

Article

Using Lactic Acid Bacteria as Silage Inoculants or Direct-Fed Microbials to Improve In Vitro Degradability and Reduce Methane Emissions in Dairy Cows

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Abstract: The current study has two objectives: (1) To determine the effect of different lactic acid bacteria (LAB) strains' inoculant on silage quality of fresh ryegrass (FR) and rain-treated ryegrass (RTR), and (2) to find the optimal way (silage inoculant vs. direct-fed microbial (DFM)) to use LAB strains in order to improve nutrient digestibility and reduce methane emission (CH₄) in ruminant production. Five LAB strains were tested, *Lactiplantibacillus plantarum* AGR-1, *L. plantarum* AGR-2, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* AGR-3, *L. lactis* subsp. *lactis* AGR-4 and *L. lactis* subsp. *lactis* AGR-5. Each LAB strain was inoculated at 10⁶ cfu/g fresh weight into the FR and the RTR and ensiled for 60 days. After ensiling, the effect of LAB strains included as a DFM or silage inoculant on rumen digestibility and CH₄ production were measured using an in vitro gas production system with three separate runs. The in vitro experiment consisted of 24 treatments (2 grasses (FR and RTR) × 2 ways (inoculant or DFM) × 6 strains (5 LAB strains + 1 Control)). The results indicated that the LAB strains' inoculant treatments reduced ($p < 0.0001$) the dry matter (DM) losses, the NH₃ concentration ($p < 0.0001$) and the pH ($p = 0.0019$) upon ensiling in both the FR and the RTR. The lowest values in dry matter (DM) loss and NH₃ concentration were found in the *L. plantarum* (AGR-2) and *L. lactis* (AGR-5). The in vitro CH₄ production was lower for silages inoculated with *L. plantarum* (AGR-1, $p = 0.0054$), *L. lactis* (AGR-4, $p = 0.026$), *L. lactis* (AGR-5, $p = 0.029$) and *L. plantarum* (AGR-2, $p = 0.090$), compared to the control. Methane production was lower ($p = 0.0027$) for LABs when used as silage inoculants, compared to being used as DFM. Lactic acid bacteria used as silage inoculants increased ($p \leq 0.0001$) the in vitro DM and organic matter (OM) degradability both in the FR and the RTR, whereas LAB strains used as DFM showed no such effect. The DM and OM digestibility were highest in the *L. plantarum* (AGR-1, $p = 0.0175$). Among the five LAB strains used in the current study, *L. plantarum* (AGR-2) was the best candidate to improve silage quality. Our observations suggest that these LAB strains are most promising when used as silage inoculants and to be confirmed in vivo.

Keywords: lactic acid bacteria; inoculant; direct fed microbial; methane; digestibility

1. Introduction

According to the European Council, greenhouse gas (GHG) emissions have to be decreased over 80% in developed countries by 2050 [1]. In this context, methane (CH₄) is the second most important gas involved in greenhouse emissions, with CH₄ from livestock accounting for 6.3% of the global

anthropogenic CO₂ equivalent production [2]. Among livestock, ruminants are the main contributors, accounting for 65% of the emissions. A decrease in CH₄ emissions' intensity (CH₄ per unit meat or milk) from ruminants can be achieved by improving forage (either fresh, hay or silage) quality and digestibility [3].

In European countries such as the Netherlands, ensiling is the most important method to preserve moist forage used as feed for ruminants [4]. This method is based on natural fermentation, in which lactic acid bacteria (LAB) ferment water-soluble carbohydrates in the plant material into organic acids, mainly lactic acid (LA), under anaerobic conditions. In consequence, the pH quickly drops, inhibiting detrimental anaerobes and preserving the nutritional value and palatability of the moist forage.

To realize an optimal preserved ensiled fodder, forages are not directly harvested and preserved. They need a wilting period before ensiling to reduce the moisture concentration, to increase concentration of water-soluble carbohydrates, to reduce water activity and to prevent effluent losses from the silo [5]. However, during the wilting, unexpected rainfall might occur and often is unavoidable. The most important negative effect of rain damage on the nutritive value of a forage during wilting is the loss of water-soluble carbohydrates through direct leaching or due to prolonged respiration by plant enzymes. Both mechanisms limit the availability of water-soluble carbohydrates required for the ensiling process and reduce silage quality [6]. After rainfall during wilting, dry matter (DM) digestibility of forage has been shown to reduce in *in vitro* [7] and *in situ* studies [8].

Although plant materials contain native LAB, the number of viable LAB on plant materials can be deficient and this delays the decline in pH during ensiling, resulting in higher nutrient losses and an increased development of detrimental microbes during the ensiling process. Therefore, to improve silage fermentation and prevent silage from spoilage, lactic acid bacteria as microbial silage inoculants have been used over several decades [9–12]. Lactic acid bacteria have not only been reported to improve silage quality and reduce fermentation losses, but also to improve animal performance by increasing milk yield and feed efficiency [9–13]. Cao et al. [14–16] reported that LAB also reduced CH₄ emission in ruminants. In contrast, other studies reported that LAB neither changed digestibility nor did they reduce CH₄ emissions [17,18]. Doyle et al. [19] showed that the effect on CH₄ emissions is clearly dependent on the LAB strains used.

Lactic acid bacteria are also known to have probiotic properties, such as improved establishment of beneficial gut microflora and competitive exclusion of pathogenic microorganisms [20]. Incorporating LAB in ruminant diets showed an increase in milk yield, improved feed efficiency, increased daily weight gain, increased carcass weight and reduction in fecal shedding of *Escherichia coli* O157:H7 [21–23].

The first objective of the current study was to determine the effect of different LAB strains' inoculant on grass silage quality of fresh grass and rain-treated grass. The hypothesis was that LAB strains will improve silage quality of both rain-treated grass and the fresh grass. Based on a survey on scientific publications, the authors found that this is the first study to determine the effect of different LAB strains which were selected based on their bacteriocins-producing properties. These LAB strains were used as a silage inoculant or a direct-fed microbial (DFM) at the same level of concentration, in the same condition experiment on rumen degradability and methane production. Therefore, the second objective of the current study was to find the optimal way to use LAB strains in order to improve nutrient digestibility and reduce methane emission in ruminant production.

