World Journal of Agricultural Sciences 8 (6): 547-559, 2012
ISSN 1817-3047

DOI: 10.5829/idosi.wjas.2012.8.6.1685

Physicochemical Screening of *Lactobacillus plantarum* and its Effects on the Fermentation of *Panicum maximum* Grass for Silage Production

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Abstract: In this study, 20 different isolates of lactic acid bacteria (LAB) were physicochemically screened for the ability to grow at low pH and produce lactic acid. The best isolates were selected for fermentation of *Panicum maximum* in order to produce silage. The grass was harvested at 6 weeks re-growth and ensiled at 15, 30, 45 and 60 days. Samples were taken on each of these days for microbial changes, physical and chemical composition and acceptability by ruminants. Afterwards, silage was exposed to air at 45 and 60 days of ensiling and its effects on microbial succession were determined. *Lactobacillus plantarum* produced the highest concentration of lactic acid (18.72) in MRS broth and has the highest optical density of 0.949 when grown at pH 2 was selected. The LAB count in the inoculated silage increased to 10.26 log cfu/g on day 45 and 10.87 cfu/g on day 60 compared to an increase of 8.46 log cfu/g and 7.48 log cfu/g respectively on the same day for the uninoculated silage. Spoilage organisms like yeast decreased to 2.94 log cfu/g and 2.97 log cfu/g on days 45 and 60 respectively in the inoculated silage. The pH of the inoculated silage decreased to 3.97 within the first 30 days of ensiling and the crude protein content increased to 9.84 by the end of day 60. The NDF content of the inoculated silage also decreased to 55.00 on day 15 while that of the control had a value of 63.00 on this same day. When exposed to air on day 45, the inoculated silage was less stable having an increase of spoilage bacteria like mould from 2.04 log cfu/g on day 1 to 3.97 log cfu/g by day 2. The uninoculated silage was more preferred with coefficient of preference value of 1.04, 1.16 and 1.21 on days 15, 30 and 60 respectively. Therefore silage inoculation with *L. plantarum* can improve the fermentation process of silage but not aerobic stability of the silage.

Key words: *Lactobacillus plantarum* · *Panicum maximum* · Silage · Inoculation · Ensiling

INTRODUCTION

Silage has been defined as fermented plant material with increasing palatability and nutritional value for animals, which can be stored for extended periods [1]. Silage production is usually achieved by ensiling, which is a method of preserving moist forage that is widely used in North America, Europe, Israel and other parts of the world [2]. Ensiling is a preservation method for moist forage crops. It is based on lactic acid bacteria (LAB) converting water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid, under anaerobic conditions. As a result, pH decreases and the moist forage are preserved from spoilage microorganisms [3]. The fermentation quality of silage is influenced by the size, diversity and activity of epiphytic LAB [3, 4]. However, a large proportion of these bacteria, being heterofermentative, may not be the most effective organisms for promoting predominant lactic acid fermentation in the silo [4, 5, 6].

It is possible to apply bacterial inoculants at ensiling in order to promote adequate fermentation patterns. Inoculants, comprising homofermentative LAB such as *Lactobacillus plantarum*, *Enterococcus faecium* and *Pediococcus* species, are often used to control the ensiling fermentation by rapid production of lactic acid and the consequent decrease in pH [7, 8, 9, 10]. Generally, lactic acid is preferred in the silo because it is a stronger acid than acetic acid [11]. Lactic acid reduces pH faster, thereby reducing plant respiration and enzyme activity as well as inhibiting other bacteria. However, acetic acid is a better inhibitor of yeast and it also maintains better

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aerobic stability than lactic acid [12]. Homofermentative LAB are the most common inoculants on the market. Initially, the main goal of using these inoculants was to preserve the quality of the ensiled plants as near to original levels as possible. Homofermentative bacteria accomplish this goal by decreasing pH, reducing dry matter losses to a minimal level (2-3%), reducing proteolysis (the breakdown of protein) and ammonia formation and increasing lactic acid and dry matter digestibility [13]. A fast decline in pH can also inhibit clostridial bacteria that produce butyric acid, a product of a bad fermentation that causes malodor. In addition, homofermentative bacteria have the potential to improve animal performance [14].

The objective of this study is to inoculate *P. maximum* grass with *L. plantarum* which has been clearfully selected by carrying out of different physicochemical test on different isolates with a determination of its effects on microbial succession, chemical composition, aerobic stability and acceptability by ruminant.

MATERIALS AND METHODS

Screening of Isolates

Culture Medium: In order to carry out the various physiochemical tests on each of the different isolates, De Man Rogosa and Sharpe (MRS) medium [15]was used.

Growth at Different Temperature: Exactly 0.1ml of the suspension containing LAB species with a concentration of 2.8 x 10^8 cfu was inoculated into sterilized MRS broth. It was then incubated in an incubator set at different temperatures of 25°C, 35°C, 37°C, 45°C for 48hrs. The cell growth was monitored by measuring the absorbance at 570nm using a spectrophotometer (Spectrumlab 752S).

Growth at Different pH: Exactly 0.1ml of the bacteria suspension containing the LAB species with a concentration of 2.8 x 10^8 cfu was inoculated into sterilized MRS broth whose pH had been adjusted to 2, 4, 6 and 8 with phosphate buffer and incubated for 48hrs. Growth was monitored by measuring the absorbance at 570nm with a spectrophotometer (Spectrumlab 752S).

Growth in Aerobic and Anaerobic Environments: Exactly 0.1ml of the LAB suspension containing 2.8 x 10^8 cfu was inoculated into sterilized MRS broth. It was then incubated in a microaerophilic environment at 37°C for the anaerobic environment and the other was incubated at 37°C in an incubator for the aerobic environment. After 48hrs of incubation, growth was monitored at 570nm with a spectrophotometer (Spectrumlab 752S).

Quantity of Lactic Acid (g/l) Produced in MRS Broth by Tested Isolates: The organisms were grown in sterile MRS broth incubated at 37°C in a microaerophilic environment at 37°C for 48hrs. It was then put in a centrifuge at 3000rpm for 15min. The supernatant was then titrated with 0.25molL^{-1} NaOH and 1ml of phenolphthalein indicator (0.5% in 50% alcohol). The titratable acidity was calculated as lactic acid (%). Each milliliter of 1N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acid was then calculated according to A.O.A.C method [17].

$$\text{Titratable acidity} = \frac{\text{Ml NaOH x NaOH x M.E x 100}}{\text{Volume of sample used}}$$

where;

Ml NaOH = Volume of NaOH used,
N NaOH = Normality of NaOH Solution
M.E = Equivalent factor

Preparation of Silage Inoculants: The *Lactobacillus plantarum* that was isolated from *Panicum maximum* silage with identification and characterization in Environmental and Biotechnology Laboratory of the Department of Microbiology, University of Ibadan, Ibadan, Nigeria, was inoculated into 500ml of prepared MRS broth in Erlenmeyer's flask. It was then incubated on a shaker incubator for 2-3 days at 250rpm. Afterwards, it was diluted with 2500ml of sterile distilled water. 1ml was pipetted out for plate count on MRS plate.

Schedule of Ensiling Experiment: *Panicum maximum* grass that was established at the Teaching and Research Farm of the University of Ibadan, Ibadan, Nigeria was harvested in the month of November, 2008 after six weeks of re-growth. The grass was chopped to 2-3 cm long and wilted for 24 hrs. Samples of the wilted grass were taken for chemical and microbiological analysis. The chopped grass was then divided into equal portions of 1kg, packed into polythene bags and properly sealed to prevent air for anaerobic conditions. Afterwards, they were inoculated with 10ml of approximately 10^6 cfu/ml of the *L. plantarum* and later ensiled. The silage was opened after 15, 30, 45 and 60 days and samples were taken for microbiological, chemical and aerobic stability as well as acceptability by

ruminant. The uninoculated samples ensiled along with the inoculated samples served as the control silage. The experiment was replicated three times to reduce error.

Microbial, Physical and Chemical Analyses of the Silage: Exactly 1g of the silage was mixed with 9ml of sterile distilled water each day the silage was opened. This was used for serial dilution to 10^{-8}. The dilutions were then plated on the following media: De-Man-Rogosa-Shape agar (MRS) to detect LAB, Nutrient Agar (NA) to detect aerobic bacteria, Yeast extracts Agar (YEA) for yeast, Potato Dextrose Agar (PDA) to detect Mould and Eosin Methylene Blue (EMB) to detect coliforms. Aerobic bacteria and Coliform plates were incubated at 37°C for 1-2 days. LAB was incubated at 37°C in a microaerophilic environment for 2 days. Mould and Yeast were cultivated at 25°C for 3 days. Numbers of colony formed were expressed in log cfu/ml/g.

Temperature of the silage was measure-d with a thermometer. 25g of the silage was later taken from the silage on each of the days of opening the silage to mix with 100ml of distilled water and shake in a shaker at 250rpm for 15min. It was then filtered with a filter paper and the filtrate was used for the determination of the pH with a pH meter. Dry matter (DM) contents of the silage were determined by oven drying at 80°C for 48hrs. Ash, crude fibre and, ether extract contents of the silage were determined according to A.O.A.C (1990). Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were determined according to Goering *et al* [16]. Crude protein (CP) content of the silage was also determined by the Kjeldahl method [17].

Aerobic Stability Test: At the end of the 45 and 60 days of ensiling, the silage was subjected to aerobic stability test that lasted for 7 days. Samples of the silage was put in a petri dish and placed in dessicators. Samples were taken on days 0, 2, 5 and 7 for the microbiological analyses of the silage. Moreover, the counts of the LAB, yeast, mould, aerobic bacteria and coliforms were determined as mentioned above.

Acceptability by Ruminant: Eight West African dwarf sheep weighing 12-14kg and about two years old were used to evaluate the free choice intake of the silage (control and LAB treated) on each of the days of termination of the experiment i.e. 15, 30, 45 and 60 days. 2.5kg each of the silage, both the LAB treated silage and the control, was placed in strategic feeding troughs measuring 1m x 2m. The sheep were allowed to feed for 6 hours per day on each day of the feed out. Consumption was measured by deduction of remnants from the amount of silage served after 4hrs. The silage preferred was assessed from the coefficient of preference (COP) value, calculated from the ratio between the intake for the individual silage treated with LAB or control divided by the average intake of the silage treated with LAB or control [18]. On the basis, a silage treatment was inferred to be relatively acceptable if COP was greater than unity.

Statistical Analysis of Silage: The data were analyzed by analysis of variance and means were separated by Duncan's multiple range [19].

RESULTS

Physiochemical Screening of LAB Isolates: Eighteen isolates of *Lactobacillus plantarum* and two isolates of *Lactobacillus brevis* were subjected to various physicochemical tests i.e. growth of LAB at different temperatures, pH, different salt concentrations, production of lactic acid in MRS broth and growth in anaerobic and aerobic conditions. The results of these tests are shown in Tables 1, 2, 3 and 4 respectively. Organism 19 identified as *L. plantarum* produced the highest amount of lactic acid of 18.72 in MRS broth. None of the organisms was able to grow at 40°C and 45°C. Organisms 32, 33, 36, 38 and 55 showed poor performance in at least each of the physiochemical test. LAB 19, also has the highest optical density (OD) of 0.949 at pH 2 in MRS broth. It was therefore selected for the inoculation of the silage.

Microbial Succession in Silage on Different Days of Ensiling: The result of microbial succession in silage on different days is shown in Table 5. Total aerobic bacteria count in the control silage was increased from 3.6 log cfu/g on the fresh wilted grass to 7.95 log cfu/g, 7.94 log cfu/g on days 15 and 30, respectively, while it was decreased to 4.95 log cfu/g at the end of day 60 of ensiling. In the inoculated silage minimal increase was observed from 3.6 log cfu/g on fresh wilted grass to 4.69 log cfu/g and was decreased to 3.74 log cfu/g, 2.86 log cfu/g and 2.84 log cfu/g at day 30, 45 and 60, respectively. Significant difference ($P < 0.05$) was observed on all the days of ensiling between the control silage and LAB inoculated silage except on day 15.

Table 1: Growth of LAB in MRS broth at different temperatures

Organisms	25°C	35°C	37°C	40°C	45°C
8	1.592	1.678	1.805	0.200	0.074
12	1.604	1.689	1.799	0.234	0.046
13	1.599	1.629	1.795	0.486	0.064
16	1.609	1.702	1.803	0.269	0.044
18	1.606	1.746	1.805	0.123	0.089
19	1.606	1.728	1.800	0.156	0.059
22	1.615	1.524	1.800	0.060	0.097
24	1.603	1.706	1.808	0.153	0.078
25	1.611	1.716	1.811	0.268	0.042
32	1.566	1.584	1.748	0.156	0.043
33	1.569	1.499	1.749	0.174	0.086
36	1.559	1.684	1.734	0.134	0.094
37	1.612	1.704	1.800	0.057	0.068
38	1.307	1.468	1.749	0.184	0.094
45	1.614	1.689	1.804	0.269	0.309
46	1.600	1.659	1.809	0.234	0.031
55	1.579	1.589	1.779	0.060	0.046
56	1.576	1.409	1.803	0.268	0.035
58	1.606	1.704	1.794	0.156	0.084
60	1.609	1.784	1.806	0.400	0.015

All the numbers on the organisms column represent *L. plantarum* except numbers 33 and 45 which represent *L. brevis*

Table 2: Growth of LAB isolates in MRS broth at different pH

Organisms	pH 2	pH 4	pH 6	pH 8
8	0.094	0.869	1.054	1.142
12	0.894	1.504	1.084	1.162
13	0.569	1.276	1.082	1.172
16	0.679	1.504	1.083	1.189
18	0.498	1.294	1.080	1.124
19	0.949	1.582	1.082	1.194
22	0.094	0.974	1.034	1.096
24	0.848	1.352	1.079	1.162
25	0.749	1.248	1.088	1.082
32	0.046	0.948	1.046	1.084
33	0.079	0.826	1.058	1.094
36	0.569	0.848	1.034	1.129
37	0.569	1.489	1.085	1.136
38	0.086	0.879	1.047	1.084
45	0.824	1.369	1.082	1.085
46	0.846	1.568	1.084	1.099
55	0.794	1.114	1.068	1.169
56	0.084	0.926	1.076	1.092
58	0.569	1.462	1.085	1.168
60	0.942	1.698	1.081	1.268

All the numbers on the organisms column represent *L. plantarum* except numbers 33 and 45 which represent *L. brevis*

Table 3: Growth of LAB isolates in MRS broth at different NaCl concentrations

Organisms	3%	5%	7%	9%
8	1.133	0.832	0.799	0.832
12	1.133	0.832	0.805	0.851
13	1.137	0.836	0.804	0.854
16	1.134	0.837	0.803	0.856
18	1.135	0.838	0.808	0.852
19	1.134	0.840	0.809	0.854
22	1.136	0.835	0.811	0.853
24	1.132	0.836	0.812	0.859
25	1.102	0.784	0.704	0.819
32	1.135	0.803	0.764	0.840
33	1.101	0.791	0.755	0.808
36	1.102	0.783	0.752	0.812
37	1.137	0.839	0.810	0.854
38	1.123	0.810	0.805	0.836
45	1.138	0.837	0.798	0.853
46	1.136	0.838	0.804	0.855
55	1.116	0.828	0.805	0.819
56	1.114	0.805	0.769	0.816
58	1.134	0.835	0.805	0.851
60	1.133	0.836	0.809	0.857

All the numbers on the organisms column represent *L. plantarum* except numbers 33 and 45 which represent *L. brevis*

Significant difference ($P < 0.05$) was observed on all the days of ensiling for the coliform count between the control and LAB inoculated silage. Decrease was observed in the count of coliform bacteria from 4.30 log cfu/g on 15 days and 30 to 3.65 log cfu/g on day 45 while increase to 3.84 log cfu/g was observed on day 60 of ensiling in the control silage. In the inoculated silage decrease from 4.60 log cfu/g on day 15 to 4.27 log cfu/g on day 30 was observed.

The LAB count increased from 5.0 log cfu/g on the fresh wilted grass to 8.20 log cfu/g on the control silage, while that of the inoculated silage was the highest having a value of 8.98 log cfu/g at the end of the first 15 days. The count was increased to 9.84 log cfu/g on day 30 in the control silage which then dropped to 8.46 log cfu/g on day 45 and 7.48 log cfu/g on day 60. In the inoculated silage the count was increased to 9.86 log cfu/g on day 30 and 10.87 log cfu/g at the end of the 60th day of ensiling. Significant difference was only observed between the LAB count of the control and the inoculated silage on day 45 and day 60 of ensiling.

Significant difference at $P < 0.05$ was also observed in the yeast count between the control and inoculated silage on all the ensiled days. Decrease in the yeast count was observed in the inoculated silage as days of fermentation increased from 4.94 log cfu/g on day 15 to 2.97 log cfu/g at the end of day 60. Decrease was only observed between the fresh wilted grass having a yeast count of 5.3 log cfu/g and control silage on day 15 which have a yeast count of 4.00 log cfu/g and similar result was also observed on day 30 and increase to 4.16 log cfu/g and 4.27 log cfu/g was observed on days 45 and 60, respectively.

Table 4: Quantity of lactic acid produce and growth of LAB isolates in different O_2 regimes

Organisms	Titrable lactic acid
	10.22
12	15.00
13	16.42
16	10.34
18	8.42
19	18.72
22	8.74
24	8.65
25	9.78
32	9.00
33	7.84
36	7.20
37	7.23
38	7.46
45	12.50
46	16.07
55	12.50
56	7.24
58	13.64
60	15.26

Organisms	Anaerobic	Aerobic
8	1.504	1.791
12	1.509	1.734
13	1.511	1.804
16	1.514	1.801
18	1.515	1.814
19	1.516	1.809
22	1.516	1.811
24	1.514	1.805
25	1.518	1.809
32	1.461	1.730
33	1.452	1.714
36	1.459	1.739
37	1.518	1.811
38	1.485	1.772
45	1.516	1.803
46	1.516	1.806
55	1.486	1.771
56	1.480	1.764
58	1.510	1.796
60	1.509	1.808

All the numbers on the organisms column represent *L. plantarum* except numbers 33 and 45 which represent *L. brevis*

Table 5: Microbial succession in silage on different days of ensiling and microbial count on fresh unensiled wilted *P. maximum* (log cfu/g)

		Days of ensiling			
		15	30	45	60
Total aerobic bacteria	Control	7.95^{a}	7.94^{a}	4.94^{b}	4.95^{a}
	Org 19	4.69^{a}	3.74^{d}	2.86^{c}	2.84^{c}
	Total aerobic bacteria count on fresh grass 3.6				
Coliform	Control	4.30^{b}	4.30^{a}	3.65^{c}	3.84^{d}
	Org 19	4.60^{a}	4.87^{a}	4.23^{b}	4.27^{c}
	Coliform count on fresh wilted *P. maximum* 5.0				
LAB	Control	8.20^{a}	9.84^{a}	8.46^{c}	7.48^{d}
	Org 19	8.98^{a}	9.86^{a}	10.26^{a}	10.87^{a}
	LAB count on fresh wilted *P. maximum* 5.0				
Yeast	Control	4.00^{c}	4.00^{b}	4.16^{b}	4.27^{a}
	Org 19	4.94^{b}	3.04^{c}	2.94^{d}	2.97^{c}
	Yeast count on fresh wilted *P. maximum* 5.3				
Mould	Control	3.46^{d}	4.64^{a}	4.27^{a}	4.00^{a}
	Org 19	3.84^{c}	2.85^{d}	2.04^{d}	2.64^{b}
	Mould count on fresh wilted *P. maximum* 5.0				

Lactic acid bacteria (LAB), Org 19 represents silage treated with *L. plantarum* Values which carry different letters were significantly different at $P < 0.05$

Table 6: Physical quality of silage by colour, structure and taste of silage

	Treatments		
Days ensiled	Parameters	Control	Org 19
15	Colour	Olive green	Olive green
	Smell/taste	Mild fruity smell	Slight fruity smell
	Structure	Firm and separable	Firm and separable
30	Colour	Olive-green	Greenish yellow
	Smell/taste	Vinegar	Sharp vinegar
	Structure	Firm and separable	Firm, visible and separable
45	Colour	Olive green	Olive green
	Smell/taste	Very sharp vinegar	Very sharp vinegar
	Structure	Visible, firm and separable	Visible, firm and separable
60	Colour	Olive green	Olive green
	Smell/taste	Mild vinegar	Sharp vinegar
	Structure	Visible and separable	Visible and separable

Org 19 is the silage treated with the two *L. plantarum*

The mould count in the inoculated silage also decreased drastically as the days of ensiling increased from 3.84 log cfu/g on day 15 to 2.64 log cfu/g at the end of the day 60. Decrease was also observed in the control silage as the days of ensiling increased from 4.64 log cfu/g on day 30 to 4.00 log cfu/g on day 60 which is not as dramatic as in the inoculated silage. Increase in the mould count was observed between day 15 and day 30 from 3.46 log cfu/g to 4.64 log cfu/g in the control silage. Significant difference was observed in the mould count between the control and the inoculated silages on all the days of ensiling.

Physical Assessment and Chemical Composition of the Silage on Different Days of Ensiling: The results of the physical and chemical composition of the silage on different days of ensiling are shown in Table 6. The colour of the guinea grass (silage) in all the LAB treated and

Table 7: Physical and chemical composition of silage on different days of ensiling

		Days of ensiling			
	Treatments	15	30	45	60
pH	Control	4.60[a]	4.73[a]	3.90[a]	2.90[a]
	Org 19	4.33[c]	3.97[b]	3.83[a]	3.10[a]
		pH of fresh wilted grass 6			
Temperature (°C)	Control	29.80[a]	28.00[a]	25.30[a]	25.00[b]
	Org 19	29.70[a]	24.60[b]	25.80[a]	26.70[a]
DM (g/100g DM)	Control	34.42[a]	34.08[a]	34.45[a]	34.43[a]
	Org 19	34.38[a]	31.26[c]	32.65[b]	32.24[b]
		DM of fresh wilted grass 30.43			
CP (g/100g DM)	Control	6.13[c]	7.44[c]	7.86[d]	6.78[c]
	Org 19	7.22[b]	8.53[b]	8.09[c]	9.84[a]
		CP of fresh wilted grass 6.56			
EE (g/100g DM)	Control	12.00[a]	13.00[c]	10.00[c]	11.00[c]
	Org 19	11.00[b]	16.00[a]	10.00[c]	15.00[a]
		EE of fresh wilted grass 12.00			
Ash (g/100g DM)	Control	11.00[b]	13.00[c]	10.00[c]	8.00[c]
	Org 19	10.00[b]	16.00[a]	10.00[c]	12.00[a]
		Ash of fresh wilted grass 8.00			
ADF (g/100g DM)	Control	38.00[a]	37.00[a]	37.00[a]	35.00[a]
	Org 19	34.00[c]	35.00[c]	35.00[c]	33.00[c]
		ADF of fresh wilted grass 37.00			
NDF (g/100g DM)	Control	63.00[a]	64.00[a]	59.00[c]	53.00[c]
	Org 19	55.00[c]	61.00[b]	61.00[b]	58.00[a]
		NDF of fresh wilted grass 75.00			
ADL (g/100g DM)	Control	7.89[c]	8.11[c]	8.57[a]	8.60[c]
	Org 19	8.62[a]	8.57[a]	8.57[a]	9.09[a]
		ADL of fresh wilted grass 11.00			
CF (g/100g DM)	Control	31.00a	31.00[a]	29.00[b]	27.00[b]
	Org 19	30.00a	31.00[a]	31.00[a]	30.00[a]
		CF of fresh wilted grass			

Dry matter (DM), Crude protein (CP), Ether extract (EE), Acid detergent fibre (ADF), Neutral detergent fibre (NDF), Acid detergent Lignin (ADL), Crude fibre (CF), Org 19 is silage inoculated with *L. plantarum*

Values which carry different letters were significantly different at $P < 0.05$

untreated i.e. the control silage, varies from olive-green to greenish yellow in 15, 30, 45 and 60 days of ensiling respectively. The smell/taste of 15 days silage varied from a mild fruity smell a sharp fruity smell for all treated silages. Mild vinegar smell to sharp and very sharp vinegar odor/taste were perceived for the guinea grass (control silage) and all the inoculated silage from days 30 to 60 respectively. The structure of guinea the grass silage for all the silage were firm, visible and discrete in the overall silage throughout the entire period of ensiling.

The pH of the silage was decreased gradually in the inoculated silage from 4.33 to 3.97 between the 15th and 30th day, while that of the control silage was not drastically low as the inoculated silage having a pH of 4.60 on day 15 and 4.73 on day 30 as shown in Table 7. Significant difference was not observed in the pH of the control and inoculated silage on days 45 and 60. Significant difference ($p < 0.05$) was not observed in the temperature of the silages on day 15 but was observed on day 30 and day 60. On day 15 the control silage had a temperature of 28°C while the inoculated silage had a temperature of 24.6°C. Significant difference ($p < 0.05$) was not observed in the DM of the silages i.e. the control and the inoculated silage on day 15. Difference was observed on the other days. On day 30 the control silage had a DM of 34.08 while the inoculated silage had a DM of 31.26. On day 60 of ensiling the DM of the inoculated silage was 32.24 while that of the control silage was 34.43. Significant difference ($p < 0.05$) was observed in the CP between the control and the inoculated silage. On day 15 the control silage had a CP of 6.13 g/100g DM while the inoculated silage had a CP of 7.22 g/100g DM. Increase to 9.84 g/100g DM was observed on the 60th day for the inoculated silage while that of the control increased to 6.78 g/100g DM on the same day. The control had a CP of 7.86 g/100g DM on day 45 while the inoculated had a CP value of 8.09 g/100g DM. Significant difference ($p < 0.05$) was not observed on day 45 between the control and the

inoculated silage for the ether extract (EE). On this day the control silage had EE value of 10.00 g/100 DM and the inoculated silage had the same value. On day 30 the inoculated silage had EE value of 16.00 g/100 DM where the control silage has EE value of 13.00 g/100 DM on the same day. Significant difference ($p < 0.05$) was only observed on day 30 and day 60 between the inoculated and the control silage for the ash content of the silage. The control silage had a value of 13.00 g/100 DM on day 30 while the inoculated silage had a value of 16.00 g/100 DM on the same day. The ash content decreased to 8.00 g/100 DM at the end of day 60 in the control silage and that of the inoculated silage decreased to 10.00 g/100 DM on day 45 and increased to 12.00 g/100 DM on day 60.

The ADF content of the inoculated silage was significantly lower than that that of the control silage on all the days of ensiling. On day 15 the control silage had an ADF value of 38.00 g/100 DM, while that of the inoculated silage was 34.00 g/100 DM. On the 60th day of ensiling the ADF content of the control silage was 35.00 g/100 DM while that of the inoculated silage was 33.00 g/100 DM. Significant decrease ($p < 0.05$) was also observed in the NDF content of the inoculated silage compared to the control silage. The NDF value of the control silage on day 30 was 64.00 g/100 DM while that of the inoculated silage was 61.00 g/100 DM. There was a decrease to 59.00 g/100 DM for the control silage on day 45 while a decrease to 58.00 g/100 DM was observed for the inoculated silage on day 60. Significant difference ($p < 0.05$) was observed in the ADL content of the silage between the control and the inoculated silage on all the days of ensiling except on day 45. On this day both the control and the inoculated silage had an ADL value of 8.57 g/100 DM. There was no significant difference ($p < 0.05$) in the control and inoculated silage for the crude fibre content on day 15 and day 30. The CF value on those days for the control and inoculated silage was in the range of 30 g/100 DM and 31 g/100g DM. On day 45 and day 60 significant differences ($p < 0.05$) were observed in the CF values between the control and the inoculated silage. Decrease in CF values from 29.00 g/100 DM on day 45 to 27.00 g/100 DM on day 60 was observed in the CF value while decrease from 31.00 g/100g DM on day 45 to 30.00 g/100 DM on day 60 was observed in the inoculated silage.

Microbial Succession in Silage after Exposure to Air for 45 Days of Ensiling: The results of the microbial succession after exposure of the silages to air for 45 days of ensiling are shown in Table 8. Aerobic bacteria were observed to decrease from 4.94 log cfu/g on day 1 of exposure to 4.25 log cfu/g on day 2 of exposure, while increase in aerobic microbial load to 9.84 log cfu/g on day 5 was observed. In the inoculated silage, increase in the count of aerobic bacteria was observed as the days of exposure increased in which 2.89 log cfu/g was observed on day 1 and increase to 8.98 log cfu/g was observed at the end of the fifth day. The LAB count in both silages decreased as the days of exposure increased. Decrease from 8.46 log cfu/g on the first day of exposure to 3.64 log cfu/g on the seventh day of exposure was observed in the control silage while decreased from 10.26 log cfu/g on the first day to 2.94 log cfu/g on the seventh day was observed in the inoculated silage. The yeast in the control silage decreased from 4.16 log cfu/g to 2.17 log cfu/g between the first and second day of exposure and an increase to 4.74 log cfu/g was observed at the end of the seventh day while increase from 2.94 log cfu/g to 3.04 log cfu/g and 5.68 log cfu/g on the first, second and fifth day of exposure were observed in the inoculated silage.

The mould count in the control silage also decreased between the first and second day of exposure from 4.27 log cfu/g to 2.01 log cfu/g but increased to 6.89 log cfu/g at the end of the seventh day. In the inoculated silage, drastic increase from 2.04 log cfu/g to 3.97 log cfu/g was observed between the first and second day of exposure.

Microbial Succession in Silage after Exposure to Air for 60 Days of Ensiling: The results of the microbial succession in silage after exposure to air for 60 days of ensiling are shown in Table 9. The aerobic bacteria count was increased from 4.95 log cfu/g to 9.24 log cfu/g between the first and seventh day of exposure in the control silage. In the inoculated silage minimal increase from 2.84 log cfu/g to 4.78 log cfu/g was observed between the first and seventh day of exposure. But drastic increase from 2.84 log cfu/g to 4.64 log cfu/g was observed between the first and second day of exposure compared to the control silage which had minimal increase from 4.95 log cfu/g to 4.98 log cfu/g between the first and second day of ensiling. LAB count in the control silage also decreased as the days of exposure increased. A decrease from 7.48 log cfu/g to 3.94 log cfu/g was observed between the first and seventh day of exposure. Similar decrease was also observed in the inoculated silage from 10.87 log cfu/g to 5.86 log cfu/g between the first and seventh day of exposure. Yeast count in the control silage decreased from 4.27 log cfu/g on the first day of exposure to 2.64 log cfu/g on the second day and increased to 6.98 log cfu/g at the end of the second day. Increase from 2.97 log cfu/g to 7.94 log cfu/g between the first and seventh day of exposure was observed in the

Table 8: Microbial succession in silage after exposure to air for 45 days of ensiling (log cfu/g)

		Days of exposure to air			
	Treatments	1	2	5	7
Aerobic bacteria	Control	4.94[b]	4.25[d]	9.84[a]	6.59[a]
	Org 19	2.89[c]	4.84[b]	8.98[b]	5.51[ab]
LAB	Control	8.46[c]	7.84[c]	4.87[c]	3.64[c]
	Org 19	10.26[a]	8.48[b]	5.24[a]	3.94[a]
Yeast	Control	4.16[b]	2.17[d]	5.64[b]	4.74[c]
	Org 19	2.94[d]	3.04[c]	5.68[b]	4.84[b]
Mould	Control	4.27[a]	2.01[b]	5.90[b]	6.89[a]
	Org 19	2.04[d]	3.97[a]	5.98[a]	6.47[c]

Lactic acid bacteria (LAB), Org 19 is silage inoculated with *L. plantarum*
Values which carry different letters were significantly different at $P < 0.05$

Table 9: Microbial succession in silage after exposure to air for 60 days of ensiling

		Days of exposure to air			
	Treatments	1	2	5	7
Aerobic bacteria	Control	4.95[a]	4.98[b]	8.74[a]	9.24[a]
	Org 19	2.84[c]	4.64[c]	4.94[b]	4.78[c]
LAB	Control	7.48[d]	6.94[d]	4.84[b]	3.94[d]
	Org 19	10.87[a]	9.47[c]	6.47[a]	5.86[b]
Yeast	Control	4.27[a]	2.64[d]	6.84[a]	6.98[b]
	Org 19	2.97[c]	3.89[b]	4.68[d]	7.94[a]
Mould	Control	4.00[a]	2.54[d]	5.74[a]	6.46[d]
	Org 19	2.64[b]	2.94[a]	4.89[b]	6.77[b]

Lactic acid bacteria (LAB), Org 19 is silage inoculated with *L. plantarum*
Values which carry different letters were significantly different at $P < 0.05$

Table 10: Acceptability of organism treated and untreated *P. maximum* silage by West African dwarf sheep

	Days of ensiling and acceptability							
	15		30		45		60	
Treatment	MDI	COP	MDI	COP	MDI	COP	MDI	COP
Control	2.20	1.07	2.02	1.07	2.15	1.15	2.08	1.40
Org 19	1.90	0.93	1.75	0.59	1.58	0.85	0.89	0.60

(MDI)(kgDM): mean day matter intake and (COP) coefficient of preference.
Org 19 is the silage inoculated with the *L. plantarum*

inoculated silage.The mould count also decreased from 4.00 log cfu/g to 2.54 log cfu/g between the first and second day of exposure in the control silage and increased to 6.46 log cfu/g at the end of the seventh day of exposure. Increase from 2.64 log cfu/g on day 1 to 6.77 log cfu/g on day 7 was observed in the inoculated silage.

Acceptability of Inoculated and Uninoculated *P. maximum* Silage by West African Dwarf Sheep: The result of the acceptability of the silage is shown in Table 10. The ruminant was observed to accept the control silage more than the inoculated silage on all the days of ensiling. The COP for the control silage ranged between 1.40 and 1.15, while that of the inoculated silage ranged between 0.59 and 0.93. Lowest COP value was observed for the control silage on day 30. The MDI for the control silage ranged between 2.08 and 2.20 while that of the inoculated silage ranged between 0.75 and 1.90.

DISCUSSION

Lactic acid reduces pH faster, thereby reducing plant respiration and enzyme activity and inhibiting other bacteria [12]. This is the major reason why the *L. plantarum* screened for this work was selected based on the highest production of Lactic acid in MRS broth and highest optical density at pH 2 in MRS broth. The colour in all the silage was olive green and this colour was in order because it was closer to the orginal colour of the grass which was in line with the findings of Oduguwa *et al*. [20] that good silage usually preserves the original colour of the standing plant. The aroma of the silage ranged from mild to sharp fruity smell. Oduguwa *et al*. [20] and Jianxin [21] also reported that good silage has a mild, slight acidic and fruity smell resembling that of cut bread and of tobacco due to the presence of lactic acid. The pH in the inoculated silage was observed to decrease drastically between the first 15 days and 30 days of ensiling in the inoculated silage compared to what was observed in the uninoculated silage. This observation is similar with those obtained by Filya [10], who reported that a fast initial pH reduction is observed in silage inoculated with homofermentative bacteria and a good aerobic stability later is controlled by heterofermentative bacteria producing more acetic acid. With this decrease in the pH of the inoculated silage compared to the control, it means the inoculums enhance the preservation of the silage. Seglar [22] reported that the decline in pH promotes increased population of efficient homofermentative lactic acid bacteria and these bacteria reduce silage pH faster and more efficiently by producing predominantly lactic acid. This assertion can be justified in this experiment because there was an increase in the count of lactic acid bacteria as the days of ensiling increased. Seglar [22] reported that a predominant of lactic acid bacteria (usually strains of *Lactobacillus plantarum*) relative to other enterobacteria and acetate producing bacteria creates a faster fermentation thus conserving more nutrients: water soluble carbohydrate, peptide and amino acids. The inoculation of silage with

homofermentative or heterofermentative LAB revealed the lower value of pH (between 3 and 4) compared to the results obtained by Ranjit and Kung [23], Winters *et al.* [24] and Aksu *et al.* [25]. For example, in the silage inoculated with a mixture of LAB consisting of *Pediococcus*, *Lactobacillus* and *Enterococcus* spp., the pH value of 3.76 was measured after 112 days of ensiling [26]. This can be observed in this experimental silage with an increase in the count of LAB compared to the count of coliform bacteria i.e. members of enterococcus in the silage.

In this silage the inoculated silage was observed to have more LAB as the days of inoculation increased while the LAB only increased up to day 30 in the control silage and a decrease was observed on day 45 and day 60. The LAB in the inoculated silage increased to 10.25 log cfu/g and 10.87 log cfu/g on days 45 and 60 respectively while a decrease to 9.86 log cfu/g and 7.48 log cfu/g on days 45 and 60, respectively was observed in the control silage. It therefore means that this inoculated silage preserved better compared to the control silage.

In the microbial changes in the ensiling process of silage fermentation reported by Sasaki, [27] and Woolford [28], LAB grew together with aerobic microbes such as yeasts, fungi and anaerobic bacteria in the presence of air between the plant particles. Fermentation was then promoted by an anaerobic environment creation and LAB became the predominate population. This is confirmed in this experiment with an increase in the count of LAB and a decrease in the count of aerobic bacteria, yeast and mould in the silage. Significant increase ($P < 0.05$) in the count of LAB in the inoculated silage compared to the control silage as the days of ensiling increased in this experiment is in agreement with the findings of Marcinakava [29] in which they inoculated grass and the population of spoilage microorganisms decreased with an increase in the days of ensiling compared to the control silage.

Seglar [22] indicated that as heat dissipates from the silage mass and the pH continues to decrease, the action of phase 3 lactic acid bacteria becomes inhibited and the cooler and more acidic environment increases the activity of phase 4 lactic acid bacteria. This is observed in the experiment because as the temperature of the silage decreased and stabilized between day 15 and 45 in the inoculated silage the population of the LAB increased in the silage inoculated. Moreover, it has been reported by many authors that ensiling grasses at a temperature of 42°C i.e. higher temperature results in clostridial fermentation and lower amounts of lactic acid than ensiling grass at 20°C i.e. lower temperature. Similarly, Adesogan [30], who ensiled corn in Florida, found that corn ensiled at 42°C had lower lactic and acetic acid concentrations and higher pH and ammonia concentrations than corn ensiled at a cooler temperature. Under these high temperature conditions, fermentation tends to be more heterolactic than homolactic. He also reported that high temperatures reduced LAB populations, but increased clostridial bacteria because they have a higher temperature for optimal growth than LAB. Therefore, warmer conditions seem to be more favorable for clostridial fermentation [31].

When the silo is opened for feeding, the silage is exposed to aerobic conditions, which leads to its deterioration [32]. Indicators for these spoilage processes are increasing temperature and pH, dry matter losses, surface mould growth and feed refusal by the animal [33]. Aerobic deterioration of silages is caused by rapid increases in yeasts and mould flora that oxidize lactic acid and volatile acids and result in increased temperatures and pH [34]. Most yeast can grow within the pH range 3-8 and some strains are able to withstand acidities of pH 2 or below [35].

Results obtained by Cai *et al.* [36] with the ensilage of *Sorghum bicolor* where selected strains of *Lactobacillus casei* FG 1 or *Lactobacillus plantarum* FG 10 isolated from corn and *Panicum maximum* were used at 10^5 CFU per gram of fresh matter. Both inoculants effectively improved fermentation decreasing contents of volatile fatty acids and ammonia N and reducing gas production and DM loss as compared to the control silage. Again, the LAB-treated sorghum silages which contained relatively high concentrations of residual WSC and lactic acid suffered a faster aerobic deterioration than the control silage. This also agrees with the result of this research when the silage was exposed to air to determine the aerobic stability of the silage after day 45 and 60 day of ensiling. It was observed that the count of aerobic bacteria, mould and yeast which are spoilage organisms increased drastically in the silage as the days of exposure increased. Ohyama *et al.* [37] reported that well preserved silages are considered to be more prone to aerobic deterioration than poorly fermented silages. However, when the silo is opened, aerobic conditions prevail at feeding time; the silage is subjected to aerobic microbial growth and is potentially unstable [7, 38, 39]. This is also observed in this experiment in that the silage that was inoculated exhibited the growth of spoilage

microorganism more rapidly compared to the control silage between the first and second day of exposure. Silage protein quality represents perhaps the most important determinant of silage nutritive value from an economic standpoint in North America. This is because most producers are ensiling crops at early maturity when digestibility is high. At this stage of growth, CP content and solubility are high [39, 40]. For this reason the *P. maximum* that was used for this experiment was harvested at six weeks regrowth when the crude protein is high and digestibility will be high. Research has shown many times that silage-based diets need supplementary protein when given to growing cattle [41, 42, 43], dairy cattle [44, 45] and even beef cows [46]. This is attributed to the poor efficiency with which silage protein is used in the rumen [47]. Thus, supplementation with a relatively undegraded protein source can increase production through increased UIP supply [48] and through increased silage intake [49] and digestibility [50]. However, in this experiment the inoculums were able to increase the crude protein content of the silage on each day of ensiling compared to the control and the un-ensiled wilted plant which were likely to improve the animal performance if fed with this silage.

The decrease in the ADF and NDF content of the silage compared to the control silage and the un-ensiled wilted grass agrees with the result of Ridhla *et al.* [51] where the authors inoculated rhodesgrass harvested, at the heading stage (21.8% DM, 5% WSC and 66.4% NDF in DM) ensiled in 2 litres bottle silos with *Lactobacillus casei* either alone or combined with increasing levels of cellulase. The combined treatment the authors reported reduced NDF, ADF and *in vitro* DM digestibility of silage compared with the untreated silage. Seglar [22] reported that abnormally high values of ADF and NDF of silage indicate that less free sugars are available and that silage quality has been sacrificed for decrease in digestibility by ruminants. Therefore, there is likely to be improved digestibility of the inoculums treated silage compared to the control silage in this research. There are various reports indicating that inoculants did not affect ruminal DM and OM degrabilities or digestibility of silages [9, 52, 53]; however in some studies, inoculants improved, degradability or digestibility [54]. Also, Kung and Ranjit [55] reported that treatment with an inoculant containing a blend of homolactic acid bacteria, propionibacteria and enzymes improved silage fermentation by causing more extensive homolactic fermentation and resulted in silage with lower ADF and NDF content. This means the decrease in the ADF and NDF content of the silage in this study has also resulted in an improved quality of the silage for effective animal production.

Seglar [22] also reported that ash to be the mineral content of the feedstuff which was also within the range suggested by Seglar [22] i.e. 10-20 g/100 DM in this research work. High ash levels author reported by the author may be indicative of excessive soil contamination coming in with the crop during harvest due to muddy or windy conditions, or dry matter losses from aerobic instability or Clostridia fermentation while the forage is in silo storage which did not occur in the silage used in this research work.

The inoculums was also able to recover DM in the silage compared to the fresh unwilted grass to about 1-3% reported by Weinberg and Muck [56]indicating that there was less energy loss in the silage by conserving the nutrient in the silage as reported by Jianxin [21].

Rooke [57] suggested that lactic acid may have a direct effect on palatability, since sour taste is associated with reduced palatability. In this experiment the acceptability of the silage treated with LAB was the lowest except at day 45 in which the LAB treated silage have a COP of 1.58 and the control silage has a COP of 1.21. The LAB used for the inoculation produces the highest concentration of lactic acid in MRS broth. This suggests whether too much lactic acid is accumulated in the silage preventing the animals from eating the silage. Low pH in silages is often associated with poor intakes because low pH in the rumen reduces cellulolytic activity and depresses intake. However there is no relationship between silage pH and rumen pH [57]. Silage is neutralized by saliva upon consumption. Low rumen pH is typically associated with grain-based not forage-based diets.

CONCLUSION

In conclusion, inoculation of silage with homofermentals like *L. plantarum* is advisable for *P. maximum* silage when the farmer is interested in enhancing the fermentation quality of the silage. The inoculums were able to increase the fermentation process and also increase the quality of the silage by reducing the NDF and ADF values and also increase the crude protein content of the silage. if it was observed that animals refused inoculated silage it would be advisable to open the silage for 12 to 24 hours as suggested by Seglar [22] before feeding the animal. However, rejection does not always mean that silage of low quality is produced.

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