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Effects of Combined Starters Culture of *Lactobacillus plantarum* on Fermentation Quality, Aerobic Stability and Acceptability by Ruminant of *Panicum maximum* Silage

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Abstract: This study examined the effects of two *L. plantarum* starters that are physicochemically different on *Panicum maximum* silage fermentation. The grass was inoculated with approximately 10^6 cfu/g of the starter culture. Aerobic stability, chemical composition and acceptability of the silage produced by ruminant were monitored at 15 days intervals for 60 days. The studies observed rapid drop in the pH of the inoculated silage to 3.90 at day 30, increase in the count of lactic acid bacteria (LAB) from 9.00 log cfu/g at day 15 to 10.15 log cfu/g at day 60 while decrease in spoilage microorganisms like mold from 4.09 log cfu/g at day 15 to 2.084 log cfu/g at day 60 in the inoculated silage. The crude protein (CP) of the silage was also observed to increase from 6.56 g/100 DM on fresh wilted grass to 9.63 g/100 dry matter (DM) at day 30 and 45 in the inoculated silage while the neutral detergent fiber (NDF) and acid detergent fiber (ADF) of the silage decreased in the inoculated silage. The NDF decreased from 59.00 g/100 DM at day 15 to 57.00 g/100 DM on day 60. The inoculated and the control silage were not aerobically stable when exposed to air at the 45 and 60 day of ensiling. The spoilage organism like yeast increased from 4.34 log cfu/g at day 1 of exposure to 4.4 log cfu/g at the second day of exposure at the 45 days of ensiling. The West African dwarf sheep used in this study showed low acceptability to the inoculated silage than the control silage with COP of 0.98, 0.90, 0.81 and 0.58 at days 15, 30, 45 and 60 respectively compared to the uninoculated control which has coefficient of performance (COP) of 1.01, 1.10, 1.18 and 1.42 at days 15, 30, 45 and 60 respectively.

Key words: Starter culture · Silage · Spoilage Microorganisms · *Lactobaccillus plantarum* · Sheep

INTRODUCTION

The preservation of forage crops as silage depends on the production of sufficient acid to inhibit activity of undesirable microorganisms under anaerobic conditions. The epiphytic lactic acid bacteria (LAB) present on forage crops convert sugar into lactic acid in the ensiling process. As a result, the pH is reduced, and the forage is preserved. However, when the silo is opened, aerobic conditions prevail during feeding time. The silage is subjected to aerobic microbial growth and is potentially unstable [1-3].

In order to improve the ensiling process, various chemical and biological additives have been developed. The biological additives are advantageous because they are safe, easy to use, non-corrosive to machinery, non-pollutant to the environment and are regarded as natural products [4]. Many types of homofermentative lactic acid bacteria (LAB) have been commercially prepared as silage additives. These selected strains encourage rapid pH decline and inhibit Dry Matter loss as well as clostridia fermentation, thereby improving preservation in a silo [5].

On many farms, silage is often exposed to varying degrees of aerobic deterioration before and during feeding. For example, the inability to remove sufficient quantities of silage from silos between feedings can result in prolonged exposure to air. Also,poorly packed silage is another common reason for silages spoiling before feeding. In an attempt to decrease labor, some producers choose to feed their heifers or steers once every 2 or 3 days. When silage is purchased and moved by trucks, silage may sit on an open pad and exposed to air for

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several days. Silos that are not well packed and sealed and exposed are particularly subjected to aerobic deterioration. Feeds that have undergone aerobic deterioration have their nutritional value reduced and present hazards to the environment (disposal of spoiled feed). Therefore, improving the aerobic stability of silages could confer a substantial advantage to producers [6]. Many researchers have proposed ways to improve the aerobic stability of silages. For instance, applying propionic acid-based additives have enhanced the aerobic stability of corn silage [7]. However, such additives are costly and thus microbial inoculants are substituted as economical alternatives. Also, adding of beneficial microorganisms (microbial inoculants) to feeds prior to ensiling improves fermentation [8]. *L. buchneri* has been marketed in combination with homolactic acid bacteria, which are commonly added to forage to increase lactic acid production, rapid drop of pH, and to improve DM recovery [9]. However, there are no long term studies on the effects of combination of two physicochemically different homolactic acid bacteria on silage.