2. Materials and Methods

2.1. Silage Additives

Five different lactic acid bacteria (LAB) strains were selected based on their bacteriocins-producing properties. The following were used in the experiment: *Lactiplantibacillus plantarum* AGR-1 (LMG P-20353), *Lactiplantibacillus plantarum* AGR-2 (CECT 4528), *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* AGR-3, *Lactococcus lactis* subsp. *lactis* AGR-4 and *Lactococcus lactis* subsp. *lactis* AGR-5

(DSM 33083). These LAB strains were supplied by Sacco S.R.L (Via A. Manzoni 29/A, 22,071 Cadorago (CO), Italy).

2.2. Grass Materials

A uniform pasture consisting of approximately 90% English ryegrass (*Lolium perenne* L.) was selected at Carus, the experimental farm of Wageningen University, the Netherlands (51°59'05.6" N 5°39'22.4" E). The pasture received a yearly fertilizing regime of approximately 20 m³ of animal manure per ha with an additional 250 kg of N per ha as mineral fertilizer. The perennial ryegrass was harvested on 23 May 2019 at a stage before flowering and chopped to the length of two centimeters using a forage harvester machine (Claas, Jaguar 890, CLAAS KGaA mbH, Harsewinkel, Germany).

After harvesting and chopping, the perennial ryegrass was homogenized and divided into two equal sized batches. One batch of ryegrass (fresh ryegrass = FR) was spread on a cement floor under a roof for 24 h before being ensiling and allowed to wilt. The other batch of ryegrass was similarly spread on a cement floor without roof covering, and was allowed to be wetted by rainfall for 1 h with a precipitation of about 4 mm. After getting wet by rainfall, this batch of ryegrass (rain-treated ryegrass = RTR) was moved to and spread on a cement floor under a roof covering for 24 h before ensiling. The temperature under the roof was 23 °C during daytime and 14 °C during nighttime. Before ensiling, the FR and RTR were thoroughly mixed and sampled ($n = 3$) for chemical analysis.

2.3. Silage Preparation

Each LAB strain, AGR-1, AGR-2, AGR-3, AGR-4 and AGR-5, was serially diluted in phosphate buffer solution (PBS) at room temperature to obtain solutions with a bacterial concentration of 10⁸ colony forming unit (cfu)/mL. A 10 mL aliquot of each LAB strain was sprayed onto 1.0 kg of chopped FR and/or RTR to achieve a final concentration of 10⁶ colony forming unit (cfu) per gram of grass fresh weight basis. The control was sprayed with the same volume of phosphate buffer solution. After inoculation with each LAB strain, the FR and RTR were homogenized and divided into three replicates of 300 g fresh weight each, which then was packed into plastic bags (25 × 30 cm; Papenburg, Germany). The bags were sealed with a vacuum sealer (Allpax GmbH & Co. KG 26,871 Papenburg, Germany). All bags (5 LAB strains × 3 replicates + 2 control × 5 replicates = 25 bags) were put inside a large black plastic bag and stored at 20–23 °C during daytime and 12–14 °C during nighttime, to allow 60 days of ensiling. After 60 days of ensiling, all bags were stored at –20 °C for 24 h to stop the ensiling process. Then, all bags were defrosted at 4 °C for 24 h. Each bag was weighed, opened, homogenized and sampled for further analyses. A representative grass silage sample (10 g fresh material) was taken from each bag to determine the dry matter and ash content. In addition, a 30 g sample from each bag was weighed into a stomacher bag, combined with 270 mL of distilled water and then mixed vigorously during 5 min at 230 rounds per minute using a stomacher machine (STOMACHER 400 CIRCULATOR). The liquid from each bag was filtered through 2 layers of cheesecloth and the pH was measured immediately in the filtrate using a pH meter (Mettler Toledo FE20/EL20 pH meter, Schwerzenbach, Switzerland). Thereafter, the filtrate was centrifuged at 2500 g during 10 min and 1 mL samples were taken to analyze for ammonia nitrogen.

The remaining grass silage from three replicates for each treatment was pooled together and cut with scissors to a particle size of 2–3 mm, and then ground in a coffee mill to a particle size of <2 mm. To avoid possible cross-contamination, untreated silage was treated and ground first, followed by the other treatments. In between, at all times, the utensils and coffee mill were vigorously washed with water and soap and sterilized again, before processing the next silage treatment. This procedure resulted in 12 grass silage samples (1 FR control + 1 RTR control + (5 LAB strains × 2 type of grass)), that were used in an in vitro gas production system. After being ground in the coffee mill, all grass silage samples were stored in sealable bags at 4 °C until use in the in vitro gas production system.

2.4. Experimental Design

In total, 24 different treatments were evaluated to understand the impact of LAB strains on methane production and rumen digestibility. The parameters tested were type of grass (FR or RTR), form of LAB administration (DFM or inoculant) and LAB strains (AGR-1, AGR-2, AGR-3, AGR-4, AGR-5 and 1 control). The experimental design can be summarized as 2 grasses (FR and RTR) \times 2 ways (DFM or inoculant) \times 6 strains (5 LAB strains + 1 Control). For LAB strains as inoculant, the LAB strains' inoculated grass silages (2.50 ± 0.05 g in fresh weight) were weighed into triplicate 250 mL bottles (Schott bottle, GL45, Mainz, Germany) for each grass silage within each run. When LAB strains were added as DFM, 2.50 ± 0.05 g fresh weight of the control silage (FR or RTR) were added into the 250 mL bottles (Schott bottle, GL45, Mainz, Germany), together with 2.5 mL of cultures containing 10^6 cfu/mL LAB strains. The LAB cultures were prepared by diluting each LAB strain in phosphate buffer solution (PBS; pH 7). Each DFM treatment was tested in triplicate. 2.5 mL of phosphate buffer solution was added to the control treatments, the blanks and the LAB strains' inoculant treatments. Three blanks bottles with only buffered rumen fluid were included within each run. The current experiment consisted of three separate runs. All handlings with all treatments were done under strict anaerobic conditions.

