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Selection of Lactic Acid Bacteria from Alfalfa Silage and Its Effects as Inoculant on Silage Fermentation

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Abstract: The first part of the study aimed to isolate, characterize, and identify wild lactic acid bacteria (LAB) strains from alfalfa silage produced in a tropical area. LAB strains were isolated from alfalfa silage ensiled for 1, 3, 7, 14, 28, and 56 days (d) and were identified by sequencing the 16S rRNA gene. The second part aimed to investigate the effects of wild LAB strains on the nutritive and fermentative characteristics of alfalfa silage. This trial was conducted according to a completely randomized design in a 4 × 2 factorial scheme [four inoculants (I) × two harvests (H)], (n = 4). The inoculants were: (1) no inoculant (CTRL), (2) *Lactobacillus pentosus* (AV 14.17); (3) *L. pentosus* + *Lactobacillus brevis* + *Pediococcus acidilactici* (Combo); and (4) commercial inoculant (CI). Alfalfa forage (7 kg) was ensiled in 10 L buckets and opened after 90 d. Seventy-seven strains were isolated. *Pediococcus*, *Lactobacillus*, and *Weissella* represented 52.0, 24.7, and 20.8% of the isolates, respectively. For the first harvest, Combo, CI, and all inoculated silages showed lower acid detergent fiber ADF, neutral detergent fiber (NDF), and ammonia nitrogen (NH_3-N), respectively. Silage fermented with AV14.17 presented greater residual water-soluble carbohydrate (WSC) in the second harvest and showed the lowest pH in both harvests. AV14.17 strain has potential as an inoculant for alfalfa silage production.

Keywords: antimicrobial activity; identification; isolates; legume silage; 16S rRNA

1. Introduction

In the tropical world, ruminant production systems use forage as a basic substrate for meat and milk yield. Thus, animal production follows the seasonality of forage production. In beef animals, for example, there is satisfactory weight gain during the rainy season and difficulty gaining or even maintaining weight during the dry season, increasing production cycles, with a decrease in the quality of the products. In this context, a viable strategy to overcome the effects of this productive gap would be the use of conserved forages.

Among conservation techniques, silage is the most used procedure in various parts of the world. Lactic acid bacteria (LAB) are the main group of microorganisms involved in silage conservation, being divided into homofermentative LAB and facultative and obligate heterofermentative LAB. The homofermentative LAB rapidly decrease pH and increase lactic acid relative to other fermentation products. Meanwhile, the obligate heterofermentative LAB silage additives slowly convert lactic acid to acetic acid and 1,2-propanediol during silo storage, improving aerobic stability [1]. Many species that were considered part of the homofermentative group are now in the facultative heterofermentative group, such as *Lactobacillus plantarum* and *Lactobacillus pentosus* [2].

The presence of some characteristics, such as tolerance to acid environments and high temperatures and also the production of compounds with antimicrobial action against undesirable microorganisms during fermentation, could improve the effects of LAB as microbial additives in tropical areas. The development of inoculants called inoculants of a new generation has sought the synergistic effect of LAB strains to obtain silages of different forages with microbiological safety [2]. This combined effect may occur due to the action of the strains in the different stages of fermentation.

Studies have demonstrated a variability in the effectiveness of inoculation with LAB, which may provide good preservation of silage [3–5], or even an absence of effects on silage fermentation and quality [6,7]. Thus, it suggests the need to find particular strains adapted to different forages under local conditions. Autochthonous strains of LAB have been already isolated from silages of different forage crops such as elephant grass [8], alfalfa [9], and corn [10]. Strains of *L. plantarum* and *Pediococcus acidilactici* isolated from elephant grass silage improved sorghum silage fermentation by decreasing silage pH, butyric acid, and yeast count [8]. However, only few research investigations have been conducted under tropical conditions [11,12].

Alfalfa (*Medicago sativa*) is a worldwide relevant crop because of its adaptability to several climate and soil conditions and also to the availability of large number of hybrids of high nutritional value [13,14]. However, the cv. "Crioula", which is the result of a selection process of varieties from Uruguay and Argentina, is the main cultivar of this species in Brazil [15]. As with other legumes, alfalfa has some limitations on silage production, such as low dry matter (DM) and water-soluble carbohydrate contents (WSC) [16] and high buffering capacity [17]. Because of these peculiarities, the addition of microbial inoculants is an option to improve the fermentation process while increasing the dry matter recovery.

Several studies are available on the use of LAB as inoculant on alfalfa silage production [18–20]. However, in Brazil, despite the lack of alternative silages, little research has been carried out using alfalfa. Thus, further studies are required involving this forage and LAB inoculants in tropical conditions. Selecting LAB strains in tropical climate areas could improve silage quality, since these strains probably are adapted to this condition and could dominate the epiphytic microflora during fermentation. We hypothesized that using LAB strains isolated from alfalfa silage enhances its fermentation under tropical conditions. Thus, this study aimed to isolate, characterize, and identify LAB species in alfalfa silage and to verify their effects on the fermentation profile and chemical composition of alfalfa silage.

2. Materials and Methods

2.1. Experimental Area

The first part of the study, which includes the isolation and characterization of LAB from alfalfa silage, was conducted between May and July 2013. The second part, in which the isolated strains were used as inoculant, was conducted between January and May 2016. The trials were performed at the Department of Animal Science of the Federal University of Vicosa ("Universidade Federal de Viçosa, UFV"), Viçosa (20°45′ S, 42°51′ W; precipitation of 1341 mm), Minas Gerais, Brazil, using the same alfalfa field. All procedures of silage making, similar in both parts of the study, were as follow: The alfalfa cv. "Crioula" was grown in a 1000 m^2 area at the Forages Crops Sector of the Animal Science Department and harvested at the early flowering stage, using a backpack mower (model FR 220, Stihl, São Leopoldo, RS, Brazil). After that, alfalfa forage was wilted for 6 h in the field and chopped into 1.5 cm size using a forage chopper (model PN Plus 2000, Nogueira S.A., São João da Boa Vista, Brazil), prior to ensiling.

2.2. Part 1—Isolation, Characterization and Identification of LAB from Alfalfa Forage and Its Silage

2.2.1. Ensiling and LAB Isolation

A total of 500 g of chopped and wilted alfalfa was packed into 25 cm × 35 cm bags (Doug Care Equipment, Springville, CA, USA), where the air was removed using a vacuum sealer (Eco vacuum 1040,

Orved, Italy). Triplicate bags were made for each period of fermentation. The bags were opened after 1, 3, 7, 14, 28, and 56 days of fermentation. Samples (25 g) of fresh alfalfa and its silages from all fermentation periods were homogenized in 225 mL of Ring's solution (Oxoid, Hampshire, England). Serial dilutions were prepared from this aqueous extract and inoculated into Petri dishes containing De Man Rogosa and Sharpe (MRS) agar (Difco, Sao Paulo, Brazil), which were incubated at 37 °C for 48 h. Petri dishes that presented colonies' growth between 25 and 300 CFU were used to isolate LAB strains. The number of grown colonies that were selected corresponded to the square root of the total present on the plate, and they were picked at random [21] for characterization and identification. After this stage, to obtain pure isolates and to verify their acid production, the selected colonies were streaked on plates containing MRS agar with bromocresol purple (0.04 g/L) and $CaCO_3$ (5 g/L) as indicators. The plates were incubated at 37 °C for 48 h in anaerobic jars (Permution®, Curitiba. PR, Brazil). Isolated colonies that presented a yellowish and clear zone, caused by the dissolution of $CaCO_3$, were stored at −80 °C for further physiological tests, antimicrobial activity, and genotypic identification.

2.2.2. Physiological Tests

The screening of LAB was performed based on the results of Gram staining, catalase activity, and acid production. Growth in MRS broth (Beton, Dickinson and Company, Sparks, NV, USA) was evaluated at different temperatures (15 °C and 45 °C) and pH levels (3.5, 4.0, 4.5 and 8.5) [22] after 24 h of incubation, and it was measured by the absorbance at 630 nm using a plate spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA). The growth at pH levels tested was done at 37 °C.

The metabolism classification of the isolates in heterofermentative or homofermentative was carried out by inoculating them in MRS broth with Durham tubes, which were incubated for 48 h at 37 °C. The presence of bubbles inside the tubes indicated heterofermentative metabolism while the absence of them indicated homofermentative metabolism.

2.2.3. Determination of Antimicrobial Activity

The antimicrobial activity of each isolate against the indicator bacteria *Listeria monocytogenes* ATCC 7644, *Escherichia coli* K12, *Bacillus cereus* ATCC 4904, and *Staphylococcus aureus* ATCC 25,923 was assessed by means of the deferred activity method [23]. Antimicrobial activity was defined by the formation of inhibition zones around the microdrops. The inhibition zone was measured with a ruler and calculated by subtracting the colony diameter from the external diameter of the inhibition area. This measurement was performed in duplicate, and the mean value was considered.

2.2.4. Extraction of LAB Genomic DNA

The DNA samples from the isolates obtained from fresh alfalfa and its silages at the various fermentation periods were extracted by using a commercial kit (Wizard® Genomic DNA Purification kit, Promega, Madison, WI, USA), with some modifications in the protocol steps. The isolates were grown in 5 mL of MRS broth and incubated at 37 °C for 14 h. The cells were then centrifuged (Mikro 200 R, Hettich) at 1000× *g* for 5 min and washed once with 0.85% saline solution. The cell sediment was resuspended in 480 µL of EDTA (50 mM), and 50 µL of lysozyme 50 mg mL was immediately added. The subsequent DNA extraction steps were performed according to the kit manufacturer's instructions. The concentration of extracted DNA was assessed with a Nanodrop spectrophotometer (Thermo Scientific 2000, Thermo Fisher Scientific, Wilmington, NC, USA), and the DNA was stored at −80 °C.

2.2.5. Species Identification by 16S rRNA Gene Sequencing

The 16S rRNA gene coding region was amplified using the polymerase chain reaction (PCR) with 2 µL of diluted DNA as template and the following primer pair: p027F (GAGAGTTTGATCCTGGCTCAG) and 1492R (TACGG(C/T)TACCTTGTTACGACTT) [24]. The PCR

was performed in 0.200 μL microcentrifuge tubes containing 50 μL of the reaction mixture: DNA (approximately 60 mg), 10X buffer (Tris-HCl 0.1 mol L, pH 8.0, KCl 0.5 mol L); MgCl2 1.5 mmol L, pH 8.0); dNTP mix (Promega, Madison WI USA); Taq polymerase (Promega, Madison, USA) (1 U); primers p027f (0.6 μmol L) and 1492 (0.6 μmol L). Autoclaved milli-Q water was added to attain a final reaction volume of 50 μL. The PCR was performed in a thermal cycler (Eppendorff®) under the following conditions: 94 °C/5 min; 30 cycles (denaturation: 94 °C/30 s; 60 °C/30 s); polymerization: 72 °C/2 min; final extension: 72 °C/5 min. A 4 μL aliquot of the PCR mixture was analyzed by electrophoresis on a 1.4% agarose gel in Tris-Borate-EDTA (TBE) buffer. The gel was stained with ethidium bromide 0.5 μg mL and the bands were visualized under ultraviolet light. The PCR product was sent to Macrogen (South Korea) for purification and sequencing.

The sequences were compared to references deposited in the GenBank database and aligned using the algorithm BLASTn (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST) for nucleotides. The sequences of 16S rRNA genes that exhibited ≥97% of similarity were considered to be part of the same operational taxonomic unit (OTU) [25]. Moreover, the sequences of the isolates obtained in our study were deposited in the GenBank database. The isolates identification, access code, growth characteristics, and antimicrobial activity are available in Supplementary Materials Tables S1 and S2.

2.2.6. Selection of LAB Strains for Alfalfa Silage Production

Three strains of LAB were selected for evaluation as inoculant in alfalfa silage production. *L. pentosus* AV14.17 and *Lactobacillus brevis* AV14.2 were isolated from alfalfa silage on day 14 of fermentation. *P. acidilactici* AV56.13 was isolated from silage on day 56 of fermentation (Table S1). These strains were chosen based on their performance under the diverse conditions tested and the broad spectrum of inhibition against pathogenic microorganisms. It was not our goal to choose between homofermentative and heterofermentative strains. We were looking for strains that could control undesirable microorganisms and thus favor the growth of LAB during the fermentation process. In addition, it is necessary that these strains survive in various conditions, so they could act as soon as possible in the fermentation process.

2.3. Part 2—Fermentation of Alfalfa Silage Inoculated with Wild LAB Strains

2.3.1. Experimental Design and Silage Preparation

The trial was conducted according to a completely randomized design in a 4 × 2 factorial scheme [4 inoculants (I) × 2 harvests (H)] with four replicates. Two harvests were made in January (harvest 1) and March (harvest 2) 2016. The inoculants evaluated were: (1) Control, no inoculant (CTRL), (2) *L. pentosus*, strain AV14.17 (AV14.17), (3) *L. pentosus* (AV14.17) + *L. brevis* (AV 14.2) + *P. acidilactici* (AV 56.13) (COMBO), and (4) Commercial inoculant (CI).

The commercial inoculant Sil All 4 × 4 W.S (Alltech, Sao Paulo, Brazil), which contained sucrose, *L. plantarum*, *P. acidilactici*, *Enterococcus faecium*, *Lactobacillus salivarus* ssp. *salivarus*, silicon dioxide, amylase, cellulose, hemicellulose, and xylanase in its composition, was rehydrated in 50 mL of distilled water and applied in an application rate of 1.0×10^6 CFU/g of fresh forage.

Alfalfa forage was harvested and wilted for six hours in the field reaching 331.4 and 435.2 g/kg of DM for harvest 1 and 2, respectively. The alfalfa forage characteristics before ensiling are shown in Table 1. Piles of 10 kg of forage were individually treated (each replicate) with the microbial inoculants and then approximately 7 kg of forage were packed in 10 L plastic buckets (mini-silo, 25 cm diameter and 25 cm height) sealed with tight lids. The buckets (16 buckets from each harvest) were stored in the laboratory at room temperature and opened after 90 days. Dry matter recovery was estimated by the weight and DM content of the fresh alfalfa and its silage [26].

Table 1. Chemical composition (g/kg of DM) and microbial population of alfalfa forage before ensiling.

Item	Harvest [1]	
	1	2
Dry matter, g/kg	331.4	435.2
Crude protein	146.7	149.5
Neutral detergent fiber	532.3	458.6
Acid detergent fiber	348.1	355.6
Water-soluble carbohydrates	17.3	34
pH	6.12	6.24
Microbial population, log CFU/g of fresh matter		
Lactic acid bacteria	6.72	6.14
Enterobacteria	7.15	6.42
Mold	4.58	4.90
Yeast	5.21	5.03

[1] Harvest 1: Alfalfa harvest in January 2016; Harvest 2: Alfalfa harvest in March 2016.

2.3.2. Preparation of the Isolated Strains for Use as Inoculant

L. pentosus AV14.17, *L. brevis* AV14.2, and *P. acidilactici* AV56.13 were cultivated in MRS broth for 48 h at 37 °C for cell activation, since they were kept at −80 °C before their use. After that, each inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, in 20 mL of MRS broth and kept at 37 °C for 16 h. This incubation time was used based on a pre-test, where the average time to obtain the microorganisms in their log phases was 16 h. The inoculum obtained in this last incubation was used to prepare the inoculants. The amount of this inoculum to achieve the final application rate of 1.0×10^6 CFU/g of fresh forage was centrifuged at $1000\times g \times 10$ min. The supernatant was discarded, and cells were resuspended in 50 mL of distilled water.

2.3.3. Chemical Analysis

Forage and silages samples were analyzed for chemical composition. The samples were collected and dried at 55 °C for 72 h in a forced-air oven and ground to pass a 1 mm knife mill screen. Dried ground samples were used to quantify DM content (method 934.01), crude protein (CP) (method 984.13), acid detergent fiber (ADF) (method 973.18), and mineral matter (method 942.05) [27]. Neutral detergent fiber (NDF) was determined using heat-stable α-amylase without the use of sodium sulfite and was corrected for residual ash [28] and nitrogen [29].

An aqueous extract was prepared from 25 g of sample (forage or silage) and 225 mL of sterile Ring's solution (Oxoid, Hampshire, England) homogenized for 1 min. The aqueous extract was used to measure the pH using a potentiometer (Tecnal, SP, Brazil) and then it was acidified with 1:1 H_2SO_4 diluted with distilled water for further analyzes of ammonia nitrogen (NH_3-N), using the phenol-hypochlorite method [30], WSC [31], and organic acids. Samples for organic acids quantification were treated with calcium hydroxide and cupric sulfate and analyzed by high-performance liquid chromatography (HPLC; SPD-10 AVP, Shimadzu, OR, USA) [32]. The HPLC apparatus (SPD-10 AVP, Shimadzu) was equipped with a refractive index detector and an Aminex HPX-87H column (BIO-RAD, CA, USA) with the mobile phase containing 0.005 M H_2SO_4 and a flow rate of 0.6 mL/min at 50 °C.

2.3.4. Quantification of Microbial Populations

A portion of the water extract was used to determine the microbial population. Serial dilutions were made and inoculated on MRS agar to determine the LAB count. Violet Red Bile agar (Difco) was used to quantify the enterobacteria population and Potato dextrose agar (Difco) [acidified with 1.5% of tartaric acid solution 10% wt/v] for yeasts and molds determination. Plates of LAB and enterobacteria were kept at 37 °C for 48 and 24 h, respectively. Yeast and mold plates were incubated at 25 °C for 120 h.

2.4. Statistical Analysis

Data from part 2 were analyzed as a completely randomized design, in a 4 × 2 factorial scheme. The model included the fixed effects of inoculants, harvests, and the interaction I × H, according to the following statistical model:

$$Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk, \quad (1)$$

where Yijk = dependent variable, μ = overall mean, αi = fixed effect of the ith inoculant, βj = fixed effect of the jth harvest, (αβ)ij = interaction between inoculant and harvest, and εijk = random error.

Analyses of variance and multiple comparisons of means were performed using the GLM procedure of SAS (SAS Intitute Inc., Cary, NC, USA). The means were separated by Tukey's test, and differences and tendencies were declared at the levels of $p \leq 0.05$ and $p > 0.05$ to $p < 0.10$, respectively.

3. Results

3.1. Screening and Identification of LAB from Alfalfa Forage and Its Silage (Part 1)

One hundred and two strains were isolated from alfalfa forage and its silage. Among these, 77 strains (three from plant and 74 from silage) showed similarity above 97% with sequences of others LAB available in the GenBank database. The genera *Pediococcus*, *Lactobacillus*, and *Weissella* represented 52.0, 24.7, and 20.8% of the isolates, respectively (Figure 1). *P. acidilactici* was the most frequent isolated species from alfalfa through the fermentation period, which corresponded to 28.6% of the isolates, followed by the *L. plantarum* (16.9%) (Figure 2). These genera and species represented the dominant microorganisms able to grow in MRS medium. In general, the species with the highest numbers of strains isolated from silage were *Weissella cibaria*, *Pediococcus pentosaceus*, *P. acidilactici*, and *L. plantarum* (Figure 2).

Enterococcus casseliflavus, *Weissella confusa*, *W. cibaria*, *P. pentosaceus*, *L. pentosus*, *L. brevis*, and *Lactobacillus casei* species were also identified (Table S2). All isolates were able to inhibit the growth of at least one indicator microorganism. Among the 77 isolates, 36 were able to grow under the tested conditions (Table S1).