The aim of this study is to determine the effects of inoculating physicochemically different isolates of *L. plantarum* on *P. maximum* silage. It also examines its effect on fermentation, aerobic stability and acceptability by ruminant animals.

MATERIALS AND METHODS

Screening of Isolates: The culture medium used for the screening was MRS broth. The organisms were cultivated at different temperature of 25°C, 35°C, 37°C, 40°C and 45°C in broth. The organisms were also cultivated in broth prepared into various pH of 2, 4, 6 and 8 in a micro aerophilic environment. The organisms were grown in aerobic environment i.e. in an incubator at 37°C and anaerobic environment i.e. microaerophilic environment in broth to determine the effects of oxygen on the isolates. The organisms were grown in different MRS broth prepared at different NaCl concentration of 3%, 5%, 7% and 9%. After 48 h of growth of the isolated culture, growth was monitored with a spectrophotometer (SpectrumLab 752S). The isolated culture was cultivated in MRS broth for 48hrs and the centrifuged at 3000 rpm for 15 min. The supernatant was then titrated with 0.25 mol NaOH and 1mL of phenolphathalein indicator (0.5% in 50% alcohol). The titratable acidity was calculated as lactic acid (%). The titratable acid was calculated according to A.O.A.C [10].

Preparation of Silage Inoculants: Identified and characterized *Lactobacillus plantarum* from *Panicum maximum* silage from Environmental and Biotechnology LAB, Department of Botany and Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria was inoculated into 500mL of prepared MRS broth in Erlenmeyer's flask. It was later incubated on a shaker incubator for 2-3 days at 250rpm. After which was diluted with 2500 mL of sterile distilled water. Plate count on MRS agar was carried out by pipetting 1ml into the agar plate.

Schedule of Ensiling Experiment: *Panicum maximum* grass that was establish at the teaching and research farm of the university of Ibadan, Ibadan, Oyo State, Nigeria was harvested in the month of November, 2008 after Six weeks of re-growth. The grass was chopped between 2-3 cm and wilted for 24 h. Samples of the wilted grass was taken for chemical and microbiological analysis. The chopped grass was then divided into equal portion of 1kg and packed into polythene bags and properly sealed to exclude air for anaerobic condition after inoculation with approximately 10^6 cfu/g of the *L. plantarum*. The silage was opened after 15, 30, 45 and 60 days for microbiological, chemical, and acceptability by ruminant. The aerobic stability test was carried out at 45 and 60 days of ensiling. There was a control i.e. uninoculated silage at each of the day in which the silage was opened. The experiment was replicated 3 times to reduce error.

Microbial, Physical and Chemical Analysis of the Silage: Exactly 1 g of the silage was mixed with 9 mL of sterile distilled water at each of the days of opening the silage. This was for serial dilution between 10^{-6} 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 extract Agar (YEA) for detecting yeast, Potato Dextrose Agar (PDA) to detect Mould and Eosin Methylene Blue (EMB) for coliforms. Coliforms were cultivated 37°C for 1-2 days. LAB was cultivated at 37°C in a microaerophilic environment for 2 days. The aerobic bacteria were also cultivated at 1-2 days at 37°C, Mould and Yeast were cultivated at 25°C for 3 days. Numbers of colony forming (cfu) were expressed in log cfu/mL/g.

Temperature of the silage was measured with a thermometer. After which 25 g of the silage was taken from the silage at each of the days of opening and mixed with 100 mL of distilled water. It was shaken on a shaker at 250 rpm for 15 min and filtered with a filter paper.