A mixture of rumen liquid was collected from three different rumen fistulated lactating Holstein-Friesian dairy cows per run (i.e., total 9 rumen fistulated cows for three runs). These cows were fed a grass and maize silage mixture in the morning and afternoon and 9 kg of concentrate according to their requirements. The handling of the animals was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands, and in accordance with Dutch legislation on the use of experimental animals. Rumen fluid was collected before morning feeding in pre-warmed thermos flasks, which were filled with CO₂ and transported directly to the nearby laboratory. All further manipulations were done under CO₂ to ensure anaerobic conditions. The rumen fluid was pooled and filtered through two layers of cheesecloth into a flask flushed with CO₂. Filtered rumen fluid was mixed with a buffer solution with constant stirring and continuous flushing with CO₂ and maintained in a water bath at 39 °C. Buffer solution was made as described in Reference [24].

Sixty mL of the buffered rumen liquid mixture was added to each bottle. Then, all bottles were directly placed in a shaking water bath at 39 °C and connected to an automated time-related gas production system [24,25]. The bottles were incubated, and gas and methane production were measured over 72 h. After 72 h of incubation, the fermentation fluid pH was recorded (Mettler Toledo FE20/EL20 pH meter, Schwerzenbach, Switzerland) and fermentation fluid from each bottle was collected for volatile fatty acid (VFA) and ammonia (NH₃) analysis. The DM digestibility and organic matter (OM) digestibility of silage were calculated with the DM and OM contents before and after 72 h of in vitro incubation. Organic matter content of samples was determined via measuring DM and ash content of those samples.

2.5. In Vitro Gas and Methane Production

Total cumulative gas (GP) and methane (GP_{CH₄}) production was measured using an automated gas production system at the laboratory of the Animal Nutrition Group of Wageningen University, the Netherlands [25]. Methane concentration in the headspace of the fermentation bottle was measured by gas chromatography (GC; GC8000 Top, CE Instruments, Milan, Italy). Fermentation bottles were modified [25] to enable sampling CH₄ from the headspace. In brief, bottles were fitted with a glass extension that was sealed with a screw cap and an air-tight septum (Grace, XLB-13 Septa 1/2). Ten μ L aliquots of the bottle headspace gas were sampled through the septa at distinct time points of incubation (0, 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 56 and 72 h) using a gas tight syringe (Setonic micro syringe, syr 10 μ L polytetrafluorethylene fixed needle (PTFE FN) 0.47 (G26s) D5J, made in Limenau Germany) and were directly injected into the GC. The GC was equipped with a stainless-steel column (6 m long, 0.53 mm diameter, 25 μ m film thickness and packed with PoraPack Q50–80 mesh Grace, Breda, the Netherlands)

and connected to a flame ionization detector. The temperature of the injector, column and detector were maintained at 150, 60 and 150 °C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. The CH₄ concentration was calculated by external calibration using a certified gas mixture with a known CH₄ concentration in synthetic air (Linde Gas Benelux, Schiedam, The Netherlands). Peak areas were determined by automatic integration system software (Chrom-Card data system Version 2.3.3, September 2005, Rodano Milan, Italy) for GC.

Cumulative CH₄ production was calculated according to the procedure described by Pellikaan et al. [25] by taking the sum of the increase in headspace CH₄ concentration between two successive valve openings and the amount of CH₄ vented from the bottle:

$$\text{CH}_4 = \sum_{i=1}^{i=1+n} \{V_{\text{HS}}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (1)$$

where CH₄ = cumulative CH₄ production (mL/g of incubated OM), V_{HS} = the bottle headspace volume (ml), C_i, C_{i+1} = CH₄ concentration in the bottle headspace gas at valve opening i and i + 1 respectively, and G_{i+1} = the amount of gas vented at valve opening i + 1 (mL).

2.6. Curve Fitting and Calculations

Total cumulative gas (GP) and CH₄ production curves were fitted with a biphasic and monophasic Michaelis-Menten equation respectively [26], using the non-linear least squares regression procedure in SAS (SAS Institute, Cary, NC, USA).

$$\text{OMCV} = \sum_{i=1 \text{ or } 2}^n \frac{A_i}{1 + \left(\frac{C_i}{t}\right)^{B_i}} \quad (2)$$

where OMCV = GP or CH₄ production (mL/g of incubated organic matter), A = the asymptotic gas production (mL/g of incubated OM), B = the switching characteristics of the curve, C = time at which half of the asymptotic gas production is reached (half-time, T_{1/2}, h) and t = the time (h). The maximum rate of gas production (R_{max}, mL/h) was calculated using the estimated A_i, B_i and C_i values, as described by Bauer et al. [27]:

$$R_{\text{max}} = \frac{A \times C^B \times B \times \left[\text{TR}_{\text{max}}^{(-B-1)} \right]}{\left[1 + C^B \times \text{TR}_{\text{max}}^{(-B)} \right]^2} \quad (3)$$

where TR_{max} is the time at which R_{max} occurs:

$$\text{TR}_{\text{max}} = C \times \left\{ \left[\frac{B-1}{B+1} \right]^{(1/B)} \right\} \quad (4)$$

The maximum rate of substrate degradation (R_M, %/h) was calculated from the A, B and C values as estimated from the CH₄ production curves [26]:

$$R_M = (B \times tR_M^{(B-1)}) / (C^B + tR_M^B) \quad (5)$$

where tR_M is the time at which R_{max} occurs:

$$tR_M = C \times (B - 1)^{1/B} \quad (6)$$

2.7. Chemical Analysis

The FR and RTR before making silage were air-dried, ground through a 1 mm sieve using a cross beater mill (Peppink 100 AN, Deventer, The Netherlands) and analyzed for DM [28], ash [29] and nitrogen (N) [30]. Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fiber (NDF) was analyzed according to Van Soest et al. [31] after a pre-treatment with a heat-stable amylase and corrected for residual ash. The water-soluble carbohydrate (WSC) contents of samples were determined by colorimetry after reaction with an anthrone reagent according to the method of Thomas [32]. All grass silage samples were analyzed for DM [28] and ash [29].

