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Dynamics of gas and greenhouse gases production during fermentation of barley silage with lactic acid bacteria

Yanlin Xue[1*], Nier Wu[1], Na Na[1], Juanjuan Sun[2], Lin Sun[1], Moge Qili[1], Dongyang Li[3], E. Li[3] and Baozhu Yang[4]

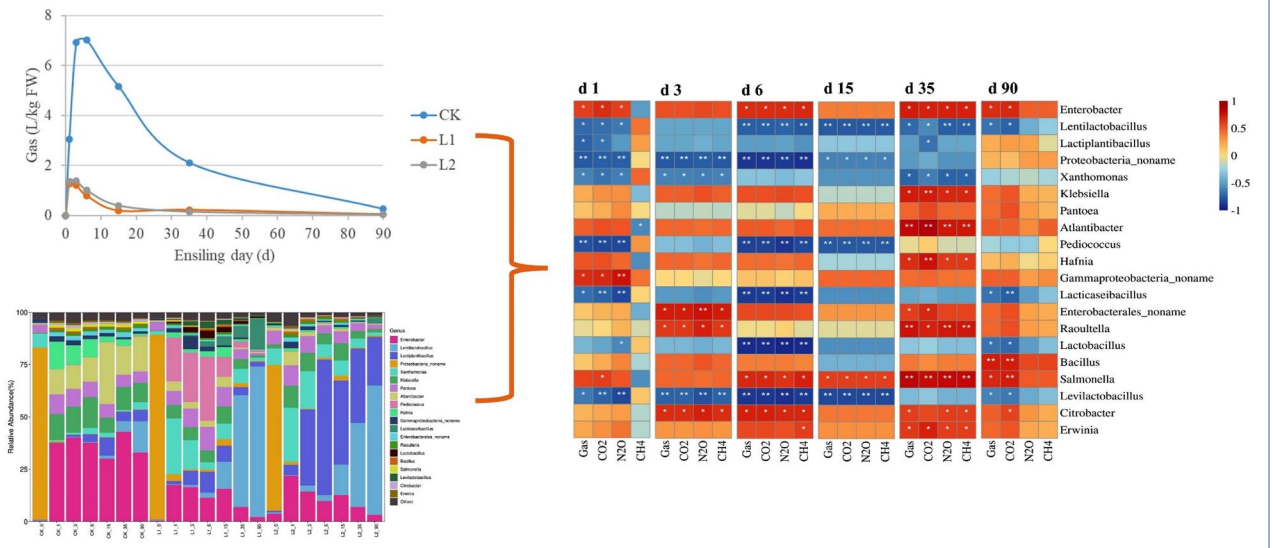
Abstract

Greenhouse gases (GHGs) are generated during fermentation in silages, especially in barley silage. However, little is known regarding the dynamics of GHG production in silages during fermentation. In the present study, GHG accumulation and reduction were assessed in barley silage. Barley was harvested at the milk stage and ensiled without (CK) or with two commercial lactic acid bacterial (LAB) additives (L1 or L2). Gas and GHG (CO_2, N_2O, and CH_4) production, fermentation quality, fermentation weight loss (FWL), and bacterial communities were analyzed at d 0, 1, 3, 6, 15, 35, and 90 after ensiling. Gas and GHG production rapidly increased in CK during the first 3 days and in L1 and L2 during the first day and then decreased ($P < 0.05$), and these values were higher in CK than in L1 and L2 from d 1 to d 35 ($P < 0.05$), with the peak production of gas and GHG observed at d 6 in CK and at d 3 in L1 and L2. Gas and GHG production were positively correlated with the count of Coliforms and the abundances of *Enterobacter*, *Klebsiella*, and *Atlantibacter* from d 0 to 6 ($P < 0.05$) but were negatively correlated with the abundances of *Lentilactobacillus*, *Lactiplantibacillus*, and *Lacticaseibacillus* from d 1 to 35 ($P < 0.05$). L1 and L2 had increasing pH and acetic acid (AA) and decreasing lactic acid after d 15 ($P < 0.05$). *Lentilactobacillus* in L1 and L2 dominated the bacterial communities from d 35 to 90 and correlated positively with pH and AA, and negatively with LA from d 6 to 90 ($P < 0.05$). FWL had a positive correlation with gas and GHG from d 1 to 35 ($P < 0.05$). The ensiling fermentation process can be divided into gas accumulation and reduction phases. Inoculation with LAB reduced gas and GHG production. The activities of enterobacteria were the main contributors to gas and GHG accumulation. *Lentilactobacillus* activity mainly caused deterioration of fermentation quality during the late fermentation phase. The GHGs generated in silage contributed to the FWL during fermentation.

Keywords Bacterial communities, Barley silage, Fermentation weight loss, Gas accumulation phase, Gas reduction phase, Greenhouse gases

*Correspondence:
Yanlin Xue
xueyanlin0925@outlook.com
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

Greenhouse gas (GHG) emissions cause global climate change, increasing temperatures, and more extreme weather events [1, 2]. To limit GHG emissions, the Paris Agreement was signed by many countries in 2016 [3]; furthermore, in 2020, China established carbon peaking (2023) and carbon neutrality (2060) goals [4]. Global GHG production is mainly accounted for by the electricity and heat production sector, the agriculture, forestry, and other land use sector, and the industry sector, with 25%, 24%, and 21% contribution, respectively [5]. Agricultural activities (crop and livestock production) contribute approximately 10–12% of GHG emissions globally [6]. For the past few years, reducing GHG emissions from crop and livestock production has become the focus of research, with remarkable results [7–10]. However, the GHGs generated in and emitted from silages, important roughages for ruminant husbandry, are not included in the official inventory [11], and there is a lack of systematic and in-depth studies in this field.

Ensiling, as a traditional method, preserves fresh forage to provide palatable roughage to ruminants all year [12, 13]. Making silage improves land use efficiency, nutrient yields of natural resources, the steady development of animal husbandry, and farmer profitability [11]. However, many deaths have been reported among men working in silos from the 1930s to 1940s, resulted from the inhalation of gases generated in silages during fermentation [14, 15]. Subsequently, attention has been given to the toxic gases (CO_2 and N_2O) generated in silages, and many related studies have been carried out. Meiering et al. [16] simulated CO_2 production in ryegrass silage, which increased during the first 7 days and then remained stable from d 7 to 21. Similarly, Williams et al. [17] reported that CO_2 production increased rapidly during early fermentation (approximately 7 days) and then decreased in ryegrass silage bankers. In baled silage, the early fermentation stage is characterized by the rapid creation of a CO_2-rich environment, and the film properties (color and type) have limited effects on the gas composition [18]. Moreover, N_2O in silage increases readily during early fermentation because of the oxidation of NO [19, 20]. However, gas production during fermentation contributes to the loss of dry matter (DM) in silages [17, 21].

With global climate change being a concern, and after the Paris Agreement was signed, the gases generated in silages have been studied from the perspective of GHG. By measuring GHG concentrations during fermentation, Schmithausen et al. [22] showed that CO_2 is the most important component of gas generated in silages. Inoculation with homofermentative lactic acid bacteria (LAB) or wilting materials can reduce gas and GHG production in silages [13, 22–24]. Nevertheless, ensiling with heterofermentative LAB results in the generation of more gas and N_2O in silages [23]. Li et al. [25] reported that CO_2 is mainly generated at the anaerobic fermentation stage and that a small amount of CO_2 dissolves in silage water during fermentation. Moreover, some studies have reported that inoculating LAB reduces gas and

CO_2 production by optimizing bacterial communities in silages [13, 24]. The activities of Enterobacteriaceae and *Lactococcus* may cause gas generation in oat silage [24]. Enterobacteriaceae may be involved in N_2O formation in sorghum—Sudan grass silage [26]. In addition, *Lactococcus* and *Citrobacter* are positively correlated with gas and CO_2 production in silage [13].