The filtrate was used for the determination of the pH with a pH meter. DM i.e. dry matter content of the silage were determined by oven drying at 80°C for 48 h. Ash, crude fibre, ether extract content of the silage was determined according to A.O.A.C. [10]. Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were determined according to Goering and Van Soest, [11], Crude protein (CP) content of the silage was also determined by the Kjeldahl method [10].

Aerobic Stability Test: At 45 and 60 days of ensiling, the silage was subjected to aerobic stability test that last for 7 days. Samples of the silage were placed in a petri dish and placed in desiccators. Samples were taken at day 0, 2, 5, and 7 for the microbiological analysis of the silage. Moreover, the count of the LAB, yeast, mould, aerobic bacteria and coliforms were determined as mentioned above.

Acceptability by Ruminant: Eight West Africa dwarf sheep weighing 12-14 kg and about two years old were used to evaluate the free choice intake of the silage (control and LAB treated) at each days of termination of the experiment i.e. 15, 30, 45 and 60 days respectively. In both the LAB treated and the control silage placed in strategic feeding troughs measuring 1 m x 2 m, 2.5 kg each of the silage was placed on it. The sheep were then allowed to feed for 6 h per day on each day of the feed out. Consumption was measured by deduction of remnants from the amount of silage served after 4 h. The silage preferred was assessed from the coefficient of preference (COP) value, calculated from the ration between the intake of the individual silage treated with LAB or control divided by the average intake of the silage treated with LAB or control [12] on this 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 mean were separated by Duncan's multiple range [13].

RESULTS

Physicochemical Screening of LAB Isolates: After the physicochemical screening of different (eighteen) isolates of *Lactobacillus plantarum* and two isolates of *L. brevis*. Two *L. plantarum* were selected for the inoculation of the *P. maximum* silage. The results of these tests were shown in Tables 1, 2, 3 and 4 respectively indicated

Table 1: Growth of LAB in MRS broth at different temperature determined by optical density

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: Optical density growth of LAB isolate 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: Optical density growth of LAB isolates in MRS broth at different NaCl concentration

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*

Table 4: Quantity of lactic acid (…) produce and growth of LAB isolates in different O_2 regime

Organisms	Titrable lactic acid	Organisms	Anaerobic	Aerobic
8	10.22	8	1.504	1.791
12	15.00	12	1.509	1.734
13	16.42	13	1.511	1.804
16	10.34	16	1.514	1.801
18	8.420	18	1.515	1.814
19	18.72	19	1.516	1.809
22	8.740	22	1.516	1.811
24	8.650	24	1.514	1.805
25	9.780	25	1.518	1.809
32	9.000	32	1.461	1.73
33	7.840	33	1.452	1.714
36	7.20	36	1.459	1.739
37	7.23	37	1.518	1.811
38	7.46	38	1.485	1.772
45	12.5	45	1.516	1.803
46	16.07	46	1.516	1.806
55	12.5	55	1.486	1.771
56	7.24	56	1.48	1.764
58	13.64	58	1.51	1.796
60	15.26	60	1.509	1.808

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

Lactobacillus plantarum (i.e. Org 60) grow over a wide range of pH having the highest growth optical density of 1.268 at pH 8 and optical density of 0.942 at pH 2 among the isolates screened. Org 19 produced the highest concentration of lactic acid of 18.72 in MRS broth. Therefore, these two *L. plantarum* were selected for the inoculation.