Fermentation fluid, sampled for volatile fatty acid (VFA) analysis (750 µL), was acidified with 750 µL of ortho-phosphoric acid solution. The ortho-phosphoric acid solution was composed of 25 mL of 85% (v/v) ortho-phosphoric acid dissolved in 200 mL Millipore water and 300 mL of a 4 g/L 2-methylvaleric acid solution. VFA concentration was analyzed by GC following the procedures of Pellikaan et al. [25], with the carrier gas modified by using hydrogen instead of helium to enhance baseline separation. Isocaproic acid was included as the internal standard. The total VFA (tVFA) concentration in the fermentation fluid was expressed as mmol/g of incubated OM. Fermentation fluid samples for NH₃ analysis (750 µL) were mixed with 750 µL of 10% trichloroacetic acid solution. Ammonia was determined using a colorimetric method [25] after deproteinizing the supernatant with 100 g/L trichloroacetic acid, and the resulting chromophore was measured at 623 nm using a UV spectrophotometer (Evolution 201-Thermo Scientific, Bleiswijk, The Netherlands).

2.8. Statistical Analysis

The effects of LAB strains included as a probiotic or silage inoculant on rumen digestibility and methane production were tested by analysis of variance using the MIXED procedure of SAS [33] as:

$$Y_{ijk} = \mu + G_i + W_j + L_k + R_f + (G \times W \times L)_{ijk} + \varepsilon_{ijkf} \quad (7)$$

where Y_{ijk} = the dependent variable, μ = the overall mean, G_i = the effect of grass type ($i = 1$ to 2), W_j = the effect of either probiotic or inoculant ($j = 1$ to 2), L_k = the effect of LAB strains, R_f = run ($k = 1$ to 3), $(G \times W \times L)_{ijk}$ = the effect of grass type and either probiotic or inoculant and LAB strains' interaction and ε_{ijkf} = the residual error term. The statistical unit was the average of triplicate in vitro bottles within a run. Differences among main effects were analyzed using Tukey–Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at a probability value of $p < 0.05$, and a trend at a probability value of $p < 0.10$.

The effects of LAB strains as a silage inoculant on DM losses, the NH₃ concentration and the pH were tested by analysis of variance using the MIXED procedure of SAS [33] as:

$$Y_{ijk} = \mu + G_i + I_j + L_k + (G \times I \times L)_{ijk} + \varepsilon_{ijk} \quad (8)$$

where Y_{ijk} = the dependent variable, μ = the overall mean, G_i = the effect of grass type ($i = 1$ to 2), I_j = the effect of either inoculant or non-inoculant ($j = 1$ to 2), L_k = the effect of LAB strains, $(G \times I \times L)_{ijk}$ = the effect of grass type and either inoculant or non-inoculant and LAB strains' interaction and ε_{ijk} = the residual error term. The statistical unit was the average of triplicate silage bags. Differences among main effects were analyzed using Tukey–Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at a probability value of $p < 0.05$, and a trend at a probability value of $p < 0.10$.

3. Results

3.1. Chemical Composition of the Grass before Making Silage and Fermentation Quality of Grass after 60 Days of Ensiling

The chemical composition of fresh ryegrass (FR) and rain-treated ryegrass (RTR) before ensiling are shown in Table 1. The dry matter (243.04 g/kg) and the WSC (90.54 g/kg DM) of fresh ryegrass were greater than those of rain-treated ryegrass. Inoculation of both FR and RTR with the LAB strains affected the silage fermentation quality, as shown in Table 2. The fermentation DM (%) loss was lower in the LAB inoculant treatments compared to the control (7.39 vs. 12.77, $p < 0.0001$). The pH (4.07 vs. 4.25, $p = 0.0019$) and the NH_3 (2.87 vs. 4.52 g/kg DM, $p < 0.0001$) content were decreased in the LAB inoculant treatments. The DM losses, NH_3 -N and pH measurement were different between the FR and the RTR (Table 2). The lowest value in DM losses and NH_3 concentration were found in the *L. plantarum* AGR-2- and *L. lactis* AGR-5-treated grasses, compared with the other LAB strains.

Table 1. Chemical composition of grass materials before ensiling.

Parameters	Unit	Rain-Treated Ryegrass	Fresh Ryegrass
DM	g/kg of fresh material	232.35	243.04
OM	g/kg DM	870.30	885.90
CP	g/kg DM	98.00	98.56
NDF	g/kg DM	628.80	596.60
WSC	g/kg DM	60.29	90.54

DM = dry matter, OM = organic matter, CP = crude protein, NDF = neutral detergent fiber, WSC = water-soluble carbohydrate.

Table 2. Effect of LAB strains on dry matter losing, ammonia concentration and pH over 60 days of ensiling.

Parameters	Grass	Control	Inoculant at 10^6 cfu/g of Fresh Material					SEM	Inoculant (I)	p-Values		G × S × I
			AGR-1	AGR-2	AGR-3	AGR-4	AGR-5			Grass (G)	Strains (S)	
DM loss (%)	Fresh ryegrass	12.05	8.71	2.16	7.5	6.61	6.27	2.1545	<0.0001	0.0635	0.1377	0.4725
	Rain-treated ryegrass	13.49	6.91	3.08	13.35	11.76	7.61					
NH_3 (g/kg DM)	Fresh ryegrass	5.376	3.297	3.092	2.791	3.449	2.987	0.8413	<0.0001	0.0053	0.9961	0.9933
	Rain-treated ryegrass	3.655	2.325	2.273	3.048	2.859	2.63					
pH	Fresh ryegrass	4.343	4.117	4.193	4.293	4.223	4.253	0.1210	0.0019	<0.0001	0.8804	0.9873
	Rain-treated ryegrass	4.163	3.927	3.82	3.857	3.963	4.090					

DM = dry matter, NH_3 = ammonia, DM loss (%) = ((DM before making silage – DM after making silage)/DM before making silage) × 100, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

3.2. Total Gas and Methane Production

The effects of grass type, LAB strain and the form LAB administration on gas production (GP), methane production (GP_{CH_4}) and methane concentration ($\text{CH}_4\%$) are shown in Table 3. The GP was lower when LAB was used as inoculant (304.57 vs. 312.76 mL/g OM incubated, $p = 0.0353$), compared to the DFM. However, the GP was greater when *L. lactis* AGR-4 was added as inoculant than as a DFM in the RTR ($p = 0.0123$). The RTR grass produced more methane than the FR ($p = 0.0016$). As a consequence, the $\text{CH}_4\%$ was lower in the fresh ryegrass ($p = 0.0301$). The GP_{CH_4} produced was lower in the *L. plantarum* AGR-1 ($p = 0.0054$), *L. lactis* AGR-4 ($p = 0.026$), *L. lactis* AGR-5 ($p = 0.029$) and *L. plantarum* AGR-2 ($p = 0.090$) treatments, compared to the control. The GP_{CH_4} was lower when the LAB was used as inoculant compared to as DFM ($p = 0.0027$).

