated by multivariate analysis, and univariate analysis on factor subjected-based scores.

Multivariate analysis	F1 Clostridia	F2 Enterobacteria, bacilli, and clostridia	F3 Yeasts and facultative heterofermentative LAB	F4 Yeasts	F5 Obligate and facultative heterofermentative LAB	F6 Lactococci	F7 Obligate hetero-fermentative bacteria
Variable							
Isoamyl acetate + propyl butyrate	97[1]	-8	-5	-2	0	1	5
Iso-valeric acid	95[1]	-3	0	-2	-3	9	3
Iso-butyric acid	93[1]	17	-4	-4	-2	10	1
Valeric acid	79[1]	55[1]	-10	-4	5	-2	-1
Butyric acid	77[1]	58[1]	-1	-5	7	-7	1
Propyl acetate	55[1]	-7	-10	2	11	15	53[1]
Ammonia nitrogen	46[1]	22	1	0	34	38	15
Ethyl butyrate	6	92[1]	17	2	0	-3	-2
2,3-Butanediol	1	87[1]	18	5	-9	0	-3
Methyl butyrate	3	84[1]	24	-6	1	9	3
2-Butanol	33	69[1]	11	7	12	-1	25
1-Butanol	51	66[1]	-5	-1	6	-12	-7
2-Methyl-1-propanol (Isobutanol)	-2	13	87[1]	13	-18	-10	-11
2-Methyl-1-butanol	-1	12	86[1]	-14	-8	18	6
3-Methyl-1-butanol	-8	20	85[1]	18	-21	7	-16
2-Phenyl ethanol	-11	10	80[1]	8	-7	9	-9
Methanol	-8	8	64[1]	-26	8	24	10
Ethanol	-2	6	10	89[1]	2	1	9
Ethyl lactate	-9	-4	-8	88[1]	15	18	1
Ethyl acetate	-1	-2	17	64[1]	15	-19	25
Acetic acid	20	-6	-16	12	74[1]	-1	40
1,2-Propanediol	-6	-1	-17	-17	72[1]	-22	-9
Lactic acid	-18	3	11	48	53[1]	37	12
2-Propanol	42	9	-8	-11	43	-29	8
pH	6	8	30	-41	-64[1]	-17	1
Dry matter	-21	-13	19	-35	-70[1]	-21	-11
2-Methyl propionaldehyde	11	1	9	15	-2	86[1]	-3
2-Methyl butyraldehyde	9	-3	21	4	4	86[1]	-7
Ethyl propionate	-7	3	-7	18	5	-13	90[1]
Propionic acid	57	5	-13	5	21	-4	70[1]
1-Propanol	62	-7	-13	1	17	12	62[1]
2-Butanone	-1	-1	16	8	6	-4	-6
2-Propen-1-ol (Allyl alcohol)	-5	3	-15	0	1	22	41
Methyl acetate	9	5	36	-20	17	-6	2
3-Methyl butyraldehyde	0	-11	23	19	-12	4	-13
Caproic acid	10	48	-22	-9	5	15	-8
Acetone	-7	5	3	-18	10	30	-5
Propionaldehyde	-6	0	-10	-15	15	35	2
1-Hexanol	-2	-4	8	23	-5	15	-3
Acetaldehyde	13	-7	7	37	15	22	1
Eigenvalue	5.92	4.36	4.04	3.12	2.93	2.61	2.60
Proportion, %	0.12	0.09	0.08	0.06	0.06	0.05	0.05
Cumulative, %	0.12	0.20	0.28	0.34	0.40	0.45	0.50

[1]Variables loaded on extracted components.
Not detected variables were: butyraldehyde, valeraldehyde, butyl acetate, hexanal, methyl valerate, ethyl valerate, 2-pentanol, methyl hexanoate, butyl butyrate, ethyl hexanoate, and 1-pentanol.

