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Greenhouse gas formation during the ensiling process of grass and lucerne silage

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ABSTRACT

Silage is an essential global feedstuff and an emitter of greenhouse gases. However, few studies have examined the formation of carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) during the ensiling process. This study aimed to record the course of gas concentrations in forage during the ensiling process and determine the temporal variation in the (microbiological) formation processes. Grass and lucerne, each with two different dry matter (DM) concentrations (four variants, each n = 3), were ensiled in laboratory-scale barrels (120 L). Gas samples were taken from the headspace of the barrels and analysed using a gas chromatograph. The measurement period included the first 49 days of the ensiling process and the measurement interval was 0.5–48.0 h. For all variants, a rapid increase in CO_2 concentration and a one-time N_2O concentration peak was observed between ensiling hours 36 and 96. Lower DM concentration led to significantly faster CO_2 production ($p < 0.05$). Lucerne forage and higher DM concentrations led to significantly increased N_2O concentrations ($p < 0.05$). The extensive measurements demonstrated that butyric acid formation by clostridia contributes to CH_4 formation; thus, lucerne silage had a significantly higher concentration from ensiling day 13 ($p < 0.05$). Therefore, malfermentation actively contributes to the formation of greenhouse gases. The method described here provides further insights into greenhouse gas formation during the ensiling process and can thus help to improve ensiling research and management.

1. Introduction

The quantification of climate-relevant emissions from agriculture is increasingly important, particularly in climate change research (Gerber et al., 2013; Grossi et al., 2019; Myhre et al., 2014). In livestock husbandry, emissions from the husbandry process itself (Mostafa et al., 2020; Philippe et al., 2011; Schmithausen et al., 2018a, b) as well as the downstream process chain (Amon et al., 2006; Kupper et al., 2020; Rodhe et al., 2015), e. g. slurry management, have been thoroughly investigated. However, emission behaviour in the upstream part of the process chain, such as during feed production, is not well studied.

To ensure that livestock are adequately fed throughout the year, one-time harvest yields must be preserved. The aim is to ensure that the final feed product suffers the smallest possible losses in quantity and quality. Consequently, feed preservation methods are used to conserve natural resources. Silage is one of the most important feeds used in global livestock production (Weinberg and Ashbell, 2003; Wilkins et al., 1999; Wilkinson and Muck, 2019), especially for ruminants. In addition, silage

Abbreviations: ANOVA, analysis of variance; DM, dry matter; FM, fresh matter; G, grass; G LW, grass longer wilted (ca. 24 h wilting); G SW, grass shortly wilted (ca. 20 h wilting); GHG, greenhouse gas; GWP, global warming potential; L, lucerne; LW, longer wilted (ca. 24 h wilting); L LW, lucerne longer wilted (ca. 24 h wilting); L SW, lucerne shortly wilted (ca. 20 h wilting); SW, shortly wilted (ca. 20 h wilting).

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is used to feed pigs (Ebertz et al., 2020; Lengling et al., 2020) and for biogas production (Jacobs et al., 2017; Weiland, 2010). It is now known that silage emits greenhouse gases (GHGs) (Krommweh et al., 2020; Schmidt et al., 2011, 2012; Zhao et al., 2016) with various global warming potentials (GWPs) (Myhre et al., 2014): carbon dioxide (CO_2; GWP = 1), methane (CH_4; GWP = 28) and nitrous oxide (N_2O; GWP = 265). CO_2 emissions are considered climate-neutral because of their biological origin, but forage production does require the use of fossil carbon reserves (e.g. fertilisers or fuels). Additionally, carbon emissions during the ensiling process or the aerobic feed-out phase (mostly due to yeast metabolism) are associated with feed and energy losses. Poor silage quality due to, for example, butyric acid production by clostridia [high risk in silages with low dry matter concentrations (DM)] can induce the degradation of proteins and amino acids to ammonia (Ohshima and McDonald, 1978). These effects are considered negative as they can exacerbate the loss of feed quality and negatively affect animal nutrition. Importantly, the conversion of forage into climate-relevant gases and the environmental impact of this process are increasingly understood to play a role in ongoing climate change. Therefore, the development of successful and efficient feed conservation processes is relevant to both animal nutrition and environmental protection. CH_4 and N_2O emissions are not considered climate-neutral because of their higher GWPs; thus, the conversion of high-value feed substances into these climate-relevant gases is a particularly important issue.

Much of the previous research has focused on silage emissions during the aerobic feed-out phase (Gerlach et al., 2018; Hafner et al., 2010; Krommweh et al., 2020; Malkina et al., 2011; Montes et al., 2010), with the focus in part on yeast activity and aerobic stability (Jungbluth et al., 2017; Shan et al., 2021a, b; Sun et al., 2015). Some GHGs are emitted once after formation during the ensiling process (Krommweh et al., 2020). Studies on GHG concentrations during the ensiling process date back several decades (Peterson et al., 1958; Wang and Burris, 1960; Weinberg and Ashbell, 1994) or do not include measurements of CO_2, CH_4 and N_2O (Franco, 2016; Peterson et al., 1958; Wang and Burris, 1960; Weinberg and Ashbell, 1994; Zhao et al., 2016). The earlier studies (Peterson et al., 1958; Wang and Burris, 1960) had methodological limitations in this context because the gas analysis technique used (mass spectrometry) was not capable of differentiating precisely between CO_2 and N_2O (Zhao et al., 2016). Furthermore, the earlier conducted studies were mostly aimed at assessing gas emissions from silage from the perspective of occupational safety (e.g. silo filler's disease caused by nitrogen dioxide) or in relation to the effects on animal nutrition. The current focus is largely on the environmental consequences of these gas emissions, especially in modern political discussions (e.g. farm-to-fork). A large proportion of the studies have been conducted using maize (*Zea mays*) silage (Peterson et al., 1958; Schmidt et al., 2012; Wang and Burris, 1960; Weinberg and Ashbell, 1994; Zhao et al., 2016), whereas studies using grass (Krommweh et al., 2020; Wang and Burris, 1960) or lucerne (*Medicago sativa*) silage (Franco, 2016; Krommweh et al., 2020; Peterson et al., 1958) are scarce. Since the anaerobic ensiling process involves several phases (Pahlow et al., 2003), the composition and metabolic activity of microorganisms change, especially during the early days of the process. Consequently, the formation and concentration of gases from the silage can also change. Although earlier studies (Peterson et al., 1958; Wang and Burris, 1960) primarily used measurement intervals of 6 h within the first 66 ensiling hours, more recent studies (Bueno et al., 2020; Schmidt et al., 2011, 2012, 2013) have mainly examined gas concentrations on single ensiling days (days 5–61). Only Franco (2016) has investigated silage gases over shorter measurement intervals (down to 0.5 h) within the first 209 ensiling hours; however, in this PhD dissertation, not all GHGs were investigated and concentration courses were not provided.

Consequently, there is still a research gap to be filled, i.e. a study of the formation of individual GHGs at different stages within the ensiling process. In the present study, detailed measurements of the gases produced were taken using precise measurement technology over short measurement intervals and a long measurement period. To this end, two different forages, namely grass and lucerne, with varying DM concentrations (following shorter and longer wilting periods) were investigated. Both DM concentrations were kept at low levels to simulate poor environmental conditions during harvesting and provoke poor silage quality. Measuring the course of the gas concentrations should provide conclusions on the (microbiological) formation processes and therefore the quality of the ensiling process itself.

To summarise, the objectives of this investigation were as follows: (1) to examine whether the method is suitable for analysing climate-relevant gases in silage at a laboratory scale; (2) to record the course of concentrations of three GHGs (CO_2, CH_4 and N_2O) from two forage types (grass and lucerne) ensiled with different DM concentrations (shorter and longer wilting periods) using short sampling intervals over a long period of the ensiling process; (3) to determine the temporal changes in microbiological gas formation during the ensiling process in relation to the chemical composition of the silage.

2. Material and methods

2.1. Forage material and silage variants

Grass and lucerne grown at Campus Frankenforst of the Rheinische Friedrich-Wilhelms-Universität Bonn (Königswinter, Germany; 50°42′50.1″N 7°12′24.9″E) were used as forage for the experiment. The crops were fertilised and managed under common practice conditions. The forage (for chemical composition, see section 3.1) was cut on the evening of the 9th May (second cut) and kept overnight on the pasture for wilting. After collection with a loading wagon (theoretical cutting length: 55 mm) at noon the following day (ca. 20 h of wilting), half of each forage material was ensiled. The other half was spread on a black

Table 1
Characteristics of silage variants within laboratory-scale barrels (120-L volume) according to wilting period and forage material.

	Unit	Silage variants[a]			
		G SW	L SW	G LW	L LW
Number of barrels		3	3	3	3
Forage material		Grass	Lucerne	Grass	Lucerne
Wilting duration		Shortly (ca. 20 h)		Longer (ca. 24 h)	
Temperature range during wilting	°C	0.8–16.8		0.8–17.9	
Temperature sum during wilting[b]	°C	151.3		219.9	
Fresh material per barrel (mean)	kg FM	85.0	81.1	72.9	63.2
Dry matter concentration (mean)	%	21.5	19.5	26.2	22.7
Silage density (mean)	(kg DM) m^{-3}	152.3	131.7	159.1	119.5

FM = fresh matter; DM = dry matter.
[a] Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW). grass longer wilted (G LW) and lucerne longer wilted (L LW).
[b] Sum of hourly mean values.

film in direct sunlight for an additional 4 h (at about 17 °C, i.e. ambient air temperature, under a clear sky) and regularly turned to ensure higher DM concentrations. Consequently, four forage material variants were produced (for details see Table 1): grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW).

After wilting, the plant materials were added to twelve 120-L barrels (high-density polyethylene) for ensiling (n = 3 for each forage variant). The fresh material was filled in layers and each layer was compacted with a hydraulic press to ensure uniform compaction within each barrel (Jungbluth et al., 2016). The density of the material was determined using the volume and weight of the barrel as well as the DM concentration (see Table 1). The plastic barrels were sealed with a modified lid, which was pressed onto the corresponding barrel using a clamping ring. A rubber septum was inserted into the plastic lids, which allowed gas sampling during the ensiling process (see section 2.3). The lids prevented the penetration of ambient air but allowed the (formed) gases inside the barrel to escape above a certain overpressure within the barrel.

After the ensiling procedure was complete, the barrels were stored indoors to ensure constant ambient air temperatures (23.2 ± 1.4 °C). Finally, the barrels were opened on day 149 to collect material samples for laboratory analysis (see sections 2.2 and 3.1).

During wilting, the outdoor air temperatures were collected from the German weather service's climate data centre (station ID 603; opendata.dwd.de, 2021); the weather station itself was positioned in Bonn-Roleber (Germany, 50°44′06.4″N 7°11′35.2″E). During forage storage, the ambient air temperature was measured using NTC thermistor sensors (TinyTag Plus 2 Logger TGP-4500; Gemini Data Loggers Ltd, Chichester, UK). The temperature measurement interval was 15 min.

2.2. Laboratory analysis of the silage material

Material samples were collected from the fresh material (on the day of ensiling, before filling barrels with SW variants) and the ensiled material (149th ensiling day). All samples were stored immediately at −20 °C until they were analysed, which is necessary to avoid further microbial activity and material composition changes before analysis. Because various types of technical equipment were required for analysis, the samples were sent to different laboratories for testing.

The first laboratory (Institute of Animal Science, Rheinische Friedrich-Wilhelms-Universität Bonn, Bonn, Germany) analysed the crude ash (see section 3.1) and crude protein concentration of samples according to specific numbered methods in the German Handbook of Agricultural Research and Analytic Methods (VDLUFA, 2012). Crude ash was determined by ashing the silage samples at 550 °C (method number 8.1). Crude protein concentration was determined using the Dumas combustion method (method number 4.1.2; using a FP328, Leco 8.1; Leco Instrumente, Mönchengladbach, Germany) in which the sample is burnt at 1,000 °C, nitrogen oxides are reduced, and other combustion products are removed. The remaining molecular nitrogen was detected using a thermal conductivity detector and the data were used to calculate the crude protein concentration. These measurement methods have been well established in previous studies (Brüning et al., 2018; Gerlach et al., 2014, 2018).

The second laboratory (Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Universität zu Berlin, Berlin, Germany) analysed the pH and concentrations of lactic acid, acetic acid, butyric acid and its (higher) homologues (in a total sum of n- and iso-butyric acids, C_4 molecules; n- and iso-valeric acids, C_5 molecules; and n-caproic acid, C_6 molecules), and ammonia-N (NH_3–N). DM was corrected for losses of volatiles during drying (Weiß et al., 2020) according to the method of Weißbach and Strubelt (2008). After pre-treatment, lactic acid levels were determined using a liquid chromatography method (via refractive index detection; LC-20 AB, Shimadzu Deutschland, Duisburg, Germany). The other acids were detected using a gas chromatograph (including a flame ionisation detector; GC-2010, Shimadzu Deutschland, Duisburg, Germany) with a free fatty acid phase column (Permabond FFAP 0.25 µm; Macherey-Nagel, Düren, Germany). NH_3–N levels were determined colourimetrically using a continuous flow analyser (San++, Skalar Analytical, Breda, Netherlands). This laboratory methodology has been described in detail in previous studies (Brüning et al., 2018; Gerlach et al., 2018; Weiß et al., 2020; Weiß and Sommer, 2012).

2.3. Sampling and analysis of fermentation gases

During the early part of the ensiling period (i.e. the first 49 days), gas samples were taken manually at regular intervals from the headspace of the silage barrels. Samples were collected using a double cannula connected to a vacuumed glass vial (20-mL volume; Jungbluth et al., 2016; Schmithausen et al., 2018b). The intervals at which gas samples were collected from the barrels varied during the ensiling process: In the first 12 h, the sampling interval was 30 min; from 12 to 48 h, the sampling interval was 2 h; and from ensiling days 3–49, the sampling interval was 2 days. No further gas samples were taken after the 49th ensiling day. The number of gas samples per barrel was 82, with 984 samples taken in total.

Subsequently, the gas samples were analysed using a gas chromatograph (equipped with an electron capture detector and a flame ionisation detector; model 8610C, SRI Instruments, Torrance, CA, USA) according to an established analytical procedure (Krommweh et al., 2020; Schmithausen et al., 2016). CO_2, CH_4 and N_2O were analysed with detection limits of 50.00, 0.08 and 0.01 ppm, respectively. If the concentrations were outside the measuring range of the gas chromatograph, the samples were diluted (1:101 with ambient air) and the corresponding original concentration was calculated.