We previously found that gas production in barley silage is higher than that in other silages (Figure S1), and we hypothesized that inoculation of LAB at ensiling barley may optimize bacterial communities to reduce gas and GHG production during fermentation. This study aimed to determine the gas and GHG production, fermentation quality, fermentation weight loss (FWL), and bacterial communities during fermentation of barley silage with commercial LAB inoculants as additives.

Materials and methods

Additives

The first commercial LAB additive (BONSILAGE) was collected from Schaumann Agricultural Trading Co., Ltd., Shanghai, China. Its main components were *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Pediococcus acidilactici* ($\geq 1.0\times10^{11}$ colony-forming units (CFU)/g). The second commercial LAB additive (Zhuanglemei) was collected from Sichuan Gaofuji Biotechnology Co., Ltd., Chengdu, China. Its main components were *Lact. plantarum* 550 and 360 ($\geq 1.3\times10^{10}$ CFU/g) and *Lent. buchneri* 225 ($\geq 7.0\times10^{9}$ CFU/g).

Preparing silage

Barley (*Hordeum vulgare*; Liangcheng barley) was harvested at the milk stage (DM content, 363 g/kg) from four fields as replicates on July 11, 2021, at an experimental farm in Huhhot, China (40° 750 N, 111° 670 E). After chopping to 1–2 cm and mixing thoroughly, the fresh forages from each field were divided into three batches for treatment. (1) CK, sprayed with 2 ml/kg fresh weight (FW) of distilled water (without any additives); (2) L1, sprayed with 2 ml/kg FW of distilled water with 2 g/t FW (recommended amount (RA)) of the first commercial LAB additive; (3) L2, sprayed with 2 ml/kg FW of distilled water with 5 g/t FW (RA) of the second commercial LAB additive. After being uniformly mixed, the forages (500 g) were packed into a polyethylene bag and sealed with a vacuum sealer. Twenty-eight bags of silage were prepared for each treatment (7 bags per field). The silages were sampled at d 0, 1, 3, 6, 15, 35, and 90 for analyses of gas and GHG production, fermentation quality, FWL, and bacterial communities.

Greenhouse gas production

The gas volume of the silage bag on each sampling day was calculated by the difference in the silage bag volume before and after ensiling [13, 24]. The gas production (L/kg FW) on each sampling day was calculated according to Eq. 1. The concentrations of CO_2, N_2O, and CH_4 were analyzed with gas chromatography (Shimadzu GC-20A, Shimadzu Co., Ltd., Kyoto, Japan) [13, 27]. The productions (L/kg FW) of CO_2, N_2O, and CH_4 on each sampling day were calculated according to Eq. 2.

$$\text{Gas production at d}x\left(\frac{\text{L}}{\text{kg}}\text{FW}\right) = \frac{\text{Gas volume in silage bag (L) at d}x}{\text{Silage weight in bag (kg) at d 0}}. \quad (1)$$

$$\text{Production of GHG at d}x\left(\frac{\text{L}}{\text{kg}}\text{FW}\right) = \text{gas production at d}x\left(\frac{\text{L}}{\text{kg}}\text{FW}\right) \times \text{concentration of GHG at d}x(\%) \quad (2)$$

dx = d 0, 1, 3, 6, 15, 35, and 90 of ensiling.
GHG = CO_2, N_2O, or CH_4.

Fermentation quality, buffering capacity, and fermentation weight loss

The silage extract was prepared according to the methods of Xu et al. [28, 29] and used for analyzing the pH, lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), and ammonia nitrogen (AN) contents of the silage. The pH of the silage extracts was measured using a pH meter (PB-10, Sartorius, Gottingen, Germany), with high-performance liquid chromatography (DAD, 210 nm, SPD-20A, Shimadzu Co., Ltd., Kyoto, Japan) for assessing the organic acid concentrations of the silage [28, 29], and with the Kjeldahl method for detecting the AN of the silage [30].

The silages were dried at 65 ℃ for 48 h to determine the DM of the silage and used for assessing the total nitrogen of the silage using the Kjeldahl method with copper as the catalyst and detecting the buffering capacity (BC) according to Playne and McDonald [31]. The FWL was assessed based on the difference in silage bag weight before and after ensiling [29].

Microbial counts and bacterial communities

Man, Rogosa and Sharpe agar, violet red bile agar, nutrient agar, and potato dextrose agar were used as media for determining LAB, coliforms, total aerobic bacteria (TAB), and yeast counts of silage according to Cai [32].

After the bacterial DNA of the silage was extracted, 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) were used as primers to amplify the V3–V4 region of the bacterial rRNA gene using the polymerase chain reaction (PCR) [33]. The purification, quantification, and sequence of PCR products, the calculation of alpha and beta diversities, and sequence alignment were carried out by LC-Bio (Hangzhou Lianchuan Biotechnology Co., Ltd., Hangzhou, China). The sequencing data were submitted to the NCBI Sequence Read Archive database (accession number: PRJNA1026762).

Statistical analyses

The effects of ensiling time and inoculants were analyzed using the GLM procedure of SAS (SAS System for Windows, version 9.1.3; SAS Institute Inc., Cary, NC, USA). The correlation heatmaps of gas and GHG production with fermentation quality, microbial counts, and bacterial communities were generated using R 3.6.1 (https://www.omicstudio.cn/space).

Results

Greenhouse gas production

The gas, CO_2, and N_2O production increased in CK during the first 6 days and in L1 and L2 during the first day and then decreased for all silages ($P<0.05$) (Table 1). CH_4 production increased in CK during the first 15 days and in L1 and L2 during the first 3 days and then decreased for all silages ($P<0.05$). Compared with L1 and L2, from d 3 to d 35, CK had higher gas, CO_2 and N_2O production from d 1 to d 35 and higher CH_4 production ($P<0.05$); moreover, L2 had more N_2O than L1 at d 3 and d 6 ($P<0.05$).

Fermentation quality

During fermentation, the CK had a decreasing pH and an increasing BC ($P<0.05$), and all silages had increasing AA, AN, and FWL ($P<0.05$) (Table 2). For L1 and L2, the pH decreased during the first 15 days and then increased ($P<0.05$), but the BC increased during the first 15 days and then decreased ($P<0.05$). The LA concentration increased in CK during the first 35 days and in L1 and L2 during the first 15 days and then decreased in all silages ($P<0.05$). Compared with L1 and L2, CK had a

Table 1 Gas and greenhouse gas production of barley silage during fermentation ($n=4$)