where F_j is the j^{th} common factor (latent variable); b_{ij} is the factor loading, which weighs the i^{th} original variable in the composition of the jth factor; m is the number of extracted factors; and e_i is the uniqueness of the i^{th} variable (i.e., the amount of variability that the i^{th} variable does not share with other variables; Krzanowski, 2000). For each variable, the proportion of variance explained by a common factor is the communality. The major result of this analysis is the separation of the total variance of each variable into its common and unique components. The unique variance consists of specific variance and random error, which usually cannot be separated. The common and specific variance components together constitute the reliable variance (Enevoldsen et al., 1996). The sum of the communalities of all original variables is the total amount of variance explained by the considered factors. The PCA was used to extract the common factor using PROC FACTOR of SAS (version 9.2). The PCA determines the best reconstruction of the correlation structure among studied variables (R*) as:

$$R^* = BB' + \psi \quad (2)$$

where B is the matrix of loading coefficients (b_{ij}), BB′ is the diagonal element that represents communality, and ψ is the diagonal matrix of uniqueness. This calculation provides extraction of new variables (latent common factors) that represent the (co)variance structure. The (co)variance in a set of variables can be subdivided into the communality, which is the (co)variance shared by all variables, and uniqueness, which is each single variable (Morrison, 1976). The number of factors to be retained (q) was determined from the eigenvalue (>1) and lower than the number of original variables p (thus $q < p$). The factor loading (correlations between the common latent factors and the original variable) and explained communalities were determined using the Kaiser Measure of Sampling Adequacy. Each variable was considered related to a factor if the absolute value of its loading was more than approximately 0.55. Then, to improve the interpretation of the extracted factors, the factor loading matrix (B) was rotated using the VARIMAX procedure of SAS. Multivariate analysis was used as a mathematical filter to detect anomalous data and outliers (Yoder et al., 2014). Thus, data were inspected by applying this multivariate analysis and focusing on loadings and subject scores. Finally, only 5 extreme values had subject scores greater than 3.0, and these samples were excluded from the database. Furthermore, for each extracted factor, individual scores were computed for each observational unit (silage or haylage) using standardized scoring coefficients extracted from the factor analysis and multiplied by the respective standardized data vector (Zvar) in the data set. This provided a direct calculation of factor scores for other observational units in an independent data set without the need for performing a new factor analysis. In other words, as indicated by the following SCORE procedure from Usage note 22554 of SAS, for a given sample:

Factor 1 = scoring var1 × Zvar1 + scoring var2 × Zvar2 + … scoring varn × Zvarn·

Factor scores for each sample on each extracted factor were treated as new variables, and the main effects of silage and haylage categories (n = 10) were analyzed using the PROC GML procedure of SAS. The means were post-hoc compared by using LSMEANS option of SAS. The significance was declared at a $P \leq 0.05$.

3. Results

3.1. Gas-chromatographic method performance

In the present study a total of 48 compounds were investigated, however the developed method showed to be potentially suitable for the identification and quantification of 46 single compounds in a unique chromatographic run. In addition, one coelution between two compounds (i.e. isoamyl acetate and propyl butyrate) was observed (chromatograms of GC/FID analysis of standard solution A and B are displayed in Supplementary Figs. 1 and 2).

The LOD was determined to be 0.30 mg/kg of fresh silage for aldehydes, methanol, methyl acetate, and acetic acid and 0.20 mg/kg of fresh silage for all the other compounds. The LOQ was of 0.90 mg/kg of fresh silage for aldehydes, methanol, methyl acetate, and acetic acid, and 0.60 mg/kg of fresh silage for all the other compounds. Retention times and RF of the investigated VOC are shown in Table 1 as well as repeatability and reproducibility parameters for each compound obtained from standard solution A and B. The rCV ranged from 0.011 to 0.133 with an average value of 0.028, whereas the RCV ranged from 0.006 to 0.022 with an average value of 0.017, respectively. Repeatability and reproducibility data obtained from grass and corn silage are displayed in Table 2 (an example of GC/FID chromatogram of VOC extracted from silage sample is shown in Supplementary Fig. 3). The rCV ranged from 0.008 to 0.051 with an average value of 0.026, whereas the RCV changed from 0.006 to 0.094 with an average value of 0.027, respectively. Mean values of recovery obtained on the standard solutions at different concentration levels of compounds (i.e., 0.1, 0.02, and 0.002 mL/L each analyte; Table 1) were in the range 0.95–1.04. No effect of analyte concentration was observed on recovery. Mean values of recovery for each analyte obtained on dry corn silage spiked with standard solutions at different compound concentration (0.1, 0.02, and 0.002 mL/L each compound) were in the range 0.55–1.06 (Table 2). A linear effect of analyte concentration ($P < 0.05$) was found on recovery of 2-methyl butyraldehyde, 3-methyl butyraldehyde, methanol, ethyl propionate, iso-butyric acid, butyric acid, and iso-valeric acid. In particular for the latter, recovery reduced by decreasing compound concentration.