Microbial Succession in Silage at Different Days of Ensiling: The result of microbial succession in silage at different days was shown in table 5. It was observed that, the spoilage organisms in the silage were inhibited in the silage. The total aerobic bacteria decreased to 3.04 log cfu/g at end of the 60th day of ensiling from 5.48 log cfu/g at day 15 of ensiling. Yeast count in the inoculated silage was also observed to be inhibited but the control silage was lower at day 15 and 30. The count decreased from 5.3 log cfu/g on the fresh wilted *P. maximum* to 4.00 log cfu/g at day 15 and 30. It was observed that, the mould count was lower in the control silage with decrease from 5.00 log cfu/g on fresh wilted grass to 3.46 log cfu/g in the control silage at day 15 while 4.09 log cfu/g was observed in the inoculated silage at the same day. But lesser count of 3.48 log cfu/g, 3.56 log cfu/g, 2.84 log cfu/g at day 30, 45, and 60 respectively was observed in the inoculated silage. The LAB count in the silage did not show significant difference ($p<0.05$) at day 15 while significant difference ($p<0.05$) was observed at day 30, 45 and 60 respectively. The coliform count in the silage did not show significant difference ($p<0.05$) at day 30 of ensiling but significant difference ($p<0.05$) was observed at the other days of ensiling. The coliform count in the inoculated silage range from 4.00 log cfu/g to 4.71 log cfu/g while that in the control silage range from 3.65 log cfu/g to 4.30 log cfu/g.

Physical Assessment and Chemical Composition of the Silage at Different Days of Ensiling: These results of the physical and chemical composition of the silage at different days of ensiling were shown in Tables 6 and 7. The colour of guinea grass (silage) in all the LAB treated and untreated i.e. the control silage varies from olive-green to greenish yellow at 15, 30, 45 and 60 days of ensiling respectively. The smell/tastes of 15 days silage were mild fruity smell to sharp fruity smell for all organism inoculated silages. Mild vinegar smell to sharp and very sharp vinegar odor/tastes were perceived for the guinea grass (control silage) and all the inoculated silage from days 30 to 60 respectively. The structure of guinea grass silage for all the silage were firm, visible and separable in the overall silage throughout the entire period of ensiling.

Table 5: Microbial succession in silage at 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+60	5.48[b]	3.74[d]	3.94[b]	3.04[b]
		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+60	4.00[c]	4.34[a]	4.71[a]	4.54[a]
		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+60	9.00[a]	8.67[c]	9.74[b]	10.15[c]
		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+60	5.30[a]	4.64[a]	4.34[a]	4.08[a]
		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+60	4.09[a]	3.48[b]	3.56[b]	2.84[b]

Mould count on fresh wilted *P. maximum* 5.0 Lactic acid bacteria (LAB), Org 19+60 represent silage treated with combination of the two *L. plantarum* Values 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+60
15	Colour	Olive green	Olive green
	Smell/tastes	Mild fruity smell	Sharp fruity smell
	Structure	Firm and separable	Firm and separable
30	Colour	Olive-green	Olive-green yellow
	Smell/tastes	Vinegar	Very sharp vinegar
	Structure	Firm and separable	Visible and separable
45	Colour	Olive green	Olive green
	Smell/tastes	Very sharp vinegar	Very sharp vinegar
	Structure	Visible, firm and separable	Separable and visible
60	Colour	Olive green	Olive green
	Smell/tastes	Mild vinegar	Very sharp vinegar
	Structure	Visible and separable	Visible and separable

Org 19+60 is the silage inoculated with the combination of the two *L. plantarum*

Significant difference ($p<0.05$) between the control and inoculated silage was observed in the pH of the silage at day 15 and 30 respectively. But no significant difference was observed in the pH at day 45 and 60 of ensiling. The pH of the control silage was 4.73 at day 30 while that of the inoculated silage was 3.90 at day 30.

No significant difference ($p>0.05$) at day 15 was observed in the temperature of the silage at day 15, 45 and 60. At day 30 where significant difference ($p<0.05$) was observed the temperature of the control silage was 28°C while that of the inoculated silage was 24.60°C.

Significant difference ($p<0.05$) was not observed in the DM of the silage at day 15 and 30. But there was increased in the DM of the silages (inoculated and uninoculated) compared to the DM of the fresh wilted grass.