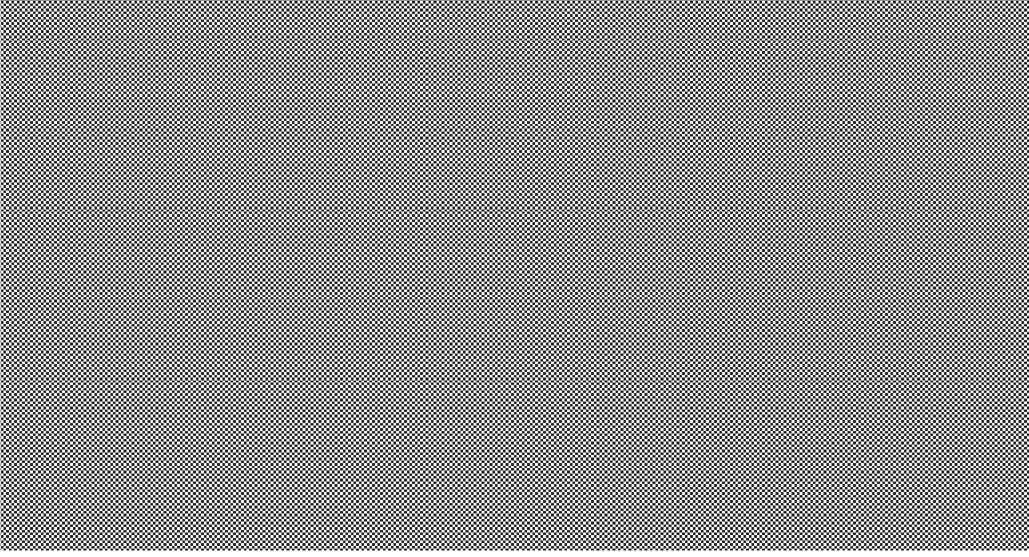

Figure 1. Frequency of lactic acid bacteria genera isolated from alfalfa silage through the fermentation period (56 days).

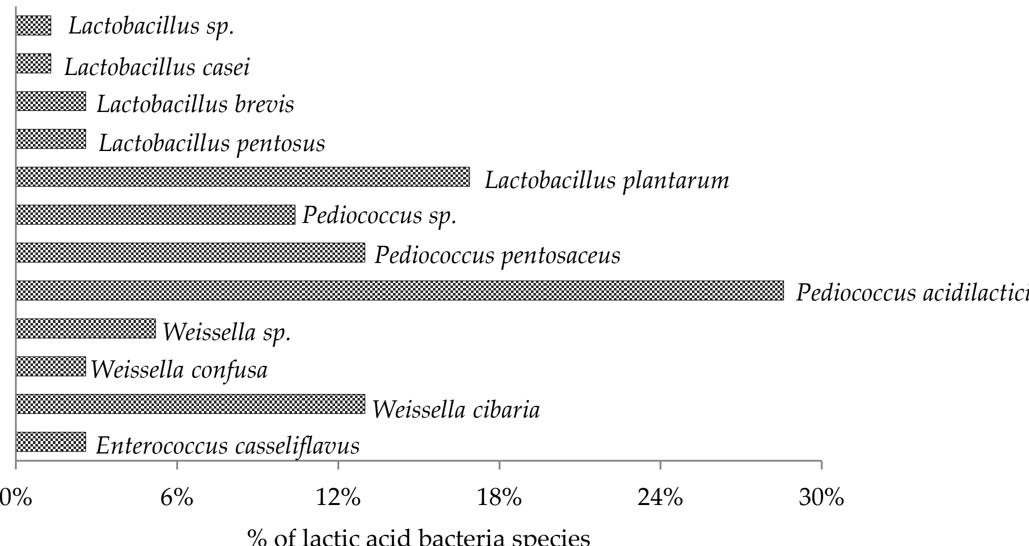

Figure 2. Frequency of lactic acid species isolated from alfalfa silage through the fermentation period (56 days).

The isolated strains, *L. pentosus* AV14.17 (GenBank access code—MK713801), *L. brevis* AV14.2 (GenBank access code—MK713790), and *P. acidilactici* AV56.13 (GenBank access code—KY613548) were selected from part 1 of the study to be used in part 2, as they showed antimicrobial activity against all indicator microorganisms, and good growth under tested conditions. The characteristics of the strains are presented in Table 2.

Table 2. Phenotypic characteristics and antimicrobial activity of lactic acid bacteria strains isolated from alfalfa silage.

Item	Strain		
	AV14.2	**AV14.17**	**AV56.13**
Species	*Lactobacillus brevis*	*Lactobacillus pentosus*	*Pediococcus acidilactici*
Access code [1]	MK713790	MK713801	KY613548
Growth at Ph [2]			
3.5	++	+++	++
4	+++	+++	+++
4.5	+++	+++	+++
8.5	+++	+++	+++
Growth at temperature (°C) [2]			
15	++	++	+
45	+++	++	+++
Growth in NaCl (g/L) [2]			
40	+++	+++	+++
65	+++	++	+++
		Radius of inhibition [3]	
Indicator microorganism			
Listeria monocytogenes	++	+++	++
Escherichia coli	+	+++	++
Staphylococcus aureus	+	+	+
Bacillus cereus	++	+++	+++

[1] GenBank database. [2] Growth measured by optical density (630 nm): <0.5 = +; >0.5 to <0.9 = ++; >0.9 = +++.
[3] Radius of inhibition (mm): − (absence of inhibition halo), + (>4 and ≤15), ++ (>15 and ≤30), +++ (>30).

3.2. Chemical Composition (Part 2)

There was an effect of I × H interaction on NDF ($p = 0.03$) and ADF ($p = 0.02$) contents. From harvest 1, silage treated with CI and COMBO showed lower ($p < 0.01$) NDF and ADF, respectively, compared to CTRL and AV14.17. Dry matter content was affected by both inoculant ($p < 0.01$) and harvest ($p < 0.01$). Alfalfa silage treated with microbial inoculants had greater DM compared with CTRL silage. Similarly, silage from harvest 2 presented greater DM content than harvest 1. Crude protein was only affected by harvest, which was greater ($p < 0.01$) for silages from harvest 2 (Table 3).

Table 3. Chemical composition of alfalfa silage treated with microbial inoculants at two harvests.

Harvest [1]	Inoculant [2]				Average	SEM [3]	*p*-Value [4]		
	Control	AV14.17	Combo	CI			I	H	I×H
	Dry matter, g/kg of Fresh Matter								
1	325	329	328	331	328 B	0.84	<0.01	<0.01	0.41
2	417	423	423	425	422 A				
Average	371 b	376 a	376 a	378 a					
	Crude protein, g/kg of DM								
1	145	146	149	146	146 B	0.07	0.16	<0.01	0.92
2	149	151	153	149	150 A				
Average	147	149	151	147					
	Neutral detergent fiber, g/kg of DM								
1	519 Aa	516 Aa	490 Aab	473 Ab	499	0.87	<0.01	<0.01	0.03
2	420 Ba	405 Ba	409 Ba	408 Ba	410				
Average	470	460	449	440					
	Acid detergent fiber, g/kg of DM								
1	422 Aa	422 Aa	369 Ab	407 Aab	405	0.65	0.01	<0.01	0.02
2	343 Ba	356 Ba	347 Aa	343 Ba	347				
Average	383	389	358	375					
	ADIN [5], g/kg of DM								
1	195	149	154	210	177	0.70	0.67	0.70	0.09
2	168	174	189	152	171				
Average	182	162	172	181					

[1] Harvest: 1 = Alfalfa harvest in January 2016, 2 = Alfalfa harvest in March 2016. [2] Inoculant: Control = No inoculant, AV14.17 = *Lactobacillus pentosus* (AV14.17), Combo = *Lactobacillus pentosus* (AV14.17) + *Lactobacillus brevis* (AV 14.2) + *Pediococcus acidilactici* (AV 56.13), CI = Commercial inoculant. [3] SEM: Standard error of mean. [4] Probability of inoculant (I), harvest (H), and interaction I with H (I × H) effects. [5] Acid detergent Insoluble Nitrogen. Means followed by same lowercase letters in the row and uppercase letters in the columns are not different according to Tukey's test ($p > 0.10$).

3.3. Fermentation Characteristics and Microbial Population (Part 2)

There was an effect of I × H interaction ($p = 0.04$) on residual WSC content and dry matter recovery ($p = 0.02$). A tendency ($p = 0.06$) of I × H interaction on NH_3-N concentration was observed. There was no difference ($p > 0.99$) among inoculants on WSC content of silages from harvest 1. However, silage treated with AV14.17 strain showed greater ($p < 0.02$) WSC content compared to CTRL and CI, in harvest 2. Although interaction effect was found for dry matter recovery, there was no difference ($p > 0.66$) among inoculants in both harvests. Ammonia nitrogen concentration was lower ($p < 0.05$) for silages treated with inoculants compared with the CTRL silage in harvest 1. There was no difference among inoculants ($p > 0.97$). The pH was affected by inoculant ($p = 0.04$) and harvest ($p < 0.01$). The silage treated with AV14.17 strain showed lower pH. Moreover, pH was lower for silages from harvest 2 compared with harvest 1. Butyric acid was not detected in the silages. Lactic and acetic acids were affected only by harvest. Silage from harvest 2 showed greater lactic acid ($p < 0.01$) and lower ($p < 0.01$) acetic acid than harvest 1 (Table 4).

Table 4. Fermentation profile of alfalfa silage treated with microbial inoculants at two harvests.

Harvest [1]	Inoculant [2]				Average	SEM [3]	*p*-Value [4]		
	Control	AV14.17	Combo	CI			I	H	I×H
	pH								
1	4.56	4.51	4.55	4.59	4.55 A	0.02	0.04	<0.01	0.73
2	4.36	4.31	4.35	4.35	4.34 B				
Average	4.46 a	4.41 b	4.45 ab	4.47 a					
	Ammonia nitrogen, g/kg of total nitrogen								
1	142 Aa	113 Ab	105 Ab	104 Ab	116	0.58	0.01	<0.01	0.06
2	64.0 Ba	56.0 Ba	55.2 Ba	64.2 Ba	59.9				
Average	103	84.3	79.9	83.9					
	Lactic acid, g/kg of DM								
1	12.5	15.0	15.1	17.6	15.1 B	0.10	0.73	<0.01	0.55
2	23.6	21.2	24.4	22.6	23.0 A				
Average	18.1	18.1	19.8	20.1					
	Acetic acid, g/kg of DM								
1	26.6	26.9	17.8	28.4	24.9 A	0.19	0.33	<0.01	0.37
2	10.2	6.10	8.25	9.2	8.44 B				
Average	18.4	16.5	13.0	18.1					
	Water-soluble carbohydrate, g/kg of DM								
1	2.68 Ba	2.76 Ba	2.53 Ba	2.53 Ba	2.63	0.04	0.02	<0.01	0.04
2	6.32 Ab	8.45 Aa	7.39 Aab	6.10 Ab	7.07				
Average	4.50	5.61	4.96	4.32					
	Dry matter recovery, g/kg of DM								
1	974 Aa	982 Aa	976 Aa	960 Aa	973	0.23	0.12	0.01	0.02
2	950 Ba	966 Aa	966 Aa	970 Aa	963				
Average	962	974	971	965					

[1] Harvest: 1 = Alfalfa harvest in January 2016, 2 = Alfalfa harvest in March 2016. [2] Inoculant: Control = No inoculant, AV14.17 = Lactobacillus pentosus (AV14.17), Combo = Lactobacillus pentosus (AV14.17) + Lactobacillus brevis (AV 14.2) + Pediococcus acidilactici (AV 56.13), CI = Commercial inoculant. [3] SEM: Standard error of mean. [4] Probability of inoculant (I), harvest (H), and interaction I with H (I × H) effects. Means followed by same lowercase letters in the row and uppercase letters in the columns are not different according to Tukey's test ($p > 0.10$).

Enterobacteria were not detected in silages. There was a tendency of I × H interaction ($p = 0.07$) on yeast count. However, the interaction study showed no differences ($p > 0.10$) among treatments. There was a harvest effect on LAB and mold counts. Lactic acid bacteria were in greater number ($p < 0.01$) and molds in lower ($p < 0.01$) for silage from harvest 1 as compared to harvest 2 (Table 5).

Table 5. Microbial population (log CFU/g of fresh matter) of alfalfa silage treated with microbial inoculants at two harvests.

Harvest [1]	Inoculant [2]				Average	SEM [3]	*p*-Value [4]		
	Control	AV14.17	Combo	CI			I	H	I×H
	Lactic Acid Bacteria								
1	8.26	8.35	8.35	8.28	8.31 A	0.11	0.54	<0.01	0.44
2	7.30	6.90	7.19	6.89	7.07 B				
Average	7.78	7.62	7.77	7.58					
	Mold								
1	2.33	2.19	2.07	1.72	2.07 B	0.22	0.70	<0.01	0.56
2	3.36	2.76	3.33	3.29	2.63 A				
Average	2.84	2.47	2.70	2.51					

Table 5. *Cont.*

Harvest [1]	Inoculant [2]				Average	SEM [3]	p-Value [4]		
	Control	AV14.17	Combo	CI			I	H	I×H
			Yeast						
1	2.34	2.21	2.26	2.45	2.32	0.19	0.12	0.02	0.07
2	2.48	2.20	1.05	0.94	1.67				
Average	2.41	2.20	1.65	1.70					

[1] Harvest: 1 = Alfalfa harvest in January 2016, 2 = Alfalfa harvest in March 2016. [2] Inoculant: Control = No inoculant, AV14.17 = *Lactobacillus pentosus* (AV14.17), Combo = *Lactobacillus pentosus* (AV14.17) + *Lactobacillus brevis* (AV 14.2) + *Pediococcus acidilactici* (AV 56.13), CI = Commercial inoculant. [3] SEM: Standard error of mean. [4] Probability of inoculant (I), harvest (H), and interaction I with H (I × H) effects. Means followed by uppercase letters in the columns are not different according to Tukey's test ($p > 0.10$).

4. Discussion

Isolation of LAB from fresh crop or silage to be used as microbial inoculant can improve silage quality [33] since epiphytic LAB are not always present in sufficient number to ensure efficient fermentation in the silo [34]. The LAB strains identified in the present study by 16S rRNA gene sequencing belonging to *W. cibaria*, *L. plantarum*, *L. pentosus*, *L. brevis*, *L. casei*, *P. pentosaceus*, and *P. acidilactici* were also found in other studies [8,35–37].

Species of the genus *Pediococcus* are known to be able to grow quickly, and as a result, they represent the largest portion of LAB when they are found in association with bacteria from the genera *Lactobacillus*, *Leuconostoc*, and *Weissella* [38]. This statement is in agreement with our findings, where *P. acidilactici* was detected in larger numbers, followed by *L. plantarum*. Several studies have reported the presence of *L. plantarum* as the main species in silage [11,34], demonstrating the importance of this species for silage preservation.

Selecting strains able to grow at high temperature and low pH and with antimicrobial activity may be essential to the effectiveness of microbial inoculants in tropical conditions. A strain identified as *L. casei* isolated from tropical forage was able to grow at low pH and was able to improve silage quality [39]. Similar results were reported for *P. pentosaceus* isolated from alfalfa silage produced under tropical conditions, which showed broad-spectrum antimicrobial activity against pathogenic and harmful spoilage organisms and the ability to grow in different pH and temperature ranges [11]. In this study, alfalfa silage treated with this specie showed greater lactic acid content and lower NH_3-N on day 28 of fermentation compared to the commercial inoculant and the lowest pH value from 14 d of fermentation. Silva et al. [40] also reported improvement in alfalfa silage fermentation when silage was prepared using *P. pentosaceus* isolated from *Stylosanthes* silage with antimicrobial activity of protein nature. Strains isolated in the present study were able to inhibit the indicator microorganisms. However, their antimicrobial activity has not been tested or characterized. Further investigation into the nature of the substance involved in inhibiting these spoilage microorganisms is required.

In the present study, the variation in characteristics of alfalfa forage between harvests resulted differences in the effect of the isolated strains associated with silage fermentation. Dry matter and WSC are the major factors affecting the fermentation process in the silo [41]. It is recognized that silage pH increases as forage DM content increases (above 40–45%) [41] because LAB metabolism is affected by the low water activity [42]. However, silages from harvest 1, which showed lower DM content compared to harvest 2, presented silages with greater pH and lower lactic acid concentration. Despite the difference on DM content and silage characteristics of the two harvests, the strain AV14.17 was effective in reducing the pH of the silage compared to that of the control silage. This result is in line with those found by Jones where inoculated silages showed higher rates of pH decline at all dry matter levels studied (330, 430, and 540 g/kg) [43]. Although the same wilting time was used for silage making in both harvests, DM was affected by weather variables, such as solar brightness (0.3 vs. 9 h; harvest 1 and 2, respectively), evaporative power (1.5 vs. 2.4 mL; harvest 1 and 2, respectively) and precipitation (2.6 vs. 0 mL; harvest 1 and 2, respectively) [44]. Furthermore, harvest alters carbohydrate metabolism

and thus stimulates the mobilization of monosaccharides in relation to sugar polymers and the hydrolysis of starch reserves in soluble sugars [45], which may explain the greater concentration of WSC in harvest 2.

As already highlighted, DM and WSC are primary in guaranteeing adequate fermentation in the silo. Although silage fermented with AV14.17 presented similar NH_3-N content compared to the COMBO and CI, these two did not differ from the control silage in some important aspects (pH, residual WSC). Addition of *L. plantarum* on silage production of several crops has shown improvement in silage fermentation [46–48]. However, few studies reported the use of *L. pentosus* on silage fermentation. It is important to emphasize, since *L. plantarum* is one of the most used species as silage inoculant, the similarity between *L plantarum* and *L pentosus*, which are genotypically related species and their sequences of the 16S rRNA gene differ only by 2 bp. Therefore, this species could also be a promising inoculant of silage.

The chemical composition of the silages was practically unaffected by inoculant. Differences in DM content were biologically insignificant. However, silage treated with COMBO showed lower ADF in harvest 1. Low ADF is desirable, as it is a fraction of the fiber that can compromise silage quality by affecting digestibility. Some meta-analysis studies showed the effect of inoculation with homofermentative and heterofermentative LAB strains on ADF and lignin silage decrease without affecting NDF and CP concentrations [5,49]. This behavior could be associated with the acidic hydrolysis of hemicellulose when organic acids are produced in higher concentration [50]. However, lactic and acetic acids were not altered by the inoculants in our study, and another unknown effect may be involved.

5. Conclusions

In conclusion, *Weissela cibaria*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Lactobacillus plantarum* were the predominant species in alfalfa silage produced in a tropical region. *Pediococcus acidilactici* dominated the microbial community throughout the fermentation period in alfalfa silage. The strain of *Lactobacillus pentosus* AV14.17 has potential to be used as inoculant on alfalfa to improve silage fermentation. However, this strain must be tested on other forage legumes and other promising strains found in the present study should also be investigated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/11/518/s1, Table S1: Characterization, identification and antimicrobial activity of lactic acid bacteria strains isolated from alfalfa forage and silages at different fermentation periods (1, 3, 7, 14, 28, and 56 days), Table S2: Identification and access code in GenBank of strains isolated from alfalfa forage end silages in different fermentation periods (1, 3, 7, 14, 28, and 56 days).

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References

1. Muck, R.R.; Nadeau, T.A.; McAllister, T.A.; Contreras-Govea, F.E.; Santos, M.C.; Kung, L. Silage review: Recent advances and future uses of silage additives. *J. Dairy Sci.* **2018**, *101*, 3980–4000. [CrossRef] [PubMed]

2. Fabiszewska, A.U.; Zielińska, K.J.; Wróbel, B. Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: A minireview. *World J. Microbiol. Biotechnol.* **2019**, *35*, 76. [CrossRef] [PubMed]
3. Li, P.; Zhang, Y.; Gou, W.; Cheng, Q.; Bai, S.; Cai, Y. Silage fermentation and bacterial community of bur clover, annual ryegrass and their mixtures prepared with microbial inoculant and chemical additive. *Anim. Feed Sci. Technol.* **2019**, *247*, 285–293. [CrossRef]
4. Yan, Y.; Li, X.; Guan, H.; Huang, L.; Ma, X.; Peng, Y.; Li, Z.; Nie, G.; Zhou, J.; Yang, W.; et al. Microbial community and fermentation characteristic of Italian ryegrass silage prepared with corn stover and lactic acid bacteria. *Bioresour. Technol.* **2019**, *279*, 166–173. [CrossRef] [PubMed]
5. Oliveira, A.S.; Weinberg, Z.G.; Ogunade, I.M.; Cervantes, A.A.P.; Arriola, K.G.; Jiang, Y.; Kim, D.; Li, X.; Gonçalves, M.C.M.; Vyas, D.; et al. Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *J. Dairy Sci.* **2017**, *100*, 4587–4603. [CrossRef]
6. Khota, W.; Pholsen, S.; Higgs, D.; Cai, Y. Natural lactic acid bacteria population of tropical grasses and their fermentation factor analysis of silage prepared with cellulase and inoculant. *J. Dairy Sci.* **2016**, *99*, 9768–9781. [CrossRef]
7. Chen, M.M.; Liu, Q.H.; Xin, G.R.; Zhang, J.G. Characteristics of lactic acid bacteria isolates and their inoculating effects on the silage fermentation at high temperature. *Lett. Appl. Microbiol.* **2013**, *56*, 71–78. [CrossRef]
8. Sifeeldein, A.; Wang, S.; Li, J.; Dong, Z.; Chen, L.; Kaka, N.A.; Shao, T. Phylogenetic identification of lactic acid bacteria isolates and their effects on the fermentation quality of sweet sorghum (Sorghum bicolor) silage. *J. Appl. Microbiol.* **2019**, *126*, 718–729. [CrossRef]
9. Zhang, Q.; Yu, Z.; Wang, X. Isolating and evaluating lactic acid bacteria strains with or without sucrose for effectiveness of silage fermentation. *Grassl. Sci.* **2015**, *61*, 167–176. [CrossRef]
10. Pang, H.; Qin, G.; Tan, Z.; Li, Z.; Wang, Y.; Cai, Y. Natural populations of lactic acid bacteria associated with silage fermentation as determined by phenotype, 16S ribosomal RNA and recA gene analysis. *Syst. Appl. Microbiol.* **2011**, *34*, 235–241. [CrossRef]
11. Agarussi, M.C.N.; Pereira, O.G.; Silva, V.P.; Leandro, E.S.; Ribeiro, K.G.; Santos, S.A. Fermentative profile and lactic acid bacterial dynamics in non- wilted and wilted alfalfa silage in tropical conditions. *Mol. Biol. Rep.* **2019**, *46*, 451–460. [CrossRef] [PubMed]
12. Leandro, E.S.; Oliveira, M.N.V.; Rufino, L.D.; Ribeiro, K.G.; Pereira, O.G. Characterization of Arachis pintoi and its silages at different fermentation periods. *Mol. Biol. Rep.* **2019**, *46*, 5019–5024. [CrossRef] [PubMed]
13. Grev, A.M.; Wells, M.C.; Samac, D.A.; Martinson, K.L.; Sheaffer, C.C. Forage accumulation and nutritive value of reduced lignin and reference alfalfa cultivars. *Agron. J.* **2017**, *109*, 2749–2761. [CrossRef]
14. Zhang, T.; Kang, J.; Guo, W.; Zhao, Z.; Xu, Y.; Yan, X.; Yang, Q. Yield evaluation of twenty-eight alfalfa cultivars in Hebei Province of China. *J. Integr. Agric.* **2014**, *13*, 2260–2267. [CrossRef]
15. Kopp, M.M.; Pereira, A.V.; Ferreira, R.P. Alfalfa cultivars in Brazil ("Cultivares de alfafa no Brasil"). In *Genetic Improvement of Alfalfa ("Melhoramento Genético Da Alfafa")*; Ferreira, R.P., Basigalup, D.H., Gieco, J.O., Eds.; Embrapa Pecuaária Sudeste: São Carlos, Brazil, 2011; pp. 309–331.
16. Dewhurst, R.J.; Fisher, W.J.; Tweed, J.K.S.; Wilkins, R.J. Comparison of grass and legume silages for milk production. Production responses with different levels of concentrate. *J. Dairy Sci.* **2003**, *86*, 2598–2611. [CrossRef]
17. McDonald, P.; Henderson, A.R. Buffering capacity of herbage samples as a factor in ensilage. *J. Sci. Food Agric.* **1962**, *13*, 395–400. [CrossRef]
18. Ertekin, I.; Kızılşimşek, M. Effects of lactic acid bacteria inoculation in pre-harvesting period on fermentation and feed quality properties of alfalfa silage. *Asian-Australas. J. Anim. Sci.* **2019**, *33*, 245–253. [CrossRef]
19. Zhao, S.S.; Wang, Y.P.; Yang, F.Y.; Wang, Y.; Zhang, H. Screening a Lactobacillus plantarum strain for good adaption in alfalfa ensiling and demonstrating its improvement of alfalfa silage quality. *J. Appl. Microbiol.* **2020**, *129*, 233–242. [CrossRef]
20. Contreras-Govea, F.E.; Muck, R.E.; Weimer, P.J.; Hymes-Fecht, U.C. In vitro ruminal fermentation of treated alfalfa silage using ruminal inocula from high and low feed efficient lactating cows. *J. Appl. Microbiol.* **2016**, *121*, 333–340. [CrossRef]

21. Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T.; Williams, S.T. *Bergey's Manual of Determinative Bacteriology*, 9th ed.; Williams & Wilkins: Baltimore, MD, USA, 1994.
22. Liu, Q.; Chen, M.; Zhang, J.; Shi, S.; Cai, Y. Characteristics of isolated lactic acid bacteria and their effectiveness to improve stylo (Stylosanthes guianensis Sw.) silage quality at various temperatures. *Anim. Sci. J.* **2012**, *83*, 128–135. [CrossRef]
23. Tagg, J.R.; Dajani, A.S.; Wannamaker, L.W. Bacteriocins of Gram-positive bacteria. *Bacteriol. Rev.* **1976**, *40*, 722–756. [CrossRef] [PubMed]
24. Heuer, H.; Krsek, M.; Baker, P.; Smalla, K.; Wellington, E.M.H. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl. Environ. Microbiol.* **1997**, *63*, 3233–3241. [CrossRef]
25. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
26. Jobim, C.C.; Nussio, L.G.; Reis, R.A.; Schmidt, P. Methodological advances in evaluation of preserved forage quality. *R. Bras. Zootec.* **2007**, *36*, 101–119. [CrossRef]
27. Association of Official Analytical Chemistry (AOAC). *Official Methods of Analysis*, 15th ed.; AOAC International: Arlington, TX, USA, 1990.
28. Mertens, D.R. Gravimetric determination of amylase treated neutral detergent fiber in feeds with refluxing in beaker or crucibles: Collaborative study. *J. AOAC Int.* **2002**, *85*, 1217–1240. [PubMed]
29. Licitra, G.; Hernandez, T.M.; Van Soest, P.J. Standardization of procedures for nitrogen fractionation of ruminant feeds. *Anim. Feed Sci. Technol.* **1996**, *57*, 347–358. [CrossRef]
30. Weatherburn, M.W. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **1967**, *39*, 971–974. [CrossRef]
31. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375–380.
32. Siegrifield, V.R.; Ruckemann, H.; Stumpf, G. Method for the determination of organic acids in silage by high performance liquid chromatography. *Landwirtsch. Forsch.* **1984**, *37*, 298–304.
33. Zhang, Q.; Li, X.J.; Zho, M.M.; YU, Z. Isolating and evaluating lactic acid bacteria strains for effectiveness of Leymus chinensis silage fermentation. *Lett. Appl. Microbiol.* **2014**, *59*, 391–397. [CrossRef]
34. Li, D.; Ni, K.; Pang, H.; Wang, Y.; Cai, Y.; Jin, Q. Identification and antimicrobial activity detection of lactic acid bacteria isolated from corn stover silage. *Asian-Australas. J. Anim. Sci.* **2015**, *28*, 620–631. [CrossRef] [PubMed]
35. Amaral, R.C.; Carvalho, B.F.; Costa, D.M.; Morenz, M.J.F.; Schwan, R.F.; Ávila, C.L.S. Novel lactic acid bacteria strains enhance the conservation of elephant grass silage cv. BRS Capiaçu. *Anim. Feed Sci. Technol.* **2020**, *264*, 114472. [CrossRef]
36. Santos, A.O.; Ávila, C.L.S.; Soares, C.; Carvalho, B.F.; Schwan, R.F.; Lima, N. Lactic acid bacteria diversity in corn silage produced in Minas Gerais (Brazil). *Ann. Microbiol.* **2019**, *69*, 1445–1459. [CrossRef]
37. Li, D.; Wang, Y.; Zhang, Y.; Lin, Y.; Yang, F. Evaluation of lactic acid bacteria isolated from alfalfa for silage fermentation. *Grassl. Sci.* **2018**, *64*, 190–198. [CrossRef]
38. Dworkin, M.; Falkow, S.; Rosenberg, E.; Schleifer, K.H.; Stackebrandt, E. (Eds.) *The Prokaryotes: A Handbook on the Biology of Bacteria: Firmicutes and Cyanobacteria*, 3rd ed.; Springer: New York, NY, USA, 2006.
39. Pholsen, S.; Khota, W.; Pang, H.; Higgs, D.; Cai, Y. Characterization and application of lactic acid bacteria for tropical silage preparation. *Anim. Sci. J.* **2016**, *87*, 1202–1211. [CrossRef]
40. Silva, V.P.; Pereira, O.G.; Leandro, E.S.; Silva, T.C.; Ribeiro, K.G.; Mantovani, H.C.; Santos, S.A. Effects of lactic acid bacteria with bacteriocinogenic potential on the fermentation profile and chemical composition of alfalfa silage in tropical conditions. *J. Dairy Sci.* **2016**, *99*, 1895–1902. [CrossRef] [PubMed]
41. Kung, L.; Shaver, R.D.; Grant, R.J.; Schmidt, R.J. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.* **2018**, *101*, 4020–4033. [CrossRef]
42. Whiter, A.G.; Kung, L. The effect of a dry or liquid application of Lactobacillus plantarum MTD1 on the fermentation of alfalfa silage. *J. Dairy Sci.* **2001**, *84*, 2195–2202. [CrossRef]
43. Jones, B.A.; Satter, L.D.; Muck, R.E. Influence of bacterial inoculant and substrate addition to lucerne ensiled at different dry matter contents. *Grass Forage Sci.* **1992**, *47*, 19–27. [CrossRef]
44. National Institute of Meteorology. Available online: https://portal.inmet.gov.br/ (accessed on 15 March 2018).

45. Tschaplinski, T.J.; Blake, T.J. Photosynthetic reinvigoration of leaves following shoot decapitation and accelerated growth of coppice shoots. *Physiol. Plant.* **1989**, *75*, 157–165. [CrossRef]
46. Guo, L.; Yao, D.; Li, D.; Lin, Y.; Bureenok, S.; Ni, K.; Yang, F. Effects of lactic acid bacteria isolated from rumen fluid and feces of dairy cows on fermentation quality, microbial community, and in vitro digestibility of alfalfa silage. *Front. Microbiol.* **2020**, *10*, 2998. [CrossRef] [PubMed]
47. Mugabe, W.; Yuan, X.; Li, J.; Dong, Z.; Shao, T. Effects of hexanoic acid, Lactobacillus plantarum and their combination on the fermentation characteristics of Napier grass. *Anim. Feed Sci. Technol.* **2019**, *253*, 135–140. [CrossRef]
48. Zhao, J.; Dong, Z.; Li, J.; Chen, L.; Bai, Y.; Jia, Y.; Shao, T. Evaluation of Lactobacillus plantarum MTD1 and waste molasses as fermentation modifier to increase silage quality and reduce ruminal greenhouse gas emissions of rice straw. *Sci. Total Environ.* **2019**, *688*, 143–152. [CrossRef]
49. Blajman, J.E.; Páez, R.B.; Vinderola, C.G.; Lingua, M.S.; Signorini, M.L. A meta-analysis on the effectiveness of homofermentativeand heterofermentative lactic acid bacteria for corn silage. *J. Appl. Microbiol.* **2018**, *125*, 1655–1669. [CrossRef] [PubMed]
50. McDonald, P.; Henderson, A.R.; Heron, S.J.E. *Biochemistry of Silage*, 2nd ed.; Marlow: Chacombe, UK, 1991.

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