Items		Ensiling time (d)							*P* value	SEM
		0	1	3	6	15	35	90		
Gas (L/kg fresh weight (FW))	CK	0	3.08Ac	7.00Aa	7.06Aa	5.16Ab	2.22Ad	0.177e	< 0.001	0.207
	L1	0	1.26Ba	1.20Ba	0.785Bb	0.192Bc	0.224Bc	0.096cd	< 0.001	0.047
	L2	0	1.37Ba	1.38Ba	1.07Bb	0.461Bc	0.165Bd	0.099d	< 0.001	0.056
	P value	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.061		
	SEM		0.105	0.172	0.189	0.172	0.078	0.023		
CO_2 (L/kg FW)	CK	0	1.83Ad	4.36Ab	4.94Aa	2.91Ac	1.31Ae	0.095f	< 0.001	0.130
	L1	0	0.705Ba	0.673Ba	0.441Bb	0.108Bc	0.127Bc	0.050cd	< 0.001	0.026
	L2	0	0.790Ba	0.773Ba	0.631Bb	0.272Bc	0.082Bd	0.053d	< 0.001	0.033
	P value	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.0534		
	SEM	–	0.062	0.106	0.130	0.097	0.046	0.012		
N_2O ($\times 10^{-3}$ L/kg FW)	CK	0	5.41Ab	7.43Aa	7.48Aa	3.35Ac	1.15Ad	0.081e	< 0.001	0.212
	L1	0	3.55Ba	2.73Cb	1.70Cc	0.199Bd	0.185Bd	0.055d	< 0.001	0.114
	L2	0	4.91Aa	3.43Bb	2.63Bc	0.551Bd	0.147Be	0.061e	< 0.001	0.124
	P value	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.314		
	SEM	–	0.222	0.208	0.248	0.121	0.042	0.012		
CH_4 ($\times 10^{-5}$ L/kg FW)	CK	0	0.888e	2.38Ab	3.12Ac	5.85Aa	4.53Ab	0.502fe	< 0.001	0.175
	L1	0	0.984ab	1.06Ba	0.836Bb	0.334Bd	0.654Bc	0.377d	< 0.001	0.053
	L2	0	0.991ab	1.21Ba	1.09Bab	0.847Bb	0.501Bc	0.369c	< 0.001	0.080
	P value	–	0.2875	< 0.001	< 0.001	< 0.001	< 0.001	0.423		
	SEM	–	0.0480	0.070	0.104	0.207	0.162	0.077		

SEM: standard error of the mean. Values with different lowercase letters (a, b, ……, f) indicate significant differences among the ensiling times of each treatment. Values with different uppercase letters (A, B, and C) indicate significant differences among treatments at the same ensiling time. CK: control; L1: ensiled barley with *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Pediococcus acidilactici*; L2: ensiled barley with *Lact. plantarum* and *Lent. buchneri*

Table 2 PH, lactic acid (LA), acetic acid (AA), ammonia nitrogen (AN), buffering capacity (BC), and fermentation weight loss (FWL) of barley silage during fermentation ($n=4$)

Items		Ensiling time (d)							*P* value	SEM
		0	1	3	6	15	35	90		
pH	CK	6.56a	6.02Ab	5.44Ac	4.86Ad	4.57Ae	4.41Ae	4.39Ae	< 0.001	0.070
	L1	6.53a	4.15Bc	3.92Bd	3.88Bd	3.84Cd	3.92Cd	4.22Bb	< 0.001	0.022
	L2	6.48a	4.19Bc	3.90Bd	3.92Bd	3.90Bd	4.22Bc	4.50Ab	< 0.001	0.017
	P value	0.165	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001		
	SEM	0.030	0.019	0.078	0.063	0.020	0.121	0.035		
LA (g/kg dry matter (DM))	CK	0	8.72Bfe	17.9Cde	26.3Bcd	38.4Bab	43.9a	32.1Abc	< 0.001	3.26
	L1	0	36.0Ab	61.7Aa	60.8Aa	63.9Aa	42.9b	24.1ABc	< 0.001	3.35
	L2	0	36.8Ab	48.7Ba	51.4Aa	51.5Aa	46.5a	15.4Bc	< 0.001	2.75
	P value	–	< 0.001	< 0.001	< 0.001	< 0.001	0.805	0.022		
	SEM	–	2.53	1.77	3.82	4.15	3.97	3.42		
AA (g/kg DM)	CK	0	7.96cd	8.94cd	9.11cd	14.5bc	21.0Ab	30.6a	< 0.001	3.01
	L1	0	5.99b	6.37b	8.33b	9.50b	9.00Bb	25.5a	< 0.001	1.65
	L2	0	7.83c	6.20c	10.6c	9.39c	24.5Ab	30.9a	< 0.001	1.73
	P value	–	0.409	0.314	0.293	0.1375	< 0.001	0.690		
	SEM	–	1.11	1.34	0.991	1.84	1.74	4.91		
AN (g/kg total nitrogen)	CK	10.6c	31.5Ab	38.1Aa	37.7Aa	38.1Aa	35.8Aa	36.7a	< 0.001	1.42
	L1	9.00d	19.2Bc	27.2Bb	27.3Bb	26.2Bb	28.7Bb	33.6a	< 0.001	1.60
	L2	10.0f	14.6Ce	23.5Bd	28.3Bc	27.8Bc	32.9Ab	37.0a	< 0.001	1.19
	P value	0.481	< 0.001	0.003	< 0.001	< 0.001	0.013	0.230		
	SEM	0.891	1.43	2.19	1.18	1.05	1.31	1.43		
BC (mEq/kg DM)	CK	186e	215Cd	288Bc	334Bb	393Aa	394Aa	405Aa	< 0.001	3.66
	L1	189f	288Ae	332Ac	364Ab	385Aa	365Bb	305Bd	< 0.001	3.21
	L2	195e	275Bd	330Ab	361Aa	368Ba	313Cc	281Cd	< 0.001	
	P value	0.210	< 0.001	< 0.001	< 0.001	0.003	< 0.001	< 0.001		
	SEM	3.02	2.60	3.31	2.88	3.81	3.15	3.98		
FWL (% fresh weight)	CK	0	0.560Ae	1.75Ad	2.36Ad	4.51Ac	9.43Ab	13.5Aa	< 0.001	0.260
	L1	0	0.319Bfe	0.702Be	1.33Bd	2.69Bc	6.63Bb	11.0Ba	< 0.001	0.133
	L2	0	0.371Bfe	0.805Be	1.40Bd	2.87Bc	6.82Bb	10.9Ba	< 0.001	0.183
	P value	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.0011		
	SEM	–	0.018	0.132	0.094	0.150	0.3050	0.366		

SEM: standard error of the mean. Values with different lowercase letters (a, b, ……, e) indicate significant differences among the ensiling times of each treatment. Values with different uppercase letters (A, B, and C) indicate significant differences among treatments at the same ensiling time. CK: control; L1: ensiled barley with *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Pediococcus acidilactici*; L2: ensiled barley with *Lact. plantarum* and *Lent. buchneri*

higher pH between d 1 and d 5, higher AN between d 1 and d 15, higher FWL between d 1 and d 90, higher BC between d 35 and d 90, and lower LA between d 1 and d 15 and higher BC between d 1 and d 6 ($P<0.05$). L2 had lower BC than L1 at d 1 and CK and L1 from d 15 to d 90 ($P<0.05$). Compared with CK and L2, L1 had a lower pH from d 15 to d 90 and less AA and AN at d 35 ($P<0.05$). At d 90, there was less LA in L2 than in CK ($P<0.05$).