The CP of the silage increased from 6.56 g/100 DM observed in the fresh wilted grass to 9.63 at day 15 and 30 in the inoculated silage which decreased to 8.53 g/100 DM and 8.31 g/100 DM at day 45 and 60 respectively. In the uninoculated silage the CP of the silage increased up to 7.86 g/100 DM at day 45 but decreased to 6.78 g/100 DM at day 60.

Significant difference ($p<0.05$) was observed in the Etylene extract(EE) in all the days of ensiling between the control and inoculated silage.

The ADF content of the silage was observed to be significantly difference (p<0.05) between the control and inoculated silage in all the days of ensiling. The ADF was observed to be lower (36.00 g/100 DM at day 30 and 45) in the inoculated silage compared to the uninoculated control (37 g/100 DM at day 30 and 45). Similarly trend was also observed in the NDF value in all the silage.

The ADL of the control silage at day 15 was 7.89 g/100 DM while that of the inoculated silage was 8.57 g/100 DM and increase to 8.60 g/100 DM at day 60 was observed in the control while increase to 8.82 g/100 DM was observed at day 60 in the inoculated silage.

The *L. plantarum* did not have any effect on the crude fiber (CF) of the silage because significant difference (p<0.050) was not observed in the silage (control and inoculated) at all the ensiled days.

Microbial Succession in Silage after Exposure to Air at 45 Days of Ensiling: The results of the microbial succession after exposure of the silages to air at 45 days of ensiling were shown in Table 8. The count of the LAB was observed to decrease from 9.74 log cfu/g at the first day of exposure to 7.24 log cfu/g at the 2nd day and down to 3.44 log cfu/g at day 7. Decrease was also observed in the control silage and decrease to 3.64 log cfu/g was observed on 7th day of exposure.

The yeast in the inoculated silage increase drastically to 4.4 log cfu/g at 2nd day of exposure from 4.34 log cfu/g at the 1st day Increased to 5.88 log cfu/g was observed at the 5th day of exposure. In the control silage similar increase was also observed but 2.17 cfu/g was observed in the yeast count on the 2nd day.

Table 7: Physical and chemical composition of silage at 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+60	4.43[bc]	3.90[b]	3.83[a]	3.03[a]
		pH of fresh wilted grass 6			
Temperature (^{0}C)	Control	29.80[a]	28.00[a]	25.30[a]	25.00[b]
	Org 19+60	30.10[a]	24.60[b]	26.30[a]	25.30[b]
DM (g/100g DM)	Control	34.42[a]	34.08[a]	34.45[a]	34.43[a]
	Org 19+60	33.36[a]	32.72[a]	33.27[b]	31.75[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+60	9.63[a]	9.63[a]	8.53[b]	8.31[b]
		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+60	10.67[b]	14.00[b]	11.00[b]	14.00[b]
		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+60	10.00[b]	14.00[b]	11.00[b]	11.00[b]
		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+60	35.00[b]	36.00[b]	36.00[b]	34.00[b]
		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+60	59.00[b]	59.67[c]	61.00[b]	57.00[b]
		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+60	8.57[b]	8.33[b]	8.33[b]	8.82[b]
		ADL of fresh wilted grass 11.00			
CF (g/100g DM)	Control	31.00a	31.00a	29.00[b]	27.00[b]
	Org 19+60	31.00[a]	30.00[a]	29.00[b]	28.00[ab]

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+60 was silage inoculated with combination of the two *L. plantarum*. Values carry different letters were significantly different at p < 0.05

Table 8: Microbial succession in silage after exposure to air at 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 + 60	3.94[b]	5.54[a]	5.99[d]	3.64[b]
LAB	Control	8.46[c]	7.84[c]	4.87[c]	3.64[c]
	Org 19 + 60	9.74[b]	7.24[d]	4.99[b]	3.44[d]
Yeast	Control	4.16[b]	2.17[d]	5.64[b]	4.74[c]
	Org 19 + 60	4.34[a]	4.44[a]	5.88[a]	4.94[a]
Mould	Control	4.27[a]	2.01[b]	5.90[b]	6.89[a]
	Org 19 + 60	3.56[b]	3.94[a]	4.98[c]	6.89[a]