3.3. Fermentation Parameters and Kinetics

The maximum rate of gas production in the second phase (GP-R2max) and the maximum rate of methane production (GP_{CH₄}-Rmax) were affected by the type of grass used (Table 4). The GP-R2max (13.41 vs. 12.65 mL/g OM/h, $p = 0.0003$) and the GP_{CH₄}-Rmax (2.49 vs. 2.38 mL/g OM/h, $p = 0.0025$) were greater in the RTR than those in the FR. The half-time of gas production for the second phase (GP-T₂_{1/2}) and the GP-R2max were affected by the different LAB strains (Table 3). The GP-T₂_{1/2} produced in the *L. plantarum* AGR-1, *L. lactis* AGR-3 and *L. lactis* AGR-4 treatments was lower compared to the control treatment ($p = 0.0006$). When LAB was used as the inoculant, the GP-R2max was lowered to the DFM application (12.82 vs. 13.25 mL/g OM/h, $p = 0.0384$). The half-time of methane production (GP_{CH₄}-T_{1/2}) tended to be lower ($p = 0.0608$) when LAB was used as an inoculant. The rate of substrate degradation (R_M) as derived from the CH₄ production curves tended to be greater (4.75 vs. 4.49 %/h, $p = 0.0686$) when LAB was used as an inoculant. The effect of interaction between type of grass, LAB strains and the way to use LAB was found for GP-T₂_{1/2} ($p < 0.0001$), GP-R2max ($p = 0.0312$), GP_{CH₄}-T_{1/2} ($p = 0.0388$) and GP_{CH₄}-Rmax ($p = 0.0019$).

Table 3. Effect of LAB strains on gas and methane production.

Parameters	Grass	Way	Strains					SEM	<i>p</i> -Values				
			Control	AGR-1	AGR-2	AGR-3	AGR-4		AGR-5	Grass (G)	Strains (S)	Way (W)	G × S × W
GP (mL/g OM)	Fresh ryegrass	DFM	325.15	307.02	329.1	313.68	323.24	323.34	9.442	0.2975	0.3599	0.0353	0.0004
		INO	312.48	287.63	309.02	289.43	283.58	276.12					
	Rain-treated ryegrass	DFM	309.15	313.23	306.15	302.08	300.78	300.74					
		INO	313.74	300.58	306.04	318.38	337.16	320.74					
GP _{CH₄} (mL/g OM)	Fresh ryegrass	DFM	65.55	64.17	62.72	64.24	62.87	64.16	1.707	0.0016	0.0621	0.0027	<0.0001
		INO	63.94	56.85	61.02	57.54	54.5	55.33					
	Rain-treated ryegrass	DFM	65.02	62.13	62.7	64.26	61.18	60.09					
		INO	62.7	59.75	62.17	66.35	66.8	66.63					
CH ₄ % (% of GP)	Fresh ryegrass	DFM	20.18	20.96	19.07	20.73	19.48	19.87	0.508	0.0301	0.1428	0.3281	0.4636
		INO	20.56	19.87	19.79	19.92	19.32	20.14					
	Rain-treated ryegrass	DFM	21.14	19.84	20.52	21.29	20.72	20.03					
		INO	20.01	19.88	20.41	20.88	19.81	20.81					

GP = gas production, GP_{CH₄} = methane production, CH₄% = percentage of methane production in total gas production, DFM = as a direct-fed microbial, INO = as an inoculant, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

Table 4. Effect of LAB strains on fermentation characteristic.

Parameters	Grass	Way	Strains						SEM	p-Values								
			Control	AGR-1	AGR-2	AGR-3	AGR-4	AGR-5		Grass (G)	Strains (S)	Way (W)	G × S × W					
GP-T _{1/2} (h)	Fresh ryegrass	DFM	13.52	12.19	13.12	12.53	13.53	12.91	1.186	0.159	0.0006	0.3831	<0.0001					
		INO	14.04	12.57	16.19	12.52	11.96	12.43										
	Rain-treated ryegrass	DFM	16.04	12.48	14.56	13.33	12.1	12.78										
		INO	14.03	11.18	12.24	12.48	11.98	22.55										
GP-R2max (mL/g OM/h)	Fresh ryegrass	DFM	13.3	13.1	12.53	13.52	12.87	13.05						0.504	0.0003	0.0602	0.0384	0.0312
		INO	12.79	11.83	12.44	12.68	11.44	12.24										
	Rain-treated ryegrass	DFM	14.23	13.4	12.76	13.96	13.05	13.23										
		INO	12.6	11.96	13.34	14.21	14.86	13.43										
GP _{CH₄} -T _{1/2} (h)	Fresh ryegrass	DFM	20.74	21.04	18.32	18.09	17.69	18.74	1.329	0.8196	0.366	0.0608	0.0388					
		INO	18.48	16.54	18.99	17.28	17.31	16.43										
	Rain-treated ryegrass	DFM	20.55	16.4	19.51	20.51	16.31	17.14										
		INO	17.38	16.8	16.71	17.53	17.27	22.03										
GP _{CH₄} -Rmax (mL/g OM/h)	Fresh ryegrass	DFM	2.33	2.27	2.41	2.5	2.46	2.47						0.09	0.0025	0.6117	0.7467	0.0019
		INO	2.54	2.39	2.28	2.32	2.19	2.35										
	Rain-treated ryegrass	DFM	2.37	2.6	2.38	2.35	2.55	2.43										
		INO	2.5	2.45	2.57	2.73	2.69	2.26										
R _M (%/h)	Fresh ryegrass	DFM	3.97	4.1	4.68	4.59	4.6	4.48	0.342	0.9068	0.4229	0.0686	0.1147					
		INO	4.6	5.08	4.36	4.8	4.9	5.27										
	Rain-treated ryegrass	DFM	4.04	4.99	4.48	4.07	5.08	4.91										
		INO	4.64	4.87	4.91	4.97	4.87	3.79										

GP-T_{1/2} = The half-time of gas production for the second phase, GP-R2max = The maximum rate of gas production in the second phase, GP_{CH₄}-T_{1/2} = The half-time of methane production, GP_{CH₄}-Rmax = The maximum rate of methane production, R_M = The rate of substrate degradation, DFM = as a direct-fed microbial, INO = as an inoculant, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis biovar diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

3.4. Fermentation End-Products

The type of grass used affected total VFA (tVFA), the molar proportion of individual VFA (acetate (HAc), propionate (HPr), butyrate (HBu), valerate (HVa), branched chain VFA (HBc)), non-glucogenic to glucogenic VFA (NGR) ratio, as well as NH₃ and pH ($p \leq 0.0018$, Tables 5 and 6). The tVFA, HAc and HVa were greater ($p < 0.0001$) in the RTR than in the FR, whereas the HPr and HBu were lower in the RTR than in the FR. This resulted in a greater NGR ratio in the RTR than in the FR ($p = 0.0118$). The HBc and the NH₃ were lower in the FR than in the RTR ($p < 0.0001$).

Table 5. Effect of LAB strains on fermentation end-products.

Parameters	Grass	Way	Strains						SEM	p-Value								
			Control	AGR-1	AGR-2	AGR-3	AGR-4	AGR-5		Grass (G)	Strains (S)	Way (W)	G × S × W					
tVFA (mmol/g OM)	Fresh ryegrass	DFM	14.87	14.34	14.65	14.83	14.78	14.64	0.325	<0.0001	0.2836	<0.0001	<0.0001					
		INO	14.68	13.97	13.23	12.99	12.82	13.23										
	Rain-treated ryegrass	DFM	14.58	14.40	14.90	14.86	14.86	14.68										
		INO	14.55	14.06	15.23	15.16	15.24	14.24										
HAc (% of tVFA)	Fresh ryegrass	DFM	60.94	60.17	60.77	60.63	60.73	60.58						0.525	0.0164	0.0906	0.0121	0.001
		INO	60.58	62.29	60.38	60.81	62.29	60.32										
	Rain-treated ryegrass	DFM	60.55	60.47	61.63	61.28	61.35	61.24										
		INO	60.92	62.76	63.63	61.80	60.46	60.64										
HPr (% of tVFA)	Fresh ryegrass	DFM	19.40	19.64	19.50	19.56	19.52	19.55	0.245	<0.0001	0.8376	0.0406	0.0025					
		INO	19.40	18.75	20.13	19.00	18.86	19.39										
	Rain-treated ryegrass	DFM	19.06	19.08	18.70	18.92	18.96	19.00										
		INO	18.93	18.81	18.00	18.84	19.19	19.10										
HBu (% of tVFA)	Fresh ryegrass	DFM	14.13	14.50	14.24	14.31	14.27	14.26						0.208	0.001	<0.0001	0.0008	<0.0001
		INO	14.42	13.45	13.85	14.56	13.20	14.66										
	Rain-treated ryegrass	DFM	14.19	14.38	13.78	14.00	13.89	13.94										
		INO	14.16	12.70	12.85	13.65	14.55	14.35										

tVFA = total volatile fatty acid, HAc = acetate acid, HPr = propionate acid, HBu = butyrate acid, DFM = as a direct-fed microbial, INO = as an inoculant, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis biovar diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

Table 6. Effect of LAB strains on fermentation end-products.

Parameters	Grass	Way	Strains						SEM	p-Values			
			Control	AGR-1	AGR-2	AGR-3	AGR-4	AGR-5		Grass (G)	Strains (S)	Way (W)	G × S × W
HVa (% of tVFA)	Fresh ryegrass	DFM	1.85	1.91	1.86	1.85	1.85	1.9	0.038	0.0006	0.3713	0.3656	0.1505
		INO	1.88	1.84	1.96	1.88	1.87	1.89					
	Rain-treated ryegrass	DFM	2.05	2.00	1.95	1.91	1.91	1.93					
		INO	1.98	1.90	1.84	1.89	1.91	1.94					
HBc (% of tVFA)	Fresh ryegrass	DFM	3.67	3.78	3.63	3.65	3.63	3.71	0.075	<0.0001	0.0772	0.3127	0.1534
		INO	3.71	3.67	3.68	3.75	3.77	3.74					
	Rain-treated ryegrass	DFM	4.14	4.07	3.94	3.89	3.89	3.89					
		INO	4.02	3.83	3.67	3.81	3.89	3.97					
NGR ratio	Fresh ryegrass	DFM	4.08	4.02	4.06	4.05	4.06	4.04	0.089	0.0118	0.904	0.2663	0.1398
		INO	4.08	4.19	3.88	4.16	4.14	4.08					
	Rain-treated ryegrass	DFM	4.09	4.11	4.2	4.16	4.15	4.13					
		INO	4.14	4.12	4.48	4.15	4.11	4.11					
NH ₃ (mmol/g OM)	Fresh ryegrass	DFM	4.57	4.55	4.41	4.57	4.41	4.55	0.106	<0.0001	0.0595	<0.0001	0.0148
		INO	4.44	4.36	3.96	4.2	4.29	4.11					
	Rain-treated ryegrass	DFM	4.73	4.67	4.78	4.78	4.77	4.66					
		INO	4.66	4.39	4.6	4.87	4.91	4.54					
pH	Fresh ryegrass	DFM	6.447	6.451	6.453	6.462	6.451	6.462	0.009	0.0042	0.011	0.1433	0.0001
		INO	6.449	6.471	6.419	6.476	6.5	6.489					
	Rain-treated ryegrass	DFM	6.472	6.467	6.48	6.483	6.464	6.469					
		INO	6.464	6.474	6.472	6.486	6.469	6.461					

HVa = Valeric acid, HBc = Branch chain acid, NGR = non-glucogenic to glucogenic volatile fatty acid ratio (NGR = (HAc + 2 × HBu + 2 × iso-butyric + HVa + iso-valeric)/(HPr + HVa + iso-valeric)), DFM = as a direct-fed microbial, INO = as an inoculant, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

The LAB strains had an effect on HAc, HBu, HBc and NH₃, as well as pH (Tables 5 and 6). The *L. plantarum* AGR-2 yielded the lowest HBu (13.68 mol/100 mol), HBc (3.73 mol/100 mol), NH₃ (4.43 mmol/g OM incubated) and pH (6.45), compared to the other LAB strains. *L. lactis* AGR-5 generated the lowest HAc (60.69 mol/100 mol), compared to the other LAB strains.

The form of LAB application affected tVFA ($p < 0.0001$), HAc ($p = 0.0121$), HPr ($p = 0.0406$), HBu ($p = 0.0008$) and NH₃ concentration ($p < 0.0001$). The tVFA, HPr, HBu and NH₃ were greater in the DFM application compared to the inoculant one. In contrast, the HAc was lower in the DFM way than in the inoculant way. There was an interaction effect between grass type, LAB strain and LAB application on tVFA, HAc, HPr, HBu, NH₃ and pH ($p = 0.0148$).

3.5. Dry Matter and Organic Matter Digestibility

Dry matter and organic matter (OM) digestibility were greater ($p < 0.0001$) in the FR than in the RTR (Table 7). Inoculant application increased ($p < 0.0001$) DM and OM digestibility both in the FR and the RTR. However, the DM and OM digestibility were not changed when LAB strains were applied as a DFM. The LAB treatments increased ($p < 0.041$) DM and OM digestibility in both grasses, compared to the control treatment. The DM and OM digestibility reached the highest value for the *L. plantarum* AGR-2 ($p = 0.0175$) and *L. plantarum* AGR-1 ($p = 0.0187$) treatments, compared to the other LAB strains.

Table 7. Effect of LAB strains on dry matter and organic matter digestibility.

Parameters	Grass	Way	Strains						SEM	p-Values								
			Control	AGR-1	AGR-2	AGR-3	AGR-4	AGR-5		Grass (G)	Strains (S)	Way (W)	G × S × W					
DM digestibility (g/kg)	Fresh ryegrass	DFM	612.5	606.27	600.6	613.3	606.42	610.14	7.327	<0.0001	0.0004	<0.0001	<0.0001					
		INO	617.02	659.73	652.8	672.31	649.33	670.15										
Rain-treated ryegrass	DFM	569.23	559.41	543.35	551.98	557.66	554.76											
	INO	560.06	619.37	609.24	596.7	563.99	564.19											
OM digestibility (g/kg)	Fresh ryegrass	DFM	596.9	590.39	584.41	598.07	590.85	593.3						12.655	<0.0001	0.041	<0.0001	0.0074
		INO	601.52	644.27	637.95	658.19	633.72	655.92										
Rain-treated ryegrass	DFM	559.46	549.27	572.24	540.72	546.49	529.67											
	INO	549.98	613.09	600.89	586.84	551.91	556.73											

DM = Dry matter, OM = organic matter, DFM = as a direct-fed microbial, INO = as an inoculant, AGR-1 = *Lactiplantibacillus plantarum* (LMG P-20353), AGR-2 = *Lactiplantibacillus plantarum* (CECT 4528), AGR-3 = *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, AGR-4 = *Lactococcus lactis* subsp. *Lactis*, AGR-5 = *Lactococcus lactis* subsp. *lactis* (DSM 33083). SEM = Standard error means.

4. Discussion

4.1. LAB Inoculant Improved Silage Quality of Rain-Treated Ryegrass and Fresh Ryegrass

Rainfall at harvest or wilting are known to reduce the quality of forage silage. The concentrations of NH₃ and ethanol increased when corn was wetted by rain before ensiling [34]. The effect of rainfall at harvest increased proteolysis [35] and effluent production [5], increasing the DM losses. In the current experiment, the LAB inoculant treatments improved the silage fermentation quality in both fresh ryegrass and rain-treated ryegrass by reducing DM losses, NH₃ concentration and silage pH. Filya [36] also found reduced NH₃ concentrations in sorghum and corn ensiled with *L. plantarum* or a mixture of *L. plantarum* and *L. buchneri* after ensiling for 90 days, compared to untreated silage. Oliveira et al. [37] selected among 130 scientific papers on the effect of LAB inoculation on silage quality. The authors also found that inoculation with LAB reduced silage pH, the DM losses and NH₃ concentration. The decrease in NH₃ concentration has been partly explained by growth inhibition of clostridia by LAB inoculation [38]. Moreover, during ensiling, the reduction of pH ≤ 4.0 inhibits proteolysis. Heron et al. [39] found that the optimal pH for proteolysis in ryegrass was ca. 6. The LAB strains inoculated to the FR and the RTR in the current experiment are homofermentative LAB. During the ensiling process, homofermentative bacteria ferment the WSC to lactic acid without CO₂ production, and can therefore reduce DM losses compared to untreated silage [35]. In addition, in this study, the pH of LAB-treated silage varied between 3.8 and 4.3. The pH values below 4.5 can inhibit the growth of clostridia, heterofermentative bacteria, yeasts and molds that contribute to DM losses by producing CO₂ during or after hexose fermentation [35].

4.2. Nutrient Digestibility

Reduction of dry matter digestibility in forage by rain damage has been reported in several previous studies [7,8,40]. During rainfall, the highly digestible components are lost through direct leaching or prolonged respiration by plant enzymes. However, fiber components such as NDF and lignin remain stable, therefore decreasing nutritive value and digestibility. In the current study, the DM and OM digestibility were lower in the rain-treated ryegrass than in the fresh ryegrass, and inoculation with LAB strains of both the FR and the RTR resulted in increased DM and OM digestibility in both grass silages.