3.2. Interpretation of extracted factors

Loading vectors of silage and haylage chemical parameters, and VOC on varimax rotated extracted factors are shown in Table 3. The interpretation of the extracted factors by multivariate analysis, based on loading vectors, permitted to identify 7 factors with biological meaning. The latter factors were named throughout the text as follows: “clostridia” for F1, “enterobacteria, bacilli, and

clostridia" for F2, "yeasts and facultative heterofermentative lactic acid bacteria (LAB)" for F3, "yeasts" for F4, "obligate and facultative heterofermentative LAB" for F5, "Lactococci" for F6, and "obligate hetero-fermentative bacteria" for F7.

In particular, some VFA (i.e., iso-valeric, iso-butyric, valeric, and butyric acids), isoamyl acetate with propyl butyrate, propyl acetate, and ammonia nitrogen loaded on the "clostridia" factor. The loading compounds on "enterobacteria, bacilli, and clostridia" factor were valeric and butyric acids, methyl and ethyl butyrate, 2,3-butaneldiol, 1-butanol, and 2-butanol. Some alcohols (i.e. 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenyl ethanol, and methanol) were correlated on the "yeasts and facultative heterofermentative LAB" factor whereas ethanol together with ethyl lactate and ethyl acetate were the loading variables on the "yeasts" factor. Acetic and lactic acids together with 1,2-propanediol were positively correlated on the "obligate and facultative heterofermentative LAB" factor whereas pH and DM were negatively correlated on the same factor. The 2-methyl propionaldehyde and 2-methyl butyraldehyde loaded on the "Lactococci" factor. The loading compounds on the "obligate hetero-fermentative bacteria" factor were ethyl propionate, propionic acid, 1-propanol, and propyl acetate.

3.3. Principal factor extraction

Seven factors showing biological relevance were extracted from PCA explaining 0.50 of the total variability of the original data (Table 3). Means and root mean square errors of the extracted factors for the different silage and haylage categories, and effect of silage or haylage category on extracted factors are shown in Table 4. Means and standard errors of the chemical and fermentative parameters (i.e. VOC) are displayed in Supplementary Table 1.

The alfalfa haylage followed by ryegrass silage differed ($P < 0.05$) from the other categories on the "enterobacteria, bacilli, and clostridia" factor for higher factor means. Silage and haylage containing legume crops (i.e., alfalfa and legume haylage followed by alfalfa silage and mixture crop haylage), except for mixture crop silage, showed higher ($P < 0.05$) factor values compared to the other groups on the "yeasts and facultative heterofermentative LAB". The fermented products containing grass crops, except for mixture crop and sorghum silage, differed ($P < 0.05$) from the other categories on the "yeasts" factor for higher factor means. Within "obligate and facultative heterofermentative LAB" factor, all silage based on or including grass crops had higher ($P < 0.05$) factor values than the other fermented products. Ryegrass and mixture crop silage together with grass and mixture crop haylage showed lower ($P < 0.05$) factor means in comparison with the other categories on the "Lactococci" factor.

Table 4
Means and root mean square errors of the extracted factors for the different silage and haylage categories, and effect of silage or haylage category on extracted factors.

Item	F1 Clostridia	F2 Enterobacteria, bacilli, and clostridia	F3 Yeasts and facultative heterofermentative LAB	F4 Yeasts	F5 Obligate and facultative heterofermentative LAB	F6 Lactococci	F7 Obligate hetero-fermentative bacteria
Alfalfa haylage (n = 11)	-0.04	1.88	1.09	-0.47	-0.38	0.54	0.12
Mixture crop haylage (n = 19)	-0.08	-0.17	0.52	0.82	-0.66	-0.26	-0.27
Grass haylage (n = 20)	-0.17	-0.13	-0.31	0.05	-0.56	-0.32	-0.25
Legume haylage (n = 8)	-0.13	-0.27	0.77	-0.65	-0.31	0.08	0.05
Mixture crop silage (n = 10)	-0.05	-0.08	-0.28	-0.31	0.93	-0.23	-0.14
Small grain silage (n = 10)	-0.15	-0.03	-0.61	0.33	0.38	0.60	0.09
Ryegrass silage (n = 8)	0.58	0.14	-0.04	0.18	0.72	-1.01	-0.17
Corn silage (n = 10)	-0.15	-0.12	-0.70	0.10	0.49	0.40	0.02
Alfalfa silage (n = 8)	-0.22	-0.29	0.67	-0.84	-0.11	0.14	0.20
Sorghum silage (n = 7)	0.98	-0.06	-0.74	-0.25	0.73	0.71	1.12
$\sqrt{MSE}$[1]	0.99	0.91	0.85	0.91	0.85	0.93	0.98
P value	0.29	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.21

[1] $\sqrt{MSE}$, root of mean square error.
Significance was considered at $P \leq 0.05$.

4. Discussion

4.1. Gas-chromatographic method performance

The precision and accuracy of the method for the quantification of all VOC in the standard solutions at different compound concentration were closed to the values reported for these parameters by the literature (rSD and RSD < 0.1, European Commission, 2013; recovery range of 0.70–1.10, Linsinger, 2008). Furthermore, these findings suggested that VOC investigated in the present study showed a good solubility in water medium up to the 0.1 mL/L concentration. Regarding to recovery values obtained on dry corn silage spiked with standard solutions, the method accuracy was in the acceptability range above mentioned for acetaldehyde and 2-methyl propionaldehyde, 2-butanone, alcohols (except methanol, 3-methyl-1-butanol, and 1-hexanol), butyl acetate, butyl butyrate, methyl hexanoate and ethyl lactate, glycols, and all VFA, whereas for the other VOC was in the range of 0.55–0.68. However, rSD and RSD were less than 0.1 for all the investigated compounds showing that the method precision was not affected by matrix effect unlike the method accuracy. The precision and accuracy parameters obtained for all detected compounds can be considered satisfactory for the purpose of the analysis, especially taking into account the relatively high volatility of the analyzed compounds (Ortega et al., 2001).

4.2. Principal factor extraction and interpretation

The F1 was defined "clostridia" because the variables loading on this factor were associated to *Clostridium* fermentation. Generally, the presence of clostridia was linked at the lower decrease of pH value, the pH value can subsequently increase again due to the fermentation of lactic acid to weaker acids like acetic and butyric acid, with a concomitant degradation of proteins and amino acids (Wilkinson, 2005). The risk of secondary fermentation is increased in crops (Wilkinson, 2005) when we have lower DM content (DM<20%), relatively low sugar content (< 30 g of water-soluble carbohydrates/ kg of fresh weight), high buffering capacity (> 400 mEq/ kg DM), and/ or low concentration of nitrates (< 10 g NO_3/ kg total N).

Both saccharolytic (*Clostridium tyrobutyricum*, *C.butyricum* and *C.beijerinckii*) and to a lesser extent proteolytic *Clostridium* spp. (*C. bifermentas*, *C.sporogenes)* can produce butyric acid (Buxton and O'Kiely, 2003; Dunière et al., 2013). In particular, butyric acid (Vissers et al., 2007) and valeric acid (Gómez-Torres et al., 2015) were associated to *Clostridium tyrobutyricum* fermentation. This species, among all genome sequenced butyric acid bacteria has the smallest genome and is the most studied butyric acid production bacterium, however this species has a relatively limited substrate spectrum and cannot use polysaccharides or most disaccharides. Compared to *Clostridium tyrobutyricum*, *Clostridium butyricum* has larger genome and can use a wider spectrum substrate, including starch, most disaccharides and glycerol (Jiang et al., 2018). This species usually produces 1,3-propandiol as the main product with much lower levels of butyrate and acetate as by products. Some strains of the *C. butyricum* group produce 2-propanol and butanol (Hippe et al., 1992), but it is unclear if this occurs in silage. Similarly, *C. tyrobutyricum* has been observed to produce small quantities of butanol in fermenting L-lactic acid (Hippe et al., 1992). In addition, Takeda and Furusaka (1975) reported that *Clostridium* spp. produced branched-chain fatty acids such as iso-valeric and iso-butyric acid. The same authors found that from the metabolism of leucine and proline (or glycine), *Clostridium indologenes*, *Clostridium putrefaciens*, and *Clostridium capitovale* produced iso-valeric and ammonia by the Stickland reaction (Takeda and Furusaka, 1975). Le Bourhis et al. (2007) in a study in cheese matrix found that the association between *Clostridium tyrobutyricum* and *C. sporogenes,* and *C. beijerinckii* enhanced the butyric fermentation and the cheese defects. Similar interaction could also occur in silage. During the ensiling process, silage microbial community strongly affect the silage quality and, in this sense, clostridial fermentation, caused by *Clostridium* spp, should be avoided because it is responsible for silage deterioration as well as undesirable rancid odor development and accumulation of ammonia and amines (Li et al., 2020). Our results of multivariate analysis did not highlight differences in clostridial fermentation among the investigated silage and haylage groups. However, 1-butanol together with butyric and valeric acids loaded on the "enterobacteria, bacilli, and clostridia" factor. It is known that clostridial fermentation also has pathways to produce butanol production (McDonald et al., 1991). The pH influenced the activity of NADH-dependent butanol dehydrogenase, but generally the undissociated butyric acid could be the major driving force for butanol production (Yang et al., 2013; Al-Shorgani et al., 2018) *C.beijerinckii* and *C.acetobutylicum* produce large quantities of solvent like butanol, acetone and ethanol, and can also produce butyrate and acetate. Our finding showed that clostridial fermentation occurred in alfalfa haylage and ryegrass silage. On the other hand, these crops are especially challenging to ensile due to high buffering capacity and low sugar content for alfalfa (McDonald et al., 1991), critical moisture (Yan et al., 2014) and soil contamination (Queiroz et al., 2018) for alfalfa and ryegrass. In addition to the organic compounds mentioned above, other VOC loaded on "enterobacteria, bacilli, and clostridia" factor linked to enterobacteria and bacilli fermentation. The biosynthesis of 2,3-butanediol in bacterial cells is accompanied by mixed-acid fermentation, which is characteristic of most members of the family Enterobacteriaceae. Generally, bacteria produce 2,3-butanediol as strategy to counter the acidic conditions. It is well known the LAB ability of produce 2,3-butanediol by pyruvate-diacetyl-acetoin pathway, that is after converted in 2-butanol (Ghiaci et al., 2014; Hieke and Vollbrecht, 1974). da Silva et al. (2019) observed a strong correlation between the 2,3 butanediol concentration and the inoculation of *Lentilactobacillus buchneri (LB)* in high-moisture corn and rehydrated corn grain silage. Therefore, it cannot be ruled out the presence of LAB that possess the ability of synthesize 2,3 butanediol.

The F3 was named "yeasts and facultative heterofermentative LAB" because VOC loading on this factor could be related to yeasts and heterofermentative LAB activity. In particular, some branched-chain alcohols (i.e. 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenyl ethanol) are produced by LAB via amino acid catabolism (Edwards and Peterson, 1994; Silva and Malcata, 1998; Hafner et al., 2013). Higher alcohols (such as branched-chain alcohols) can be produced from 2-keto acids by employing the last two steps of the Ehrlich pathway for the biosynthesis of fusel alcohols originally found in yeast (Hazelwood et al.,

2008). Amino acids that are assimilated by the Ehrlich pathway (valine, leucine, isoleucine, methionine, and phenylalanine) are taken up slowly throughout the fermentation time. After the initial transamination reaction, the resulting α-keto acid cannot be redirected into central carbon metabolism. Before α-keto acids are excreted into the growth medium, yeast cells convert them into fusel alcohols or acids via the Ehrlich pathway (Hazelwood et al., 2008).

However, to date there are no information about methanol production by silage bacterial or fungal species (Hafner et al., 2013). Generally, the presence of this alcohol in ensiled products is ascribed to activity of plant enzymes prior to ensiling. The pectin demethylation, that occurs in leaf expansion and cell wall synthesis, brings about the methanol production (Fall and Benson, 1996). Methanol is a major end-product of pectin metabolism by microorganisms (Schink and Zeikus, 1980), and by certain bacteria of the genus *Enterobacter* and *Clostridium* (Lindinger et al., 2006). Pectin is strongly fermented by all strains of *Clostridium butyricum* tested. Products from the fermentation of pectin are large amounts of methanol, acetate, H_2, and CO_2, and moderate amounts of butyrate and ethanol (Rainey et al., 2015). It has been severally reported that microbial fermentation of substrates rich in pectin can result in the formation of methanol (Siragusa et al., 1988; Nakagawa et al., 2000; Alvarenga et al., 2011). Contaminating yeast has been demonstrated to produce methanol during traditional fermentation (Dato et al., 2005). Recent studies have also shown that the ethanol fermenting yeast, *S. cerevisiae* has several strains with slightly different metabolism (Jespersen, 2003; Okunowo et al., 2005; Stringini et al., 2009) with some strains possibly producing methanol.

However, it is likely that methanol formation in silage occurs through the action of pectinesterase and polygalacturonase, which are responsible for de-esterification of pectin in higher plants (Birch et al., 1981; Hou et al., 2008). Also, it is possible that damage to plant cells during harvest and chopping of corn plants for silage triggered the action of pectinesterase and polygalacturonase, which facilitate the degradation of pectins and, thus, the formation of methanol (Pedrolli et al., 2009). Differences on yeasts and facultative heterofermentative LAB factor were observed between all the fermented products containing legume crops (except mixture crop silage) and the other categories. Legume crops are known to be difficult to ensile due to their high buffering capacity (Henderson, 1962) and the low concentration of water-soluble carbohydrates that can lead to a risk of butyric acid fermentation, thus probably modifying the fermentation pathways occurring in those silage with respect to grass (Hattori et al., 1996).

The "yeasts" component was so denominated because variables loading on this factor could be linked to the yeast activity. In particular, ethanol is one of the products of yeast fermentation using sugar as a substrate (Driehuis and Elferink, 2000), whereas ethyl acetate could be synthesized by certain yeast species (Nordström, 1966; Fredlund et al., 2004; Park et al., 2009; Kruis et al., 2017) that are known to be present in silage (Pahlow et al., 2003). Three types of enzymes were associated with ethyl acetate formation by yeasts: esterases, hemiacetal dehydrogenases, and alcohol acetyl transferases (Park et al., 2009). These enzymes also metabolize ethanol to produce ethyl acetate (Kruis et al., 2017). Brüning et al. (2018) reported that the concentrations of ethyl acetate and ethyl lactate could be increased by delayed sealing silage because yeasts could multiply during delayed sealing causing direct formation of ethyl ester (ethyl acetate and ethyl lactate) or produce ethanol for esterification. The VOC loading on the "yeasts" component showed lower mean values in the fermented products containing legume crops (except for mixture crop haylage). This could be attributed to the excessive sugar availability stimulating the growth of yeasts (Adesogan and Newman, 2010) and, as mentioned above, legume crops are characterized by low concentration of water-soluble carbohydrates (Hattori et al., 1996). Ethyl lactate according to many authors is formed by chemical synthesis from lactate and ethanol (Weiss et al., 2016; Brüning et al., 2018; Weiss et al., 2019). In silage the LAB and in particular obligate homofermenters are the main producers of lactate. This chemical reaction take place slowly when there are a surplus of lactic acid and ethanol (Peter and Vollhardt, 1988). Biochemical production of esters probably occurs in silage as well. LAB have acyltransferase and esterase enzymes that produce esters from carboxylic acids and alcohols (Liu and Siezen, 2006).