Microbial counts

For all silages, the counts of LAB and yeasts increased during the first 3 days, the TAB increased on the first day, and then they decreased (except for yeasts in CK) ($P<0.05$) (Table 3). The count of Coliforms in CK increased during the first 3 days and then decreased ($P<0.05$) and was not detected at d 35 and d 90. However, the count of Coliforms in L1 and L2 decreased on the first day ($P<0.05$) and was not detected from d 6 to d 90. In comparison to L1 and L2, CK had fewer LAB on the first day ($P<0.05$), and L2 had higher LAB than CK between d 3 and d 6 ($P<0.05$). Moreover, L1 had fewer LAB than L2 on d 1, and CK and L2 had fewer LAB from d 15 to d 90 ($P<0.05$). The CK had higher TAB than L1 and L2 from d 15 to d 35 ($P<0.05$), and the L1 had lower TAB than CK and L2 from d 35 to d 90 ($P<0.05$). The CK contained fewer yeast than L1 and L2

Table 3 Counts of lactic acid bacteria (LAB), coliforms, total aerobic bacteria (TAB), and yeasts of barley silage during fermentation ($n=4$)

Items		Ensiling time (d)							*P* value	SEM
		0	1	3	6	15	35	90		
LAB (lg colony-forming units (cfu)/g fresh weight (FW))	CK	4.03Be	8.76Cc	9.22Ba	9.07Bab	9.00Ab	8.79Ac	7.62Ad	< 0.001	0.054
	L1	5.91Af	9.23Bab	9.37ABa	8.94Cb	8.45Bc	7.83Bd	6.78Be	< 0.001	0.101
	L2	6.00Ae	9.48Aa	9.43Aa	9.21Aa	8.91Ac	9.02Ac	7.69Ad	< 0.001	0.042
	P value	< 0.001	< 0.001	0.041	0.002	0.006	< 0.001	< 0.001		
	SEM	0.054	0.070	0.051	0.037	0.096	0.074	0.094		
Coliforms (lg cfu/g FW)	CK	7.45c	9.08Ab	10.2Aa	7.62Ac	4.12Ad	0	0	< 0.001	0.090
	L1	7.50a	5.82Bb	0	0	0	0	0	< 0.001	0.061
	L2	7.23a	6.09Bb	0	0	0	0	0	< 0.001	0.057
	P value	0.151	< 0.001	< 0.001	< 0.001	< 0.001	–	–		
	SEM	0.093	0.096	0.043	0.117	0.043	–	–		
TAB (lg cfu/g FW)	CK	7.77e	9.42a	9.08b	9.11b	8.53Ac	8.12Ad	7.82Ae	< 0.001	0.087
	L1	7.68b	9.26a	9.22a	8.94a	7.90Bb	6.53Cd	7.20Bc	< 0.001	0.095
	L2	7.80c	9.53a	9.33a	9.03b	7.79Bc	7.61Bc	7.73Ac	< 0.001	0.089
	P value	0.167	0.154	0.333	0.166	< 0.001	< 0.001	< 0.001		
	SEM	0.044	0.094	0.112	0.057	0.069	0.143	0.081		
Yeasts (lg cfu/g FW)	CK	6.40b	8.71Ba	9.08Ba	9.05a	8.68Ba	8.84ABa	8.83Aa	< 0.001	0.114
	L1	6.61d	9.15Aa	9.39Aa	9.11a	8.35Bb	8.56Bb	7.30Bc	< 0.001	0.124
	L2	6.40e	9.43Aa	9.48Aa	9.19b	9.11Ab	8.94Ac	8.73Ad	< 0.001	0.056
	P value	0.297	0.006	0.001	0.360	0.004	0.033	< 0.001		
	SEM	0.103	0.118	0.053	0.066	0.115	0.088	0.145		

SEM: standard error of the mean. Values with different lowercase letters (a, b, ……, e) indicate significant differences among the ensiling times of each treatment. Values with different uppercase letters (A, B, and C) indicate significant differences among treatments at the same ensiling time. CK: control; L1: ensiled barley with *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Pediococcus acidilactici*; L2: ensiled barley with *Lact. plantarum* and *Lent. buchneri*

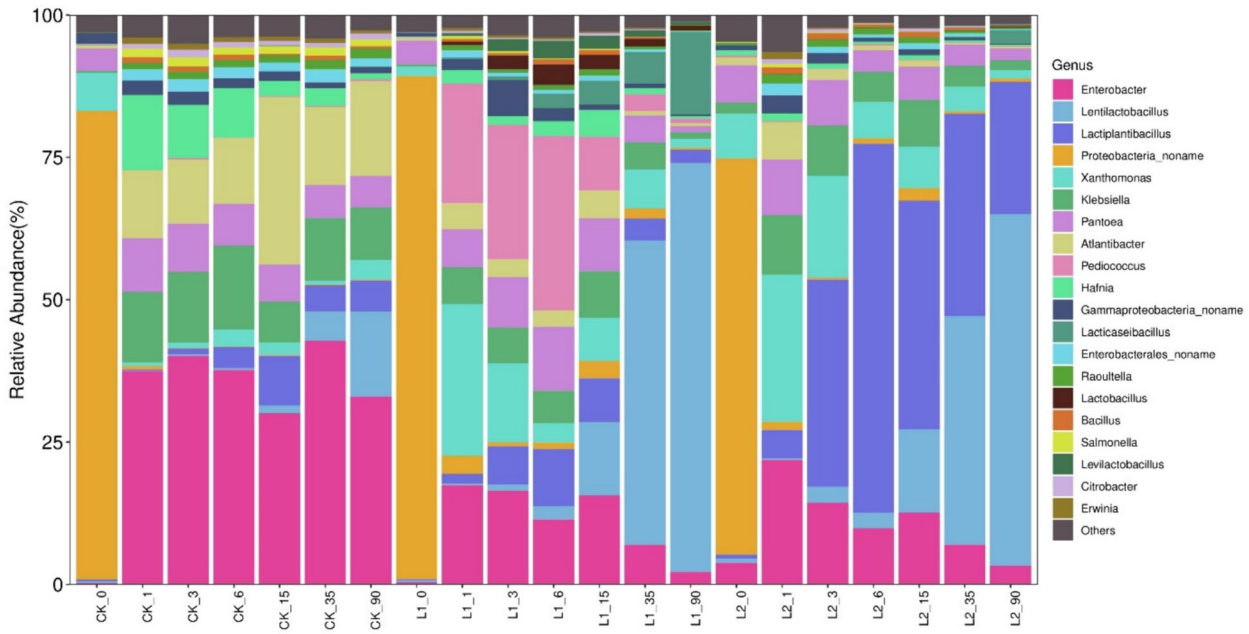


Fig. 1 Bacterial communities of barley silage during fermentation (genus level, $n=4$). CK: control; L1: ensiled barley with *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Pediococcus acidilactici*; L2: ensiled barley with *Lact. plantarum* and *Lent. buchneri*

between d 1 and d 3 ($P < 0.05$). L2 had more yeast than CK and L1 at d 15 and L1 at d 35 ($P < 0.05$). There were fewer yeasts in L1 than in CK and L2 at d 90 ($P < 0.05$).

Bacterial communities

The abundance of *Lentilactobacillus* increased during fermentation in CK, L1, and L2 (from 0.32% to 14.9%, from 0.28% to 71.8% and from 0.70% to 61.7%, respectively) (Fig. 1). *Lactiplantibacillus* in CK increased from 0.27% to 8.74% during the first 15 days and then decreased to 5.43% at d 90. Moreover, its abundance in L1 and L2 increased from 0.26% to 10.0% and from 0.75% to 64.8% during the first 6 days, respectively and then decreased to 2.28% and 23.3% at d 90, respectively. *Pediococcus* in CK and L2 had low abundances (less than 0.50%) during fermentation. However, its abundance in L1 increased from 0.21% to 30.6% during the first 6 days and then decreased to 7.09% at d 90. *Lacticaseibacillus* had a low abundance in CK during fermentation (< 0.20%) and in L2 during the first 15 days (< 0.05%) and then increased to 2.40% at d 90. However, its abundance in L1 increased from 0.05% to 14.5% during fermentation.

The abundance of the Noname Proteobacteria in CK_0, L1_0, and L2_0 was 82.3%, 88.3%, and 70.0%, respectively and then rapidly decreased to 0.60%, 3.18%, and 1.45%, respectively on the first day, after which the abundance kept lower level. The abundance of *Enterobacter* in CK, L1, and L2 rapidly increased on the first day (37.5%, 17.5%, and 21.9%, respectively) and then remained high in CK (> 33% from d 1 to d 90) and decreased to 2.21% in L1 and to 3.30% in L2 at d 90. *Xanthomonas* in CK rapidly decreased in abundance on the first day (6.69% to 0.61%) and then increased to 3.47% at d 90. However, its abundance in L1 and L2 rapidly increased on the first day (1.65% to 26.6% and 7.86% to 25.8%, respectively) and then decreased to 1.58% in L1 and to 1.48% in L2 at d 90. The abundance of *Klebsiella* in CK increased during the first 6 days (from 0.34% to 14.8%) and then decreased to 9.22% at d 90. Moreover, its abundance increased to 8.20% in L1 at d 15 and to 10.5% in L2 at d 1 and then decreased to 1.17% and 1.74% at d 90, respectively. The abundance of *Pantoea* in CK and L2 increased on the first day (from 3.96% to 9.41% and from 6.58% to 9.74%, respectively) and then decreased to 5.51% and 2.07%, respectively, at d 90. Moreover, its abundance in L1 increased during the first 6 days (from 4.20% to 11.2%) and then decreased to 1.03% at d 90. *Atlantibacter* in CK and L1 increased during the first 15 days (from 0.54% to 29.5% and from 0.48% to 4.89%, respectively) and then decreased to 0.48% and 0.57%, respectively, at d 90. Moreover, its abundance in L2 increased from 1.36% to 6.61% on the first day and then decreased to 0.37% at d 90. Hafnia in CK and L2 increased from 0.09% to 13.2% and 0.91% to 1.38%, respectively, on the first day and then decreased to 1.06% and 0.20%, respectively, at d 90. Moreover, its abundance in L1 increased from 0.11% to 4.68% in the first 15 days and then decreased to 0.43% at d 90.

Correlation between gas production and fermentation quality

The pH correlated positively with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 6, d 15, and d 35 (except CO_2) ($P < 0.05$) (Fig. 2). LA was negatively correlated with gas, CO_2, N_2O, and CH_4 at d 1 (excluding N_2O and CH_4), d 3, d 6, and d 15 ($P < 0.05$). The AN had a positive correlation with gas at d 1 and with gas, CO_2, N_2O, and CH_4 at d 3 (except N_2O), d 6, and d 15 ($P < 0.05$). BC correlated negatively with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 3, and d 6 and positively with gas, CO_2, N_2O, and CH_4 at d 35 ($P < 0.05$). The FWL correlated positively with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 3, d 6, and d 15 ($P < 0.05$).

Correlation between gas production and microbial counts

LAB were negatively correlated with gas and CO_2 at d 1 ($P < 0.05$) and positively correlated with gas, CO_2, N_2O, and CH_4 at d 15 ($P < 0.05$) (Fig. 2). The coliforms were positively correlated with CO_2 and N_2O and negatively correlated with CH_4 at d 1 ($P < 0.05$) and positively correlated with gas, CO_2, N_2O, and CH_4 at d 3, d 6, and d 15 ($P < 0.05$). The yeast correlated negatively with gas at 1 d ($P < 0.05$) and with total gas, CO_2, N_2O, and CH_4 at 3 d ($P < 0.05$).

Correlation between gas production and bacterial communities

Enterobacter had a positive correlation, and *Lentilactobacillus* had a negative correlation with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 6, d 35, and d 90 (except N_2O and CH_4) ($P < 0.05$) (Fig. 4). *Lactiplantibacillus* was negatively correlated with gas and CO_2 at d 1 and with CO_2 at d 35 ($P < 0.05$). Noname Proteobacteria was negatively correlated with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 3, d 6, and d 15 ($P < 0.05$). *Xanthomonas* was negatively correlated with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 3, and d 35 ($P < 0.05$). *Klebsiella*, *Atlantibacter*, and *Hafnia* were positively correlated with gas, CO_2, N_2O, and CH_4 at d 35 ($P < 0.05$); moreover, *Atlantibacter* was negatively correlated with CH_4 at d 1 ($P < 0.05$). *Pediococcus* was negatively correlated with

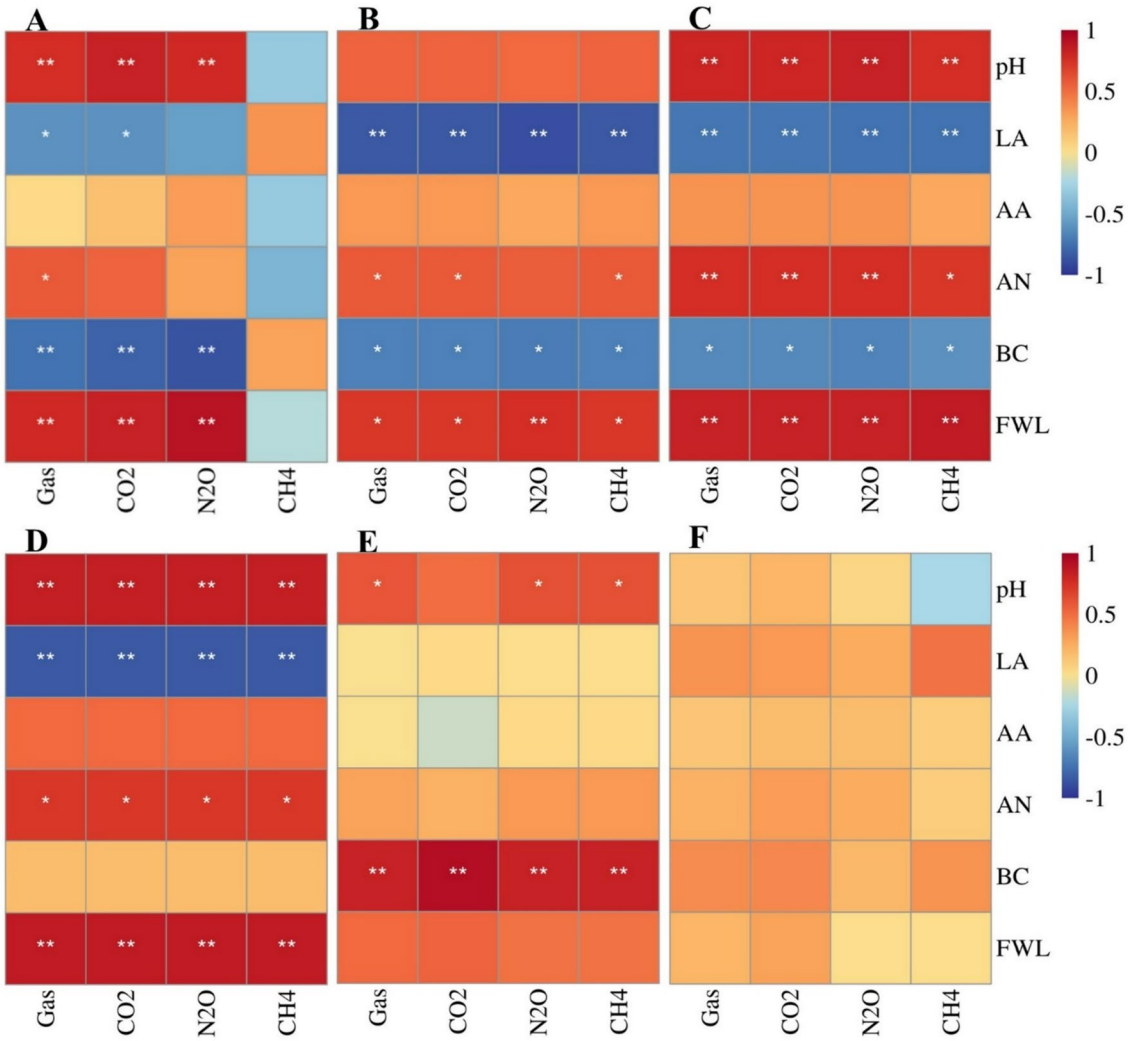


Fig. 2 Correlation heatmap of gas, CO_2, N_2O, and CH_4 with pH, lactic acid (LA), acetic acid (AA), ammonia nitrogen (AN), and fermentation weight loss (FWL) of barley silage at d 1 (**A**), d 3 (**B**), d 6 (**C**), d 15 (**D**), d 35 (**E**), and d 90 (**F**) after ensiling ($n = 12$). *$P < 0.05$, **$P < 0.01$

gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 6, and d 35 ($P < 0.05$). *Lacticaseibacillus* was negatively correlated with gas, CO_2, N_2O, and CH_4 at d 1 (except CH_4), d 6, and d 90 (except N_2O and CH_4) ($P < 0.05$).

Discussion

Gas and GHG production

Gas production increased in CK during the first 3 days and in L1 and L2 during the first day and then decreased (Table 1 and Figure S2). In our other study (unpublished), gas production increased during the first 4 days and then decreased in barley silage without any additives (Figure S1). Similar dynamics of gas production were also detected in stylo, rice straw, and oat silages [13, 24]. Furthermore, gas production reached its peak at d 6 in CK but at d 3 in L1 and L2 (Table 1 and Figure S2). A previous study also detected gas peak production at d 3 and d 7 in silage with and without inoculation, respectively [13]. These results indicated that the fermentation process in silage can be divided into a gas accumulation phase and a gas reduction phase according to gas production, and inoculating LAB at ensiling can shorten the gas accumulation phase of silage.

CO_2, N_2O, and CH_4 are the main GHGs generated in silages [22, 34, 35]. Although CO_2 has one global warming potential (1 GWP), N_2O has 265 GWP, and CH_4 has 28 GWP, the average production of CO_2 was 564 times that of N_2O and 64,577 times that of CH_4 during fermentation (Table 1). Schmithausen et al. [22] reported that the CO_2 concentration was more than 50% of the gas at d 12 of ensiling, with peak concentrations of N_2O and

CH_4 less than 1200 ppm and 100 ppm, respectively. Furthermore, other studies have shown that in silages, CO_2 accounts for more than 60% of the gas produced [13, 17, 20] and was one of the main components of gases generated in silages [16, 19, 23]. These results indicated that CO_2 is the most important GHG generated in silage.

The peak productions of gas and CO_2 in L1 (d 1) and L2 (d 1) were less than 20% of those in CK (6 d) (Table 1). Previous studies also detected lower gas production in oat silage with the same LAB additives (*Lact. plantarum* as one major component) [24] and in silages of stylo and rice straw with *Lact. plantarum* [13]. However, Gomes et al. [23] reported higher gas production in wilted oat silage inoculated with *Lent. buchneri*. The differences in gas production might be caused by differences in the metabolic pathways of *Lact. plantarum* (homofermentative LAB) and *Lent. buchneri* (heterofermentative LAB). The homofermentative LAB converts carbohydrates into lactic acid, but the heterofermentation of glucose produces CO_2 in the silage system during anaerobic fermentation [11, 36]. These results suggest that inoculation of LAB with *Lact. plantarum,* as the main component, can reduce gas and GHG production in silages.

Gas accumulation phase

The gas in silage is mainly generated by the respiration of raw materials and microorganisms during the initial aerobic phase and by the activities of microorganisms during the initial anaerobic fermentation phase [13, 20]. Generally, silos quickly reach anaerobic conditions within a few hours of ensiling [16–18, 20]. Sun et al. [37] reported that in whole-plant corn silages with similar laboratory silos, the initial aerobic phase lasted less than 2 h. Previous studies have shown that most of the CO_2 in silage was generated during the anaerobic period [22, 25]. There was no change in the volume of any of the silages after 4 h of ensiling, and gas production rapidly increased during the first 3 days in CK and on the first day in L1 and L2 (Table 1 and Figure S2). These results indicated that the gas in silage accumulates mostly through the activities of microorganisms during the initial anaerobic phase rather than during the aerobic phase.

Enterobacteriaceae dominated the bacterial communities in untreated barley silage (CK) with high gas production during fermentation (Table 1, Figure S2, S3, and S4). During the first 6 days (gas accumulation phase), the CK had higher gas and GHG production, pH, coliforms, *Enterobacter*, *Klebsiella*, *Atlantibacter*, and *Hafnia*, and

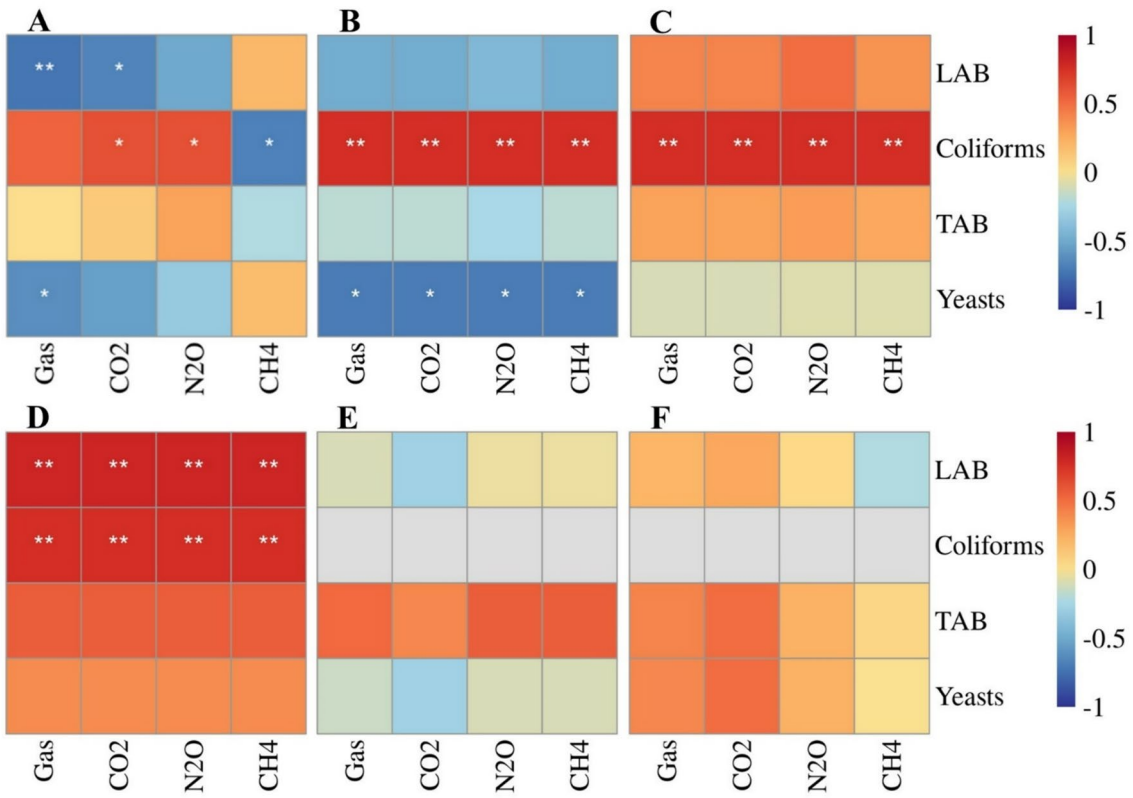


Fig. 3 Correlation heatmaps of gas, CO_2, N_2O, and CH_4 with lactic acid bacteria (LAB), coliforms, total aerobic bacteria (TAB), and yeasts of barley silage at d 1 (**A**), d 3 (**B**), d 6 (**C**), d 15 (**D**), d 35 (**E**), and d 90 (**F**) after ensiling ($n = 12$). *$P < 0.05$, **$P < 0.01$

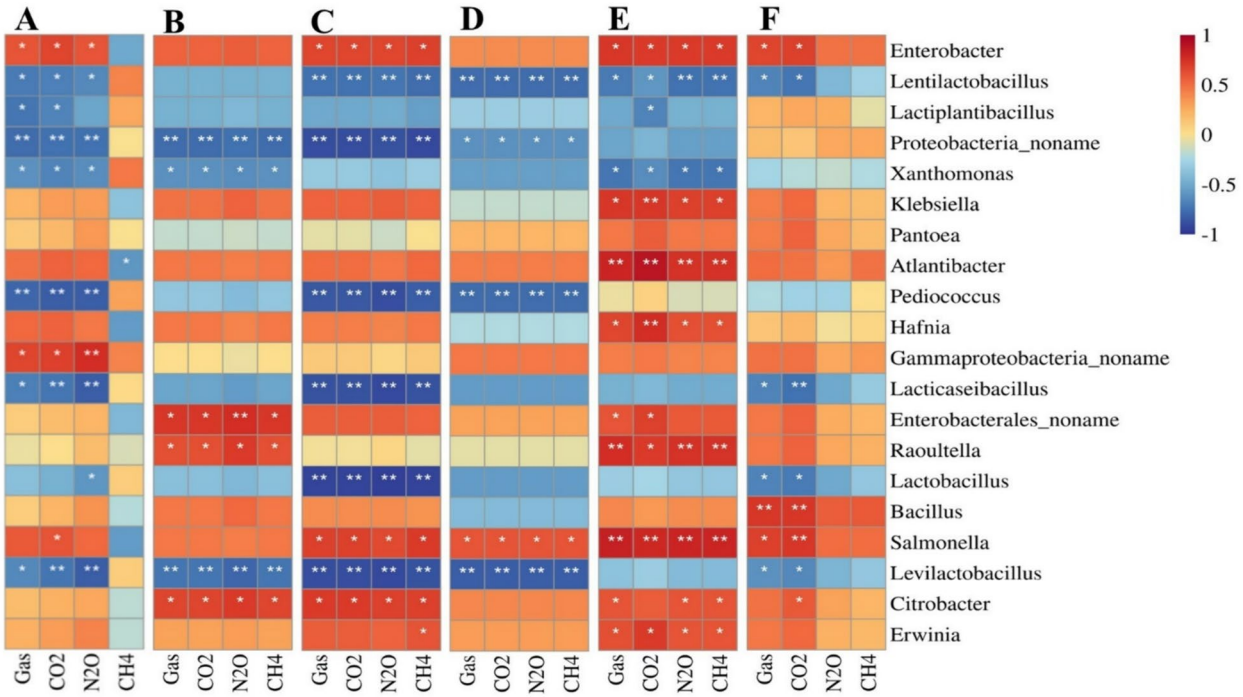


Fig. 4 Correlation heatmap of gas, CO_2, N_2O, and CH_4 with bacterial communities (genus level, top 20) of barley silage at d 1 (**A**), d 3 (**B**), d 6 (**C**), d 15 (**D**), d 35 (**E**), and d 90 (**F**) after ensiling ($n = 12$). $^*P < 0.05$, $^{**}P < 0.01$

lower LA than L1 and L2 (Tables 1, 2, and 3, and Fig. 1, S2, and S5). Gas, CO_2, and N_2O had positive correlations with pH and the microbes mentioned above and negative correlations with LA from d 1 to d 6, and CH_4 had the same correlation with those at d 3 and d 6 (Figs. 2, 3, and 4). However, Chen et al. [13] reported a positive correlation of gas and CO_2 production with *Lactococcus*, *Leuconostoc*, and *Lachnoclostridium* in stylo silages and with *Prevotella*, *Citrobacter*, and *Massilia* in rice straw silages during fermentation. Sun et al. [24] showed a positive correlation of gas production with Enterobacteriaceae and *Enterobacter* at d 1, with Enterobacteriaceae, *Enterobacter*, and *Lactococcus* at d 3, and with *Lactococcus* at d 6 in oat silages. These different correlations might have resulted from the different silage having unique microbial communities during fermentation.

Enterobacter, *Klebsiella*, and *Atlantibacter* are Enterobacteriaceae that can utilize glucose as a substrate to produce AA, ethanol, and CO_2 in silages [38]. *Hafnia* also can ferment glucose to acid and gas [39]. After ensiling, enterobacteria, lactobacilli, and plant nitrate reductase degrade nitrate to nitrite and nitric oxide and then to form ammonia and N_2O [40, 41]. Previous studies have also reported that the enterobacteria in silage are the main microbes that degrade nitrate during fermentation [41, 42]. In addition, facultative anaerobic enterobacteria can convert enzymatic formate into CO_2 and H_2 during the initial fermentation phase [43]. Clostridia can degrade lactate into butyric and acetic acids and form H_2 [44]. H_2 can be used for methanogenesis under anaerobic conditions and converted to CH_4 by archaea with AA in silages [22]. Chen et al. [13] reported the formation of H_2 during the initial fermentation phase in stylo silage and rice straw silage. Enterobacteriaceae were the main bacteria, and clostridia were not detected in any of the silages from d 1 to d 6 (Fig. 1 and S4). Furthermore, pH was positively correlated with coliforms and *Enterobacter* at d 1; with coliforms, *Enterobacter*, *Atlantibacter*, and *Hafnia* at d 3; and with coliforms, *Enterobacter*, and *Klebsiella* at d 6 (Figure S6). LA had a negative correlation with *Hafnia* at d 1 and with coliforms, *Enterobacter*, and *Klebsiella* at d 3 and d 6 (Figure S6). These results indicate that during the gas accumulation phase, the accumulation of gas and GHG in barley silage might be mainly due to the activities of enterobacteria but was slowed by rapid fermentation in LAB-treated barley silage.

Gas reduction phase

The production of gas and GHG decreased after d 6 in CK and after d 3 in L1 and L2 (Table 1 and Figure S2). Previous studies also revealed a reduction in the production of gas and/or CO_2 in silage during the late fermentation phase [13, 24]. In a silage system, CO_2 can gradually leak out of the polyethylene film of bunker

silage during fermentation [17] and continuously escape from the silage bale during the first 25 days of ensiling [35]. Furthermore, it partially dissolves in interstitial silage water (39.2 mmol L^{-1} at 20 °C) to form H_2CO_3 [25]. CO_2, as a growth factor, is assimilated by taking part in some fundamental metabolic processes of heterotrophic bacteria [45]. LAB are heterotrophic gram-positive bacteria [46]. *Lact. plantarum* and *Lent. buchneri* contains 9 and 10 genes involved in inorganic carbon (CO_2 and HCO_3^-) assimilation, respectively [11]. The *Lact. plantarum*, *Enterococcus faecalis*, and *Enterococcus faecium*, as capnophiles, can assimilate inorganic carbon as a substrate in carboxylation reactions in silages [46]. The main LAB genera were negatively correlated with gas and GHG from d 1 to d 90 (Fig. 4). *Lact. plantarum* increased during the first 15 days in CK and during the first 6 days in L1 and L2 and then decreased (Figure S7). The abundance of *Lent. buchneri* increased in all the silages during fermentation (Figure S7). Furthermore, *Lact. plantarum* and *Lent. buchneri* was the main bacteria in all the silages during fermentation, and *P. acidilactici* was the main bacterial species in L1 from d 1 to d 35 (Figure S7). These results indicate that during the gas reduction phase, the gas and GHGs might escape from the silo into the air, dissolve in silage water, and participate in the metabolism of the main LAB (*Lentilactobacillus*, *Lactiplantibacillus*, and *Pediococcus*) in silage.

In oat silage, gas production was negatively correlated with *Lactiplantibacillus* and *Lentilactobacillus* at d 3; with *Pediococcus* and *Lactobacillus* at d 6; with *Lentilactobacillus*, *Lacticaseibacillus*, and *Pediococcus* d 15; with *Lacticaseibacillus* at d 35; and with *Lactiplantibacillus* and *Pediococcus* at d 90 [24]. Similarly, Chen et al. [13] revealed a negative correlation of gas and CO_2 production with *Serratia*, *Sphingobacterium* and *Sphingomonas* in rice straw silage and with *Pediococcus*, *Klebsiella* and *Escherichia–Shigella* in stylo silage. Previous studies have also reported that *Serratia*, *Sphingobacterium*, and *Sphingomonas* can sequester CO_2 [47–49]. Therefore, the mechanism of gas and GHG reduction in silages during late fermentation phases needs further study.

Fermentation quality

After d 15 of ensiling, L1 and L2 had increasing pH and decreasing LA, and the AA concentration increased in all silages (Table 2). Similar trends were detected in whole-plant corn silages, oat silage, barley silage, Napier grass silage, rehydrated corn kernel silage, smooth bromegrass silage, and *Leymus chinensis* silage [24, 28, 50–54]. *Lentilactobacillus* in L1 and L2 had a positive correlation with pH and AA and a negative correlation with LA from d 15 to d 90 (Figure S8). Interestingly, in our other study, *Lentilactobacillus* was the most bacterial genus at d 35 and d 90 and had a positive correlation with pH and AA and a negative correlation with LA in oat silage with/without LAB additives (L1 and L2) [24]. Moreover, Oude Elferink et al. [55] revealed that *Lent. buchneri* can convert LA into AA, 1,2-propanediol, ethanol and CO_2 under acidic and anoxic conditions. The abundance of *Lent. buchneri* in L1 and L2 increased during fermentation, and it was the most abundant bacterial species at d 35 and d 90 (Figure S7). These results indicate that during the late fermentation phase, a decrease in fermentation quality was a common phenomenon caused by *Lent. buchneri,* which dominated the bacterial communities of the silages.

The proteolysis in silage is mainly attributed to microbial activities during fermentation [56], which results in low protein utilization in the rumen [57, 58]. The AN level is an indicator of protein degradation and silage preservation [56, 57]. In the present study, all silages were well preserved according to Kung et al. [59] due to the lower level of AN (from 33.6 to 37.0 g/kg TN) in terminal silages (Table 2). Compared with CK, L1 and L2 had lower AN contents and coliforms counts from d 1 to d 15 and lower abundances of *Enterobacter* from d 1 to d 6 (Tables 2 and 3; Fig. 1 and S5). The AN in all the silages had a positive correlation with *Enterobacter* and coliforms from d 1 to d 6 and with coliforms at d 15 (Figure S6). Moreover, Enterobacteriaceae can form AN from protein degradation in silage [60]. Thus, Enterobacteriaceae activity mainly contributed to the formation of AN in barley silage during the early fermentation stage. After d 15, the CK had a high level of AN, but there was no difference, and L1 and L2 had increasing AN (Table 2). The AN in L1 and L2 had a positive correlation with *Lentilactobacillus* from d 15 to d 90 (Figure S8). Further study is needed to determine the cause of the increase in AN in barley silages treated with LAB during the late fermentation stage.

The fermentation process significantly increases the BC of silages due to the formation of lactates, acetates, and NA during fermentation [31]. However, in the present study, the BC in L1 and L2 increased during the first 15 days and then decreased, and the BC in CK increased during fermentation (Table 2). Interestingly, LA and BC displayed the same trends in all the silages (Table 2). BC had a positive correlation with LA in all silages at d 1, d 3, d 6 and d 90 (Figure S6); with LA, AA, and AN in L1 and L2 from d 1 to d 15; and with LA in L1 and L2 from d 15 to d 90 (Figure S8). Moreover, in our other studies BC and LA exhibited similar changes during fermentation in *Leymus chinensis* silage and oat silage [24, 28]. These results indicate that the LA concentration in the silages contributed most strongly to the BC of the silage during fermentation.

Fermentation weight loss

The weight loss of silage during fermentation is caused mainly by gas, volatile organic compounds (VOCs), and water escaping from the silo into the air [11]. Polyethylene has gas permeability with very low transmission rates [20]. In addition, the FWL had a positive correlation with gas and GHG in all silages from d 1 to d 15 (Fig. 2); with LA and AA in CK during fermentation and in L1 and L2 during the first 15 days; and with AA in L1 and L2 from d 15 to d 90 (Figure S8). Moreover, no effluents were found in the silos. L1 and L2 had lower FWL and less gas and GHG production than CK during fermentation (Tables 1 and 2). Previous studies have shown lower loss and less gas production in inoculated silage [13, 21, 24]. These results suggest that the losses of ensiled barley mainly resulted from gas, LA, and AA escaping from the laboratory silo (polyethylene bag) during fermentation and that inoculating LAB at ensiling can reduce the FWL by decreasing gas and GHG production in silage.

Conclusion

The fermentation process of silages can be divided into a gas accumulation phase and a gas reduction phase, with CO_2 as the main component of the gas. Inoculating LAB reduces gas and GHG production as well as the gas accumulation phase. The activities of enterobacteria strongly contribute to gas and GHG accumulation in barley silage. Gas and GHGs escape from silos into the air, dissolve in silage water, and participate in LAB metabolism in silage during the gas reduction phase. Fermentation quality deterioration is a common phenomenon caused by *Lentilactobacillus* activity during the late fermentation phase in silages. The FWL is caused by the gas produced, as well as by LA and AA escaping from the silo, but can be decreased by inoculating LAB during fermentation.

Abbreviations

AA	Acetic acid
AN	Ammonia nitrogen
BA	Butyric acid
BC	Buffering capacity
CFU	Colony-forming units
CH_4	Methane
CO_2	Carbon dioxide
DM	Dry matter
FW	Fresh weight
FWL	Fermentation weight loss
GHG	Greenhouse gases
GWP	Global warming potential
HCO_3^-	Bicarbonate radical
LA	Lactic acid
LAB	Lactic acid bacteria
NO	Nitric oxide
N_2O	Nitrous oxide
PA	Propionic acid
PCR	Polymerase chain reaction
TAB	Total aerobic bacteria
VOC	Volatile organic compounds

Supplementary Information

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Additional file1.

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Author contributions

YX contributed to securing financial support, designing the study, and preparing the first manuscript draft; YX, NW, NN, JS, LS, MQ, DL, EL, and BY contributed to do this study and revised the manuscript draft; NW, and NN performed data collection and statistical analysis. All authors have read and approved the final manuscript.

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Availability of data and materials

Data will be made available on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Inner Mongolia Engineering Research Center of Development and Utilization of Microbial Resources in Silage, Inner Mongolia Academy of Agriculture and Animal Husbandry Science, 22 Zhaojun Road, Hohhot 010031, China. [2]Institute of Grassland Research, Chinese Academy of Agricultural Sciences, Hohhot 010013, China. [3]College of Forestry and Prataculture, Ningxia University, Yinchuan 750021, China. [4]School of Life Sciences, Inner Mongolia University, Huhhot 010020, China.

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