Inoculation of LAB strains to forage increased silage digestibility both in vitro and in vivo. The OM digestibility has been reportedly increased when a mixture of *L. plantarum*, *L. buchneri* and *L. lactis* strains were inoculated to ryegrass [41]. In addition, the in vitro digestibility of DM and NDF have been reported to increase after inoculation with *Enterococcus faecium* or *L. buchneri* strains, or a combination of *L. plantarum* and *E. faecium* to whole-plant wheat or corn silage [42]. The total tract digestibility of DM and OM were shown to increase in lambs fed alfalfa silage inoculated with *L. plantarum* [43]. Daniel et al. [44] also found that DM digestibility increased in dairy cows fed corn silage inoculated with a

mixture of *L. lactis*, *L. plantarum* and *E. faecium*. However, other studies have shown variable effects of silage digestibility due to LAB inoculation. Ellis et al. [13] reported similar NDF digestibility when dairy cows were fed rye grass inoculated with *L. plantarum*, *L. buchneri*, *L. lactis* and the control silage. With inoculation of *E. faecium* and *Limosilactobacillus fermentum* to grass, the OM digestibility of grass silage was reduced, however, the OM digestibility was increased when *L. plantarum* was inoculated to grass [45]. The increase in digestibility could be due to the effect of hydrolytic enzymes which are produced by the bacteria. Kim et al. [46] reported that *L. plantarum* released cellulose, xylanase, chitinase and esterase enzymes when *L. plantarum* was used as a silage inoculant.

In the current study, the DM and OM digestibility were not changed when LAB strains were used as DFM. The results of the current study are in agreement with those of Keady and Steen [47], who found no increase in silage digestibility when *L. plantarum* was added immediately to grass silage before feeding to beef cattle. Apparent digestibility of DM was not affected when *L. buchneri* was used as a probiotic [48,49]. Ellis et al. [13] also reported that the NDF digestibility was not changed when *L. lactis* was inoculated to grass 16 h before morning feeding to dairy cows. Total-tract apparent digestibility of DM, OM and NDF was not changed when dairy cows were directly fed *Propionibacterium*, *L. plantarum* and *Lactocaseibacillus rhamnosus* [50].

In the current study, the DM and OM digestibility were greater when LAB were used as inoculants rather than as DFM. This means that the mechanisms of action of LAB differ depending on the way of administration. This finding is in agreement with reports by Robelo et al. [48,49]. Typically, LAB strains are not prevalent in the rumen of animals fed fresh forage. However, the LAB become more dominant in the rumen of animals fed large amounts of sugar or starch [51] because LAB have affinity for these substrates as well as tolerance to the rumen's low pH. Weinberg et al. [52] found that addition of glucose with LAB markedly enhanced the survival of inoculated LAB in rumen fluid. This confirms that the LAB strains can compete effectively with the ruminal microbiota for exogenous glucose. In concentrate-fed rumen, there is plenty of substrate for rapid LAB growth. However, a proportion of the WSC of grass is fermented by microbes during ensiling. Therefore, in the silage-fed rumen, the LAB grows less rapidly than in concentrate-fed rumen, explaining the likely limited substrate for LAB growth in the models used in the present study. Moreover, Robelo et al. [48,49] found that LAB grow more efficiently during fermentation in the silo than in the rumen, which can explain the improvement in nutrient digestibility when LAB was used as an inoculant but not as a DFM.

4.3. Methane Production

Studies show variable effects of LAB on CH₄ production. Cao et al. [14] studied the effect of *L. plantarum* Chikuso-1 on an ensiled total mixed ration (TMR) and showed that methane production was decreased by 8.6% and propionic acid was increased by 4.8% compared with untreated TMR silage. Cao et al. [16] reported that methane production was reduced by 46.6% when inoculant *L. plantarum* Chikuso-1 with vegetable residue silage was used, compared to the control group. The *L. plantarum* Chikuso-1 inoculated in TMR silage decreased ruminal methane emissions (24.7%) in sheep, compared with a control diet [15]. Jalc et al. [45] found significant reductions in CH₄ during inoculation of grass silage with *E. faecium* (CCM 4231), *L. fermentum* or *L. plantarum* (CCM 4000). However, for that same LAB, but as an inoculant with corn, Jalc et al. [53] found no significant changes in CH₄. Ellis et al. [41] found that after 6 h of incubation, CH₄ production was reduced in grass-clover silage inoculated with mixture of *L. buchneri* and *L. lactis*, but CH₄ production was increased in ryegrass inoculated with a mixture of *L. plantarum*, *L. buchneri* and *L. lactis*. However, the authors found no significant changes in CH₄ at 72 h of incubation. In the current study, the CH₄ production was reduced, compared to the control, when both grasses were inoculated with *L. plantarum* (AGR-1), *L. lactis* (AGR-4), *L. lactis* (AGR-5) and *L. plantarum* (AGR-2) treatments. All the above-mentioned results suggest that the methane production is clearly dependent on LAB strains. These findings are in line with the report of Doyle et al. [19]. It is hypothesized that LAB could decrease CH₄ production in ruminant in four possible ways: (1) LAB are able to alter the rumen fermentation leading to a decrease

in CH₄ production, (2) LAB are able to directly inhibit rumen methanogens, (3) LAB are able to inhibit specific rumen bacteria that produce H₂ or methyl-containing compounds, which are the substrates for methanogenesis, and (4) LAB are able to produce bacteriocins which inhibit methanogens, or they affect other rumen microbes that produce substrates necessary for methanogenesis [19]. The results in the current study show that different LAB strains differently affected GP-T2_{1/2} GP-R2max, the proportion of HAc, HBU, HBC and NH₃ concentration. This result is in agreement with the first hypothesis that LAB strains are capable of altering ruminal fermentation, leading to the reduction of enteric CH₄ production.

5. Conclusions

LAB strains inoculated into rain-treated and fresh ryegrass improved silage quality of both silage grasses. Among the 5 LAB strains used in the current study, *L. plantarum* (AGR-2) was the best candidate to increase silage quality. The LAB strains should be used as a silage inoculant to increase feed quality, digestibility and to produce less methane emission.

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