2.4. Data processing and statistics

The compositions of the different silage variants were compared using one-way ANOVA. The gas concentrations within the silage barrels during ensiling were compared using a mixed ANOVA. In all analyses, Tukey's-HSD test was used as a post hoc test if the homogeneity of variance was given; if not, a Games–Howell test was used. For all statistical analysis, P values < 0.05 were considered significant. IBM SPSS Statistics (Version 26.0) was used to conduct statistical analysis, whereas Microsoft Excel was used to perform descriptive data analysis.

3. Results and discussion

3.1. Composition of the silage

The fresh forage and the ensiled material had low DM concentrations (see Table 2) below or at the lower limit of the recommended range, which is 25%–35% DM for grass silage (Kung et al., 2018) and 30%–35% DM for lucerne silage (Kung et al., 2018; Seglar, 2003). Nevertheless, the difference in DM concentration between the SW and LW variants was significant ($p < 0.05$). The L SW variant was under the target value for the crude protein concentration of lucerne silages (however, incorrect laboratory results were possible due to NH_3–N out-gassing, as explained in the last paragraph of this section), whereas the other variants exceed the target values, i.e. <170 g (kg DM)$^{-1}$ for grass silage (Spiekers, 2012) and ~200 g (kg DM)$^{-1}$ for lucerne silage (Seglar, 2003). These characteristics are due to the early cutting time of lucerne, a leafy second grass cut, the fertiliser management process and the low external air temperature during wilting (see Table 1).

High crude protein, NH_3–N and crude ash concentrations in the silage have buffering properties and can inhibit a rapid decrease in pH at the beginning of ensiling (Kung et al., 2018; Kung and Shaver, 2001). Both grass variants had pH values at the upper end of the recommended values, i.e. 4.0–5.0; however, especially with low DM concentrations,

Table 2
Chemical compositions of fresh and ensiled materials[a] for the tested silage variants[b].

Variant	pH	Dry matter	Crude ash	Crude protein	Lactic acid	Acetic acid	Butyric acid[c]	Ammonia nitrogen	Ammonia nitrogen
		[g (kg FM)$^{-1}$]	[g (kg DM)$^{-1}$]						[g (kg total N)$^{-1}$]
G_{fresh}	6.4	258	131	172	2.2	16.2	0.5	3.3	119
L_{fresh}	6.9	237	118	217	2.0	17.7	0.7	4.2	121
G SW[d]	**4.9**^{a} ± 0.2	**215**^{b} ± 3	**131**^{a} ± 0.3	**208**^{b} ± 8.1	**47.2**^{c} ± 11.0	**11.3**^{a} ± 1.0	**41.6**^{a} ± 7.4	**6.9**^{a} ± 0.5	**206**^{a} ± 12
L SW[d]	**6.2**^{b} ± 0.0	**195**^{a} ± 3	**179**^{b} ± 3.5	**176**^{a} ± 4.0	**1.9**^{b} ± 0.1	**38.6**^{b} ± 2.3	**83.5**^{b} ± 7.2	**22.2**^{b} ± 1.2	**788**^{c} ± 66
G LW[d]	**4.8**^{a} ± 0.1	**262**^{d} ± 1	**127**^{a} ± 4.9	**225**^{c} ± 1.7	**64.6**^{c} ± 7.4	**9.6**^{a} ± 1.7	**26.5**^{a} ± 0.7	**5.8**^{a} ± 0.1	**160**^{a} ± 5
L LW[d]	**7.1**^{c} ± 0.2	**227**^{c} ± 1	**185**^{b} ± 9.0	**246**^{c} ± 8.9	**0.0**^{a} ± 0.0	**29.9**^{b} ± 4.0	**69.0**^{b} ± 7.6	**22.8**^{b} ± 0.3	**580**^{b} ± 22

Means with different superscript lowercase letters within a column differ significantly (Tukey's-HSD or Games–Howell tests, $p < 0.05$).
DM: dry matter, FM: fresh matter, N: nitrogen.
[a] Fresh material was sampled on the ensiling day before the SW variants were ensiled; ensiled material was sampled on the 149th ensiling day.
[b] Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW).
[c] Sum of n- and iso-butyric acids (C_4 molecules), n- and iso-valeric acids (C_5 molecules) and n-caproic acid (C_6 molecules).
[d] Means ± standard deviation (see Fig. 1).

pH values should be at the low end of this target range (Spiekers, 2012). L SW and L LW variants had pH values that were too high given that the maximum target value is pH 4.5 (Kung et al., 2018; Seglar, 2003). These values can be explained by high NH_3–N [partially in the form of dissolved ammonium-N (NH_4^+-N), alkaline substances], low lactic acid and high butyric acid concentrations in the lucerne silage. The butyric acid concentration was greater than the given maximum value of 3 g (kg DM)$^{-1}$ (Spiekers, 2012); thus, it can be considered an indicator of the undesirable activity of clostridia (Rooke and Hatfield, 2003), which can metabolise lactic acid to butyric acid, hydrogen (H_2) and CO_2 during anaerobic (saccharolytic) metabolism. Clostridia are particularly active in silage with pH values > 4.2–4.5 (Pahlow et al., 2003) and DM concentrations <30%–35% (Kung et al., 2018). The LW variants tested here tended to have low butyric acid concentrations ($p < 0.1$ within G variants; $p < 0.05$ within L variants).

All tested materials showed higher NH_3–N concentrations than those measured in previous analyses (Hartinger et al., 2019; Kung and Shaver, 2001; Wyss et al., 2017). Previously, Weiß (2001) reported NH_3–N concentrations of up to 30% in a grass–legume silage mix with added clostridia spores. Wet silages seem to have higher NH_3–N concentrations (Hartinger et al., 2019; Kung and Shaver, 2001) and low wilting intensity also favours high NH_3–N concentrations (Hartinger et al., 2019). Concentrations of NH_3–N at >12% of total N indicate protein degradation by enterobacteria (Pahlow et al., 2003; Spoelstra, 1987) and clostridial activity (Kung and Shaver, 2001; Pahlow and Hünting, 2012). Enterobacteria, which are particularly active during the early stage of ensiling (Heron et al., 1993), can convert nitrate first to nitrite and then to NH_3 and N_2O during denitrification at high pH values (>4.5). Nitrate is known to have an inhibitory effect on clostridia (Kaiser and Weiß, 2007; Weiß, 2001; Wilkinson, 1999), but this was apparently insufficient in the tested materials. Proteolytic clostridia metabolise various proteins and amino acids to NH_3 (Ohshima and McDonald, 1978; Weiß, 2001), among other compounds, during the second stage of anaerobic (clostridial) malfermentation (deamination, decarboxylation and Stickland reactions) (Kaiser and Weiß, 2007; Rooke and Hatfield, 2003; Weiß, 2001). However, clostridial activity has the greatest impact (Kaiser and Weiß, 2007); it causes the formation of ammonia, butyric acid and (higher) homologues (Weiß, 2001) (Table 2).

The combination of low lactic acid, high butyric acid and high NH_3–N concentrations indicates high clostridial activity (Kaiser and Weiß, 2007; Weiß, 2001), in which the saccharolytic clostridia that are active at >4.2 pH (Pahlow et al., 2003) precede the proteolytic clostridia (Weiß, 2001) that are active at >5.0 pH (Pahlow et al., 2003), during the malfermentation phase (lactic acid degradation). The conversion of high-value proteins and amino acids to NH_3–N is known to reduce feed quality (Kung and Shaver, 2001; Wilkinson, 1999) and lead to gaseous emissions, especially when the material has high pH values (i.e. when the equilibrium between volatile ammonia and nonvolatile ammonium shifts towards ammonia). These emissions could be relevant during material analysis, e.g. potential NH_3–N emissions before or during the analysis of crude protein in the L SW variant could affect the calculation of relative NH_3–N concentrations, and for determination of environmental pollution. Further research is therefore recommended because NH_3 emissions were not assessed during this investigation.

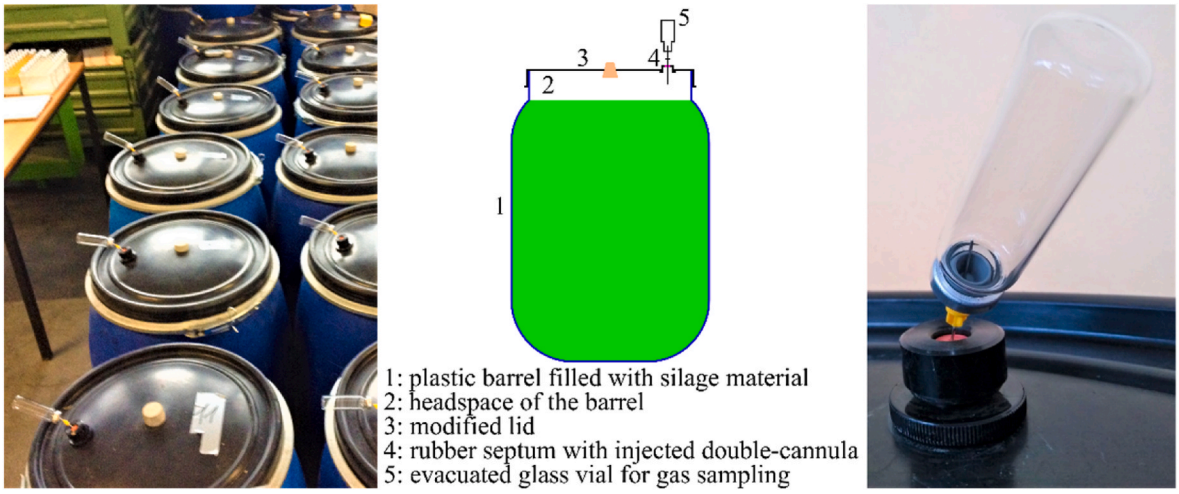


Fig. 1. Silage barrels with injected double cannulas and glass vials for gas sampling. Left: parallel gas sampling. Middle: Schematic sketch of the barrels and the gas sampling. Right: Rubber septum, injected double cannula and vial.

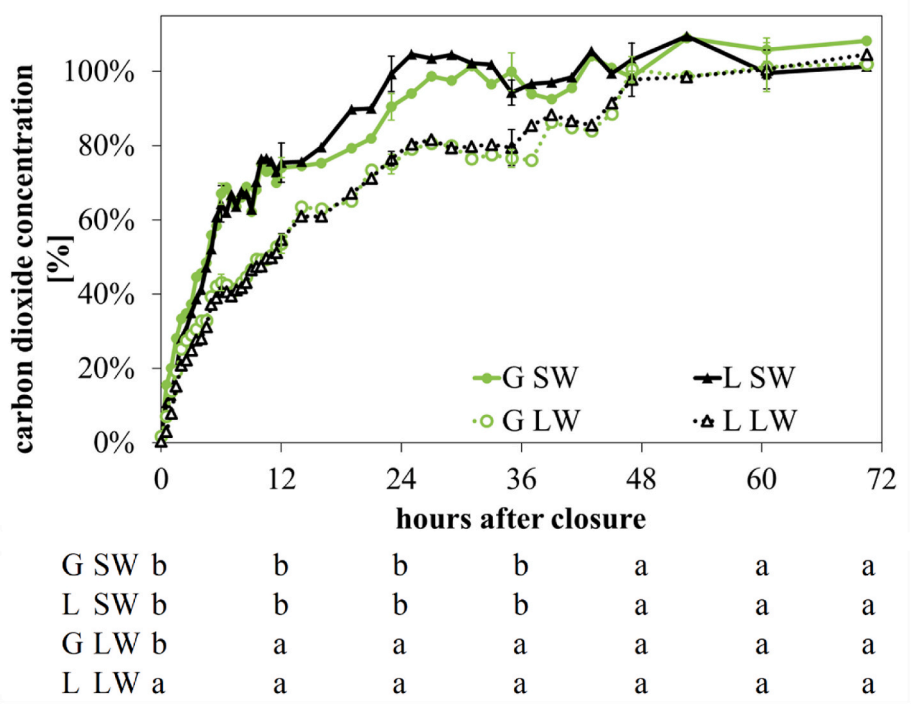


Fig. 2. Mean carbon dioxide concentration (%) within the headspace of the silage barrels (n = 3) containing each silage variant during the ensiling process. Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW). Error bars indicate standard deviations. Significant differences ($p < 0.05$) among the four variants at selected time points are indicated by different lowercase letters.

3.2. Gas formation during the ensiling process

3.2.1. CO_2

A rapid increase in CO_2 concentration was detected in all silage barrels (see Fig. 2). The G and L variants did not differ in terms of these concentrations. However, lower DM concentrations in the SW variants led to a significantly faster increase in CO_2 concentrations at the start of ensiling, i.e. between ensiling hours 4.5 to 35.0 ($p < 0.05$; exception for ensiling hour 19). CO_2 concentrations above 100% may have resulted from the analysis of diluted gas samples (see section 2.3); thus, a modified methodology (e.g. dilution with pure nitrogen or a smaller dilution ratio) should be considered in future investigations.

The course of the measured CO_2 concentrations is in agreement with data published in earlier literature, which show a rapid increase in CO_2 concentrations in silage (Li et al., 2017; Wang and Burris, 1960). Wang and Burris (1960) showed a degressive course in the measured CO_2 concentrations of maize silage (field tower silos), which is consistent with the results of the present study (see Fig. 2) and with those reported by Li et al. (2017). In contrast, Wang and Burris (1960) recorded a linear increase in a feed mix containing soybean and Sudan grass (42.6% CO_2 after 60 h). However, the measured concentrations can vary depending on the gas tightness of the silos and the remaining air inside.

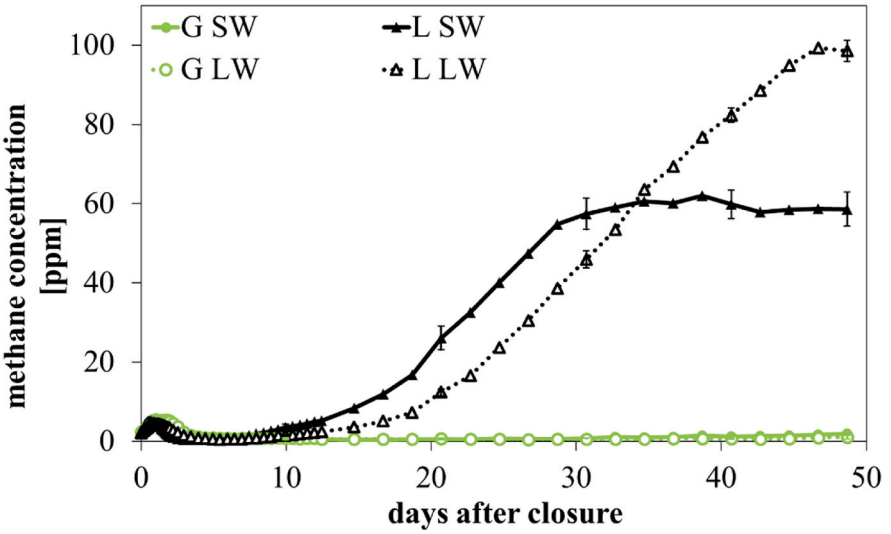
Li et al. (2017) showed that CO_2 formation starts once the silo is closed but that most of the CO_2 is formed by lactic acid bacteria after anaerobic conditions have been reached. Gomes et al. (2019) found that lower DM concentrations lead to higher gas formation, which explains the rapid increase in CO_2 concentrations in the SW variants observed in the present study between ensiling hours 4.5 to 35.0 (see Fig. 2). Higher water availability affects microbial activity, regardless of the plant material. Faster gas formation can be interpreted as a rapid start to ensiling when it is (primarily) due to lactic acid bacteria. In the present study, all variants were characterised by wet plant material that provokes malfermentation. Thus, even the low DM concentrations are not recommended and the target values should be used in practice (see section 3.1).

During CO_2 formation, the pressure inside the silage storage containers increases (Daniel et al., 2016; Daniel and Nussio, 2015b; Li et al., 2017) and the gases escape, which results in measurable (GHG) emissions (Schmidt et al., 2011, 2012). CO_2 concentrations are correlated with the microbial activity and positive pressure in the silage barrel (Daniel et al., 2016; Li et al., 2017). After completing the main fermentation phase (Pahlow et al., 2003), a pressure drop can be detected that leads to negative pressure in the silage containers (Schmidt et al., 2018). This could be attributable to the dissolution of CO_2 in the liquid phase (Li et al., 2017) or microbial activity (possibly the Wood–Ljungdahl pathway) (Schmidt et al., 2018; Vigne et al., 2019). Qualitative observations in the current study, i.e. lids slightly curved inwards, suggested negative pressure within the silage barrels containing G variants. In contrast, barrels with L variants showed overpressure during the complete anaerobic storage phase, i.e. the lid was curved outwards; moreover, when the clamping rings on the barrel were opened on ensiling day 149, gas was observed to escape immediately. This indicates that the lucerne silage never reached the stable anaerobic storage phase (Pahlow et al., 2003), which was confirmed by the butyric acid formation results (see section 3.1). Consequently, steady but varying gas formation may have occurred from the L variants over the storage period, which resulted in an outward gas flow. However, gas formation can vary widely (Daniel et al., 2015; Daniel and Nussio, 2015a; Gomes et al., 2019); therefore, it is difficult to predict the emission quantities produced in these trials.

Nevertheless, continuous gas formation is relevant for evaluating the measured CH_4 and N_2O concentrations (see sections 3.2.2 and 3.2.3). Furthermore, emission measurements should be performed for both high- and low-quality silages in future studies.

3.2.2. CH_4

The four experimental silage variants had an initial CH_4 concentration peak that varied from 4.6 ± 0.2 to 5.8 ± 0.3 ppm between ensiling hours 16.2 ± 4.8 and 39.2 ± 3.1 (Figs. 3 and 5). The G SW variant showed a significantly higher CH_4 concentration than the G LW variant



G SW	a	a	a	a	a
L SW	b	c	c	b	b
G LW	a	a	a	a	a
L LW	a	b	b	c	c

Fig. 3. Mean methane concentration (ppm) within the headspace of the silage barrels (n = 3) containing each silage variant during the ensiling process. Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW). Error bars indicate standard deviations. Significant differences ($p < 0.05$) among the four variants at selected time points are indicated by different lowercase letters.

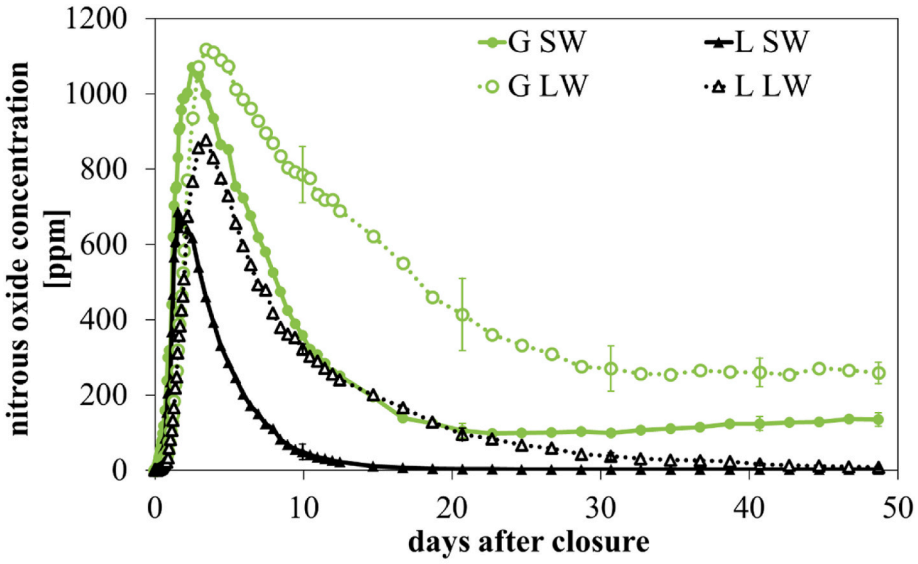
between ensiling hours 6 and 27 ($p < 0.05$) but lower concentrations between ensiling hours 40 and 106. A similar trend applied to the L SW and L LW variants for ensiling hours 7 to 21 and 31 to 94, respectively. After reaching these peaks, the CH_4 concentrations in all variants decreased to <1 ppm. The CH_4 concentrations in the G barrels were constant after ensiling day 6 (0.7 ± 0.4 ppm), whereas the CH_4 concentrations from lucerne silage substantially increased after ensiling day 12 resulting in a significant difference between the G and L variants ($p < 0.05$). Additionally, the L SW variant had significantly higher CH_4 concentrations than the L LW variant for ensiling days 9.5–10.5, 11.5–12.5 and 21.0–30.0 but lower concentrations after ensiling day 38 ($p < 0.05$).

Studies of CH_4 formation within forage, especially in silage, are scarce. Emery and Mosier (2015) studied CH_4 concentrations at the laboratory-scale using gas-tight plastic containers with aerobically stored nonforage switchgrass and corn stover. During the storage phase (59 days), these researchers were generally able to measure CH_4 concentrations at 2–15 ppm (although single peaks were up to 2,100 ppm); low moisture concentrations resulted in the highest CH_4 concentrations. The current findings are only partially consistent with those of Emery and Mosier (2015), i.e. higher CH_4 concentrations were detected in the SW variants during the first ensiling day (Figs. 3 and 5); however, the SW variants had lower CH_4 concentrations in the second phase of the initial peak. The earlier formation of CO_2 in the SW variants (see section 3.2.1) led to the release of CH_4 via outward gas mass flow. In one study, Schmidt et al. (2011) measured CH_4 concentrations at 2 ppm in sugarcane silage on ensiling days 5, 33 and 61; in another study, they detected 7 ppm CH_4 in maize silage on ensiling days 5 and 15 (Schmidt et al., 2012). Krommweh et al. (2020) measured CH_4 concentrations of 3.2–9.6 ppm within grass silage bales and of 10.2–24.4 ppm within lucerne silage bales at the time of silage opening. However, Gerlach et al. (2018) did not detect CH_4 emissions during the feed-out phase of maize and lucerne silage.

To our knowledge, a detailed course of CH_4 concentrations during the ensiling process has yet to be reported. Consequently, the curves shown in Figs. 3 and 5 represent new information on the time course of CH_4 formation. Nevertheless, further investigations with various forage types will be necessary.

An increase in CH_4 concentrations (see Fig. 3) has yet to be detected during the ensiling process. One earlier postulated explanation (Pahlow et al., 2003; Spoelstra, 1983; see section 3.1) is as follows: during lactate degradation to butyric and acetic acid, clostridia can form H_2 that is converted to CH_4 during anaerobic methanogenesis. In addition, archaea can form CH_4 (as well as CO_2 and other compounds) from H_2 and acetic acid (Aumüller-Gruber et al., 2013). Given the current lack of research related to CH_4 production, it is unclear which microorganisms within the silage contribute to methanogenesis. Likewise, it is uncertain whether the population of methanogenic microorganisms differs among different forage materials (Emery and Mosier, 2015; Yenjai et al., 2012) and among different stages of the anaerobic storage phase. However, the production of biogas has shown that obligate anaerobic methane-forming organisms are active when pH values are neutral (Aumüller-Gruber et al., 2013). Thus, the organisms likely benefit from the provision of H_2 and the rising pH values during the course of lactic acid degradation and NH_3 or NH_4^+ formation, respectively, in lucerne variants (see section 3.1). Consequently, increasing CH_4 concentrations may be useful for indicating clostridia activity. Compared with the L LW variant, the L SW variant showed an increase in CH_4 concentration at an earlier stage of the ensiling process (Fig. 3). The higher water availability seems to affect the onset of microbial activity (see section 3.2.1). However, the L LW variant subsequently shows higher CH_4 concentrations than the L SW variant. This is in line with higher pH values and lower lactic acid concentrations (Table 2), i.e. indicators of higher clostridial activity, as well as previous reports in the literature (Emery and Mosier, 2015).

Gerlach et al. (2018) stated that 'fermented forages seem to be an unlikely source of CH_4 emissions', which is contradicted by the current results, at least for lucerne silage with poor ensiling quality. At present,



G SW	b	a	b	c	b
L SW	a	a	a	a	a
G LW	c	b	b	d	c
L LW	b	a	a	b	a

Fig. 4. Mean nitrous oxide concentration (ppm) within the headspace of the silage barrels (n = 3) containing each silage variant during the ensiling process. Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW). Error bars indicate standard deviations. Significant differences ($p < 0.05$) among the four variants at selected time points are indicated by different lowercase letters.

it is not possible to determine whether CH_4 is formed at climate-relevant levels. Further investigations with different forage types and (induced) clostridial activity must therefore be conducted.

3.2.3. N_2O

The time course of N_2O concentrations in the silage barrels revealed that N_2O levels increased for all variants in the first hours of the ensiling process, peaked between ensiling hours 38.3 ± 4.2 and 94.5 ± 6.9, and then subsequently regressively decreased (Fig. 4). The grass silage produced higher N_2O concentrations than the lucerne silage from ensiling day 3 onwards. Thus, with a few exceptional individual gas samples, significant differences between the G SW and L SW variants and between the G LW and L LW variants, respectively, were consistently detected ($p < 0.05$). Additionally, higher DM concentrations in individual forage led to higher N_2O concentrations but delayed N_2O peaks. The barrels containing the LW variants had significantly lower N_2O concentrations than those containing the SW variants between ensiling hours 16 to 45, but N_2O levels were higher in the former from ensiling days 3.5–16.0 ($p < 0.05$). After the peaks had been reached, the N_2O concentrations of L variants decreased to <10 ppm until ensiling day 47. In the G variants, N_2O concentrations remained at higher levels; thus, from ensiling day 29 onwards, a significant difference in N_2O concentrations was detected between the G and L variants ($p < 0.05$).

Zhao et al. (2016) measured N_2O concentrations in maize silage (gas sampling during the first week of ensiling in a laboratory-scale silage experiment) at 1,806–1,836 ppm. Wang and Burris (1960) measured N_2O concentrations in a field silo (maize silage) at 10,000–43,500 ppm within the first 66 h of ensiling. The substantially higher values reported by Wang and Burris (1960) have already been discussed by Zhao et al. (2016) with one possible explanation: the identical mass of CO_2 and N_2O could have led to uncertain N_2O values in the course of mass spectroscopy and subsequent differentiation. The current results tend to confirm N_2O levels stated by Zhao et al. (2016). In the emission studies from a Brazilian working group, detected N_2O concentrations were 1–937 ppb in emitted gas samples from various silages (Schmidt et al., 2011, 2012). Furthermore, Franco (2016) detected emission rates of 374 mg (kg DM)$^{-1}$ up to the 120th hour of ensiling for lucerne silage, although no further emissions occurred after this point. Additionally, Gerlach et al. (2018) reported that lucerne silage with pH values of ~5.8 emitted N_2O during the feed-out phase.

Lower DM concentrations led to earlier gas formation (see sections 3.2.1 and 3.2.2). After a specific point within the first four days of ensiling (N_2O peaks; Fig. 4), no further (relevant) amounts of N_2O were produced. The formation by enterobacteria ends as soon as the nitrate and respective nitrite contents in the materials have been entirely converted or when the enterobacteria are inhibited by decreasing pH values (see section 3.1). Unfortunately, pH measurements and nitrate concentrations were not recorded for this period. Consequently, it is unclear whether additional N_2O was formed from the LW variants or whether the higher concentrations were due to other unknown effects. N_2O concentrations decreased more quickly in the SW variants than in LW, which might have been due to the higher bacterial activity and earlier CO_2 formation in the former (see sections 3.2.1 and 3.2.2). After fermentation was complete, the grass silage did not produce any additional gas; thus, the produced amounts of N_2O remained in the barrels. For the L variants, it can be assumed that the outward gas flow entirely released the produced N_2O (see section 3.2.1). Nevertheless, enterobacteria may have remained active and formed N_2O from other sources because the pH of the lucerne silage was above the critical activity limit of enterobacteria, i.e. >4.5–5.0 (Gerlach et al., 2018; Spoelstra, 1985). However, considering the increased CH_4 concentrations in the barrels (see section 3.2.2), it can be assumed that relevant amounts of N_2O were not produced (Fig. 4). Franco (2016) showed that lucerne silage emitted significantly higher amounts of CO_2 and N_2O than were emitted by maize silage. Nonetheless, further studies will be necessary to determine the emission behaviour during the ensiling process for different forage types under various conditions and ensiling management practices.

3.2.4. CH_4 and N_2O concentrations within the first four ensiling days

Fig. 5 shows that, for each silage variant, CH_4 formation occurred

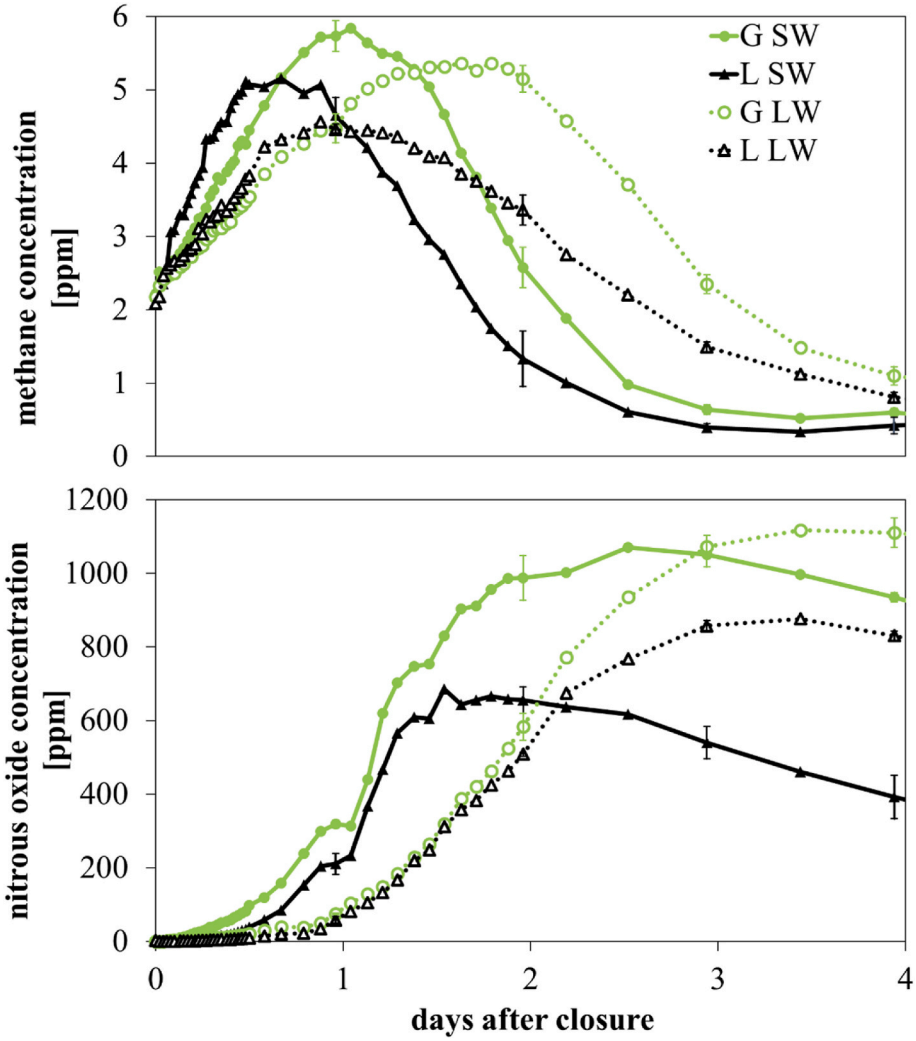


Fig. 5. Mean methane and nitrous oxide concentrations (ppm) within the headspace of silage barrels (n = 3) containing each silage variant within the first 4 days of the ensiling process. Variants: grass shortly wilted (G SW), lucerne shortly wilted (L SW), grass longer wilted (G LW) and lucerne longer wilted (L LW). Error bars indicate standard deviations at selected time points.

before N_2O formation. The time interval between the concentration peaks of the two gases was longer in the LW variants: 34.7 ± 13.3 h for G SW, 22.2 ± 5.6 h for L SW, 55.3 ± 5.0 h for G LW and 66.0 ± 2.0 h for L LW, respectively.

At this stage, a final conclusion cannot be made regarding which microorganisms and metabolic processes are involved in gas formation at the beginning of ensiling. One potential explanation follows, although other (biochemical) formation processes are also possible (e.g. degradation of cell components). It is possible that facultative anaerobic enterobacteria enzymatically convert formate (HCO_2^-) into CO_2 and H_2 during the first hours (Pahlow et al., 2003). The increase in CH_4 concentration ended when CO_2 concentrations exceeded 88.9% for G SW, 81.4% for L SW, 84.1% for G LW and 74.4% for L LW. The formed H_2 can be used for methanogenesis because of the anaerobic conditions and formation of small anaerobic pockets within aerobic plant material (Emery and Mosier, 2015; Yenjai et al., 2012). Additionally, the residual respiration of the forage and the increasing activity of the lactic acid bacteria lead to complete anaerobic conditions and a decrease in pH levels. The former condition leads to increased formation of N_2O by enterobacteria (see sections 3.1 and 3.2.3). This process releases oxygen that this is rapidly respired. The latter condition first inhibits methanogenic microorganisms, which are typically active at higher pH levels, e.g. >6.8 pH for archaea (Aumüller-Gruber et al., 2013), and then inhibits the enterobacteria, which are active at >4.5 pH (see section 3.1). Consequently, the first CH_4 peak and the N_2O peak can be attributed (indirectly) to the metabolism of the enterobacteria.

The higher measured gas concentrations suggest that the activity of enterobacteria is higher in the G variants. Unfortunately, the concentrations of formate and nitrate in the fresh material and microbial population were not analysed. Consequently, it is not possible to conclude whether additional substrate or a bigger microbial population led to increased gas formation in the grass variants. The similar CO_2 formation results for the two forages (see section 3.2.1) suggest that more CH_4 and N_2O were formed in the grass variants. Earlier release of the gases via CO_2 mass flow seems unlikely. However, it is unclear why the time interval between the gas peaks was longer in the LW variants. A more detailed investigation of gas concentrations and emission quantities could potentially clarify when the gases are released; thus, further studies evaluating GHG formation during this period should be conducted.

3.3. Examination of the methodological procedure

In this study, 82 gas samples per silage barrel were taken and analysed using a gas chromatograph; overall, 984 gas samples were analysed. To our knowledge (see section 1), this is the first study to investigate such an extended ensiling period (49 days) using such short sampling intervals (as frequent as 0.5 h). Furthermore, this study used

the largest common laboratory-scale containers available (120-L volume), whereas previous studies mainly used much smaller silos (maximum volume: 20 L) (Bueno et al., 2020; Franco, 2016; Schmidt et al., 2011, 2012).

The gas samples were taken in the headspace of the standing laboratory-scale barrels above the silage material. Although the methods used do not provide an answer to the open question (Zhao et al., 2016) of how relevant gas measurements taken at the laboratory-scale are on a practical scale, the plastic barrels used here are established laboratory silos (Jungbluth et al., 2017; Sun et al., 2015) and the gas sampling technique is comparable to that used in earlier studies (Bueno et al., 2020; Restelatto et al., 2019; Schmidt et al., 2011, 2012). In practice bunker silos, gas formation during the ensiling process is observed through inflated silo film or gas escape at ground level (for instance, reddish-brown gas clouds for nitrous gases containing nitrogen dioxide). CO_2 and N_2O have a higher molecular weight than air and can accumulate within the silage barrels. The gas measurements show a N_2O peak in the first ensiling days (no discolouration was visible), but gas concentrations may have been higher in the deeper layers of the barrel. However, gas formation (mainly CO_2) from the material led to an outward gas flow (see section 3.2.1), which escapes between the barrel and the lid. The gas sampling point (see section 2.3) was located at the edge of the lid; thus, the escaping gases, which contained quantities of N_2O, flowed past the measuring point. Given the lower molecular weight of CH_4, the concentration of this gas (see section 3.2.2) could be measured continuously. Consequently, it is assumed that the measurement results are transferable to practice silos with a comparable ensiling process.

The measurement of GHGs using a gas chromatograph is an established method that is practically suitable for use in animal houses, even when gas concentrations are low (Schmithausen et al., 2016, 2018b). However, this methodology is not applicable for measurements in practice. Therefore, additional studies are required to examine the potential use of rapid testing systems with lower measurement accuracy to determine the appropriateness of the ensiling process in practice.

In the present study, the measurements taken using the tested materials under the specific conditions showed that gas concentrations within silage vary considerably over time. On the one hand, the experiment indicated that CH_4 formation preceded N_2O formation by several hours during the first days of the ensiling process (see section 3.2.3). On the other hand, the measured values show CH_4 formation after ensiling day 12 in silages with increased butyric acid concentrations (see section 3.2.1). To our knowledge, this was the first practical measurement to show this phenomenon. Therefore, the methodology described here could provide further insights into the metabolic activity of microbiota during the ensiling process.

Unfortunately, the quantification of GHG emissions was not possible in this study because the objective was instead to record detailed concentration courses over time. Schmidt et al. (2012, 2011) concluded that GHG emissions from the ensiling process play a subordinate role compared with the emissions from cattle and dairy farming processes. This statement could be re-examined considering the varying gas concentrations observed during the ensiling process as well as calculated emission quantities. Any projections of GHG emission quantities based on one-time measurements of gas concentrations (Bueno et al., 2020; Schmidt et al., 2011, 2012) could underestimate or overestimate the emissions from silage (especially in cases with poor ensiling quality). Further studies should also include assessment of NH_3 concentrations or emissions as well as more extensive material analyses.

Finally, it can be concluded that, in addition to chemical analyses of silage material, repeated gas analyses, as described in the present study, could contribute to improving our understanding of the ensiling process and ensiling quality. Therefore, new studies addressing open research questions and comparing various types of forage are recommended.

3.4. Implications for ensiling management research

In practice, silages are too often of poor quality; hence, it is necessary to investigate these situations appropriately. In some regions of the world, weather fluctuations and relatively short harvest periods (due to precipitation) can impair harvest conditions, harvest security and fermentation capacity (Persson and Höglind, 2014). In addition, the demand for high-quality silage will increase globally over time (Wilkinson, 1999; Wilkinson and Muck, 2019). However, if high-efficiency harvesting machinery is not available, it is difficult to use shorter harvesting periods effectively. For this reason, fresh material with low DM concentration was used here to provoke malfermentation and simulate adverse harvest conditions. However, differences in the gas courses of the silage variants show that DM concentrations noticeably affect microbiological gas formation. Additional research should therefore involve experiments with varying DM concentrations.

According to current knowledge, malfermentation leads to five negative outcomes: (1) decreased feed intake (Spiekers, 2012), (2) reduced feed quality (especially energy and protein losses) (Wilkinson, 1999), (3) increased feed quantity demand to fulfil the nutritional needs of the animals, (4) higher levels of feed disposal, and (5) increased direct GHG emissions during the anaerobic storage period. The first four factors lead to a rise in indirect emission quantities and climate impacts from animal feeding and biogas production, respectively, because silage quantities must be increased to produce the final outputs. This situation may also be applicable to the ensiling of other forages (e.g. maize or whole-plant grain silage), although further experimental studies are required to confirm this. Future studies must also examine whether significant GHG emissions occur in this case.

Especially in modern times, various methods should be used to investigate and analyse the environmental impact of ensiling for livestock feed or biogas production and the optimisation of these methods should be attempted. The use of ensiling additives to control the ensiling process may be one viable option. The addition of chemical compounds or microorganisms (mostly homofermentative or heterofermentative lactic acid bacteria) could positively influence the ensiling process, which could improve ensiling quality even under unfavourable conditions. However, the production and application of these substances is associated with increased effort for which the climate impact cannot currently be quantified. A future comparison could involve the reduction in GHG emissions as result of minimising fermentation losses vs. the additional effort required to include silage additives.

This study investigated the GHG emissions of different feeds with varying DM concentrations. DM concentrations especially were found to affect the timing of microbial formation processes (see section 3.2). Thus, studies must be conducted to determine the effects of various DM concentrations and investigate larger DM differences between silage variants. In addition, DM concentrations can influence other silage parameters, such as the possible packing density of the silo, and can therefore influence aerobic stability. As stated above, several additional studies are required to improve our understanding of the multidisciplinary natural and man-made silage process chains.

4. Conclusion

Based on the execution and results of the experiment, the sampling methodology was suitable to measure the varying gas concentration courses within the silos and conclude the gas formation. This method can be used for future fundamental research concerning different silage variants for laboratory-scale measurements. However, further research should quantify gas emissions and analyse microbial populations for more detailed insights. The short measurement intervals (down to 0.5 h) demonstrated that gas formation occurs within short periods, especially during the first four ensiling days. Lower dry matter concentrations favoured an earlier onset of CO_2, CH_4 and N_2O formation. Besides, the produced gases affect each other; the ongoing formation of CO_2 forces

the other gases out of the silos. Thus, dry matter concentration plays a significant role for the measured concentration courses and should be considered in future studies. For lucerne variants, lactate degradation and butyrate formation by clostridia (malfermentation) led to the production of CH_4 at rising pH values from ensiling day 12. This phenomenon is reported here for the first time. Thus, malfermentation impairs silage quality (reduced feed value or increased disposal quantities) and actively contributes to GHG formation. An optimal ensiling process, obtained using the best possible management practices, would therefore be desirable from the perspective of animal nutrition and environmental protection. However, further research is needed to determine GHG emission quantities and the effects of various elements such as versatile forage types, DM concentration, silage additives and environmental factors.

Author contributions

Alexander J. Schmithausen and **Hauke F. Deeken** are equal first authors of this work. **Alexander J. Schmithausen**: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualisation, Supervision, Project administration, and Funding Acquisition. **Hauke F. Deeken**: Validation, Formal analysis, Data curation, Writing – original draft Preparation, Writing – review & editing, and Visualisation. **Katrin Gerlach**: Conceptualisation, Validation, Investigation, Resources, and Writing – Review & Editing. **Manfred Trimborn**: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, and Writing – Review & Editing, Supervision. **Kirsten Weiß**: Validation, Investigation, Resources, and Writing – Review & Editing. **Wolfgang Büscher**: Resources, Writing – review & editing, Project administration, and Funding Acquisition. **Gerd-Christian Maack**: Validation, Writing – review & editing, and Visualisation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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