The F5 was named as "obligate and facultative heterofermentative LAB" as among the variables loading on this factor, 1,2-propanediol and acetic acid were associated with obligate heterofermentative LAB fermentation pathway but also to the facultative heterofermentative LAB that in specific condition (low availability of glucose) produce acetate. High levels of acetic acid and 1,2-propanediol could be obtained by the silage inoculation with LB, that is one of the most common types of hetero-fermentative LAB that are used to improve the aerobic stability of silage (Schmidt et al., 2009; Oliveira et al., 2017). Homofermentative and facultative heterofermentative LAB generally produce lactic acid which in turn inhibits the growth of epiphytic undesirable microbes by pH lowering (Silva et al., 2016). In our study, the highest values of lactic and acetic acids found in silage containing grass crops agreed with the lowest pH values measured in the same samples. Moreover, DM variable negatively correlated on the "obligate and facultative heterofermentative LAB" factor. On the other hand, hetero-fermentative LAB, like LB, were associated to less efficient fermentative pathways that can cause DM losses (Pahlow et al., 2003).

The F6 was named "Lactococci" due to the variables loading on this component, specifically 2-methyl propionaldehyde and 2-methyl butyraldehyde. In particular, both van Kranenburg et al. (2002) and Liu and Siezen (2006) reported that LAB can produce branched-chain aldehydes (i.e. 3-methylbutanal and 2-methylpropanal) via branched-chain amino acid catabolism. Furthermore, the Strecker degradation, an abiotic and non-enzymatic reaction, could synthetise aldehydes. This pathway was observed during cheese production (Smit et al., 2009), however it is possible that the same reaction occurs in silage (Hafner et al., 2013). In our study, high concentrations of 2-methyl propionaldehyde and 2-methyl butyraldehyde occurred in the most part of the investigated fermented products, suggesting that Lactococci pleasantly grow and perform their activity on fermented substrates. In Lactococci, branched chain amino acids (BCAA) are primarily converted into α-keto acids by transamination (Yvon et al., 1997, Atiles et al., 2000; Yvon et al., 2000) which are further converted into hydroxyacids or into flavor compounds, such as carboxylic acids by an oxidative decarboxylation, or the methylaldehydes, 2- and 3-methylbutanal, and 2-methylpropanal by a non-oxidative decarboxylation. Non-oxidative decarboxylation of α-keto-β-methyl-n-valeric acid leads to the formation of 2-methylbutanal. Recently two 2-keto acid decarboxylases have been identified from *Lactococcus lactis* with a high level of conservation (almost 90 % amino acid sequence identity) between them and were named α-ketoisovalerate decarboxylase (Kivd), or keto acid decarboxylase (KdcA) (De La Plaza et al., 2004;

Smit et al., 2005).

Our results obtained through multivariate analysis did not show differences among the different investigated silage and haylage groups on the "obligate hetero-fermentative bacteria". The "obligate hetero-fermentative bacteria" factor was characterized by VOC such as ethyl propionate, propionic acid, 1-propanol, and propyl acetate. This result agreed with the data obtained by Raun and Kristensen (2010) that reported positive correlation between 1-propanol concentration and contents of propionic acid and propyl acetate in fermented products. The specific pathway was associated with hetero-fermented silage by previous studies (McDonald et al., 1991; Raun and Kristensen, 2010). In addition, 1-propanol and propionic acid were reported as dominant products of microbial 1, 2-propanediol degradation (Krooneman et al., 2002).

5. Conclusion

Alfalfa haylage and ryegrass silage were characterized by VOC associated to fermentations by enterobacteria, bacilli, and clostridia. Legume crops, except mixture crop silage, were characterized by higher levels of VOC can be related to yeasts and facultative heterofermentative LAB activity and lower levels of VOC deriving from yeasts activity compared with the other silage. In grass, corn and sorghum silage occurred VOC linked to obligate and facultative heterofermentative LAB fermentation. A Clostridia activity was observed for sorghum silages. The most part of the investigated fermented products were characterized by 2-methyl propionaldehyde and 2-methyl butyraldehyde associated to Lactococci activity. Despite a characterization of expected VOC for different silage categories was reported in present survey, future studies are required on a greater number of samples to better define VOC profiles characterizing the different types of silage.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115593.

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