Lactic acid bacteria (LAB), Org 19+60 is silage inoculated with the combination of the two *L. plantarum*
Value carry different letters were significantly different at $p < 0.05$

Table 9: Microbial succession in silage after exposure to air at 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 + 60	3.04[b]	5.28[a]	5.34[b]	5.68[b]
LAB	Control	7.48[d]	6.94[d]	4.84[b]	3.94[d]
	Org 19 + 60	10.15[c]	9.78[a]	6.46[a]	5.34[c]
Yeast	Control	4.27[a]	2.64[d]	6.84[a]	6.98[b]
	Org 19 + 60	4.08[a]	4.64[a]	5.78[b]	6.04[c]
Mould	Control	4.00[a]	2.54[d]	5.74[a]	6.46[d]
	Org 19 + 60	2.84[b]	2.64[c]	4.85[b]	6.54[c]

Lactic acid bacteria (LAB), Org 19+60 is silage inoculated with combination of the two *L. plantarum*. Value 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	1.01	2.02	1.10	2.15	1.18	2.08	1.42
Org 19+60	2.14	0.98	1.65	0.90	1.49	0.81	0.84	0.58

(MDI)(kgDM): mean day matter intake and (COP) coefficient of preference. Org 19+60 is the silage inoculated with the combination of the two *L. plantarum*

The mould of the silage increased from 4.27 log cfu/g at the 1st to 5.90 log cfu/g at 5th day in the control silage, while increase from 3.56 log cfu/g to 4.98 log cfu/g at 5th day was observed in the inoculated silage.

Microbial Succession in Silage after Exposure to Air at 60 Days of Ensiling: The results of the microbial succession at 60 days of ensiling were shown in Table 9. Spoilage microorganisms like yeast, mould and aerobic bacteria were observed to increase as the days of exposure of the silage increased in all the silage. The yeast increased from 4.34 log cfu/g at 1st day to 5.88 log cfu/g at 5th day in the inoculated silage, while decrease to 2.17 log cfu/g at 2nd day from 4.16 log cfu/g at 1st day was observed in the uninoculated silage.

The mould count decreased to 2.10 log cfu/g at 2nd day from 4.27 log cfu/g at1 st day in the control silage. Increase from 3.56 logcfu/g at 1 st day to 3.94 log cfu/g at 2nd day and 4.98 log cfu/g at 5th day was observed in the inoculated silage.

The aerobic bacteria increased from 4.94 log cfu/g at 1st day to 9.84 log cfu/g at 5 th day in the control silage, while increase to 5.99 log cfu/g at 5th day from 3.94 log cfu/g at 1st day was observed in the inoculated silage.

The LAB count decreased from 8.46 log cfu/g to 4.87 log cfu/g at 5th day in the control silage, while decrease to 4.99 log cfu/g at 5th day from 9.74 log cfu/g at 1st day was observed in the inoculated silage.

Acceptability of Inoculated and Uninoculated *P. Maximum* Silage by West African Dwarf Sheep: The results of the acceptability test are shown in Table 10. Data in table indicated that the sheep used consumed more of the silage from the control silage than that of the inoculated silage. Hence, as the days of ensiling increased the COP of the inoculated silage decreased while that of the control silage increased. The COP range between 1.01 and 1.42 for the control silage while that of the inoculated silage range between 0.58 and 0.98 for all the ensiled days. The MDI for the control silage range between 2.02 and 2.20 while that of the inoculated silage range between 0.84 and 2.14.

DISCUSSION

In order to improve silage quality, many LAB-containing biological additives have been developed and are currently available [3, 14, 15]. These inoculants may inhibit the growth of harmful bacteria and enhance lactic acid fermentation during ensiling studied periods. The naturally occurring LAB on plant influences the action of silage inoculants because the introduced bacteria must compete with these LAB [16]. Lin *et al.* [16] also reported that epiphytic LAB play a major role in silage fermentation, and the numbers of LAB have become a significant factor in predicting the adequacy of silage fermentation and determining whether to apply silage bacterial inoculants. Among epiphytic LAB, lactic acid-producing cocci, e.g., *Streptococci, Leuconostocs, Pediococci, Lactococci,* and *Enterococci*, start lactate producing fermentation process in silage, creating an aerobic environment suitable for the development of *Lactobacilli*, although it was shown that they grew vigorously only in the early stage of ensiling processes [17]. In contrast with these lactic acid-producing cocci, lactobacilli play an important role in promoting lactic acid fermentation for a longer time. Epiphytic lactobacilli counts on silage crops are usually low and variable when the lactobacilli reach a level of at least 10^5 cfu/g of FM silage stores well [18]. Therefore, in this study, the count of LAB on the fresh grass was low while that of the yeast was high. But it was observed that as the days of ensiling increased the count of the LAB increased at day 15 which decreased at day 30 and increased again at day 45 and 60 respectively in the inoculated silage. At 30th day of ensiling it was observed in this study that the count of LAB was higher in the control silage than in the inoculated silage. This may be as a result of competition between the microbial inoculants and the epiphytic LAB or it may be because the two inoculants are antagonizing each other in the same ecological environment since two microbial inoculants with two different physiological properties are being combined together.

Previous study with the inoculation of microbial inoculants used in this study singly i.e. not in combination shows a sharp increase in the count of LAB as the days of ensiling increased with rapid inhibition of spoilage organisms like aerobic bacteria, yeast, and Mould [19]. But the results in this study contradict the previous results because as the days of ensiling increased there was no rapid decrease in the count of spoilage organism especially yeast. This conform to report of Kung *et al*. [20] that inoculation does not affect the count of yeast and mould in whole high moisture corn silage (HMCS) when inoculated with *L. buchneri* 40788 (6.6×10^5 cfu/g of HMCS but when the application rate was 4×10^5 cfu/g of HMCS, 6×10^5 cfu/g of HMCS and 8×10^5 cfu/g of HMCS, also they reported that the count of yeast and mould was reduced compared to the uninoculated silage. Hence, in this study the count of the spoilage organism may be reduced if the microbial count used for the inoculation of the silage is increased or decreased. Nevertheless, rapid decreased in the count of yeast is not an evidence of poor fermentation since the count of LAB observed in this study is more than what Hellings *et al.,* [18] suggested should be the count of LAB in silage with good fermentation.

All silage exposed to air eventually deteriorates as a result of aerobic microbial activity [21]. The factors that influence deterioration includes oxygen (exposure time), composition of the microbial population, substrate type, and temperature [21]. Lactate-assimilating yeast (*Saccharomyces, Candida*, *Cryptococcus*, and *Pichia* spp.) are usually the initial cause of aerobic deterioration [6]. Some strains of lactate-assimilating yeast grow well in an environment in which the pH level is between 3 and 8 and the temperature is under 40°C [21]. These are conditions common in corn silage when it is first exposed

to air. As lactic acid and other residual sugars are combusted and assimilated by yeast, the temperature starts to rise [21]. Once the temperature is above 45°C, the amount of yeast present declines and other microbial organisms (bacilli) begin to accumulate. As aerobic deterioration takes place, there are changes in the chemical parameters of silage [22]. The pH level tends to increase, ammonia and amines accumulate, and the levels of organic acids (lactic and acetic acid) tend to decline [21]. In this study, it was also observed that as the day of exposure of the silage to air increased the count of spoilage organism like yeast, aerobic bacteria and mould increase in the silage. The count of the LAB which is responsible for the production of lactic acid decreased as the time of exposure increased in the silage. This probably could have resulted in the decrease in the pH of the silage and decrease in the temperature of all the silage as all these processes occurred in the silage.

Homofermentative bacteria, such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Pediococcus* spp. and *Enterococcus* spp., mainly produce lactic acid. Heterofermentative bacteria, such as *Lactobacillus buchneri*, produce lactic acid, acetic acid, ethanol, and carbon dioxide. Generally, lactic acid is preferred in the silo because it is a stronger acid than acetic acid [23]. Lactic acid reduces pH faster, thereby reducing plant respiration and enzyme activity and inhibiting other bacteria. However, acetic acid is a better inhibitor of yeast, and maintains better aerobic stability than lactic acid. This was similar to what occurred in the LAB inoculated silage with combination of *L. plantarum* in this study with a rapid reduction of the pH of the silage compared to that of the uninoculated silage. The main purpose of heterofermentative inoculants is to improve aerobic (the presence of oxygen) stability by reducing the level of yeast in the silage (high levels of yeast can cause heating). *Lactobacillus buchneri* is the main heterofermentative LAB used in forage crops in the U.S. [23]. *Lactobacillus buchneri* produces more acetic acid than homofermentative bacteria [24]. Therefore, in this study despite the combination of the two physicochemically different isolates of *L. plantarum* the aerobic stability of the inoculated and the uninoculated silage was very poor with increasing the count of spoilage microorganisms within a short period of time. The inoculated silage was even observed to be less stable than the control silage that drasticly increased in the count of the mould and yeast at the second day of exposure which was not observed in the control silage. This was similar to the report of Jonsson, [21] that some strains of lactate assimilating yeast grow well in an environment in which the pH level is between 3 and 8. Similar report was also reported in the studies of Moon, [25] and Woolford [26] that the acid tolerant yeast that assimilate lactate (and usually not bacteria) are primarily responsible for the spoilage when expose to air. The authors went further to report that lactic acid by itself is not an effective antimycotic agent. However, in this study, there was probably to be enough lactic acid in the silage at the day 45 and 60 of ensiling in the inoculated silage than the control silage readily available as substrate for the yeast.

Kung and Ranjit [27] reported that the inoculants containing a blend of homolactic bacteria, propionibacteria, and enzyme improved silage fermentation by causing more extensive homolactic fermentation and resulted in silage with lower ADF and NDF content. This was similar to what was observed in this study in which silage inoculated with the combination of the two physicochemically different *L. plantarum* resulted in silage with reduced ADF and NDF content. Whereas, Adesogan *et al*. [28] in their own study reported that the bermudagrass silage ADF and NDF was not affected with treatment with inoculants containing mixture of *P. pentosaceau, L. buchneri* and beta-glucanase, alpha-amylase, and xylanase contrary to the findings of this study. Kung and Ranjit [27] also reported that their inoculant did not affect the CP of their silage which was similar to the report of Kleinschmit and Kung [24] in their study with corn silage inoculation with *L. buchneri* 40788 and *Pediococcus pentosaceaus* R1094 but contrary to the report of this study. Therefore, the combination of inoculants used in this study was able to increase the nutritional qualities of the silage by reducing the NDF and ADF and increasing the CP of the silage.

Silage is an important source of lactic acid for the domestic ruminant. Cattle may be maintained entirely on silage and the intake of lactic acid by these animals may amount to 1 kg per day. It is difficult to evaluate the utilization of this fraction for production. A number of trials have been carried out comparing milk production or weight gains, or both, in ruminants fed silage and hay made from the same source of plant material. Results from these trials have been variable [29-35] but, in general, the slightly smaller intake of dry matter when silage was fed was offset by a higher production per unit of dry matter. The net result of production from animals fed silage was

equal to or slightly greater than production from those fed hay. Therefore, the uninoculated silage observed to be well accepted in this study could have been of advantage in that the sheep could have a good production than feeding on inoculated silage. The reason why the animals consumed less of the inoculated silage compare to the uninoculated control could not be ascertain but it has been shown that the infusion of lactic acid into the rumen [36, 37], or supplementing the ration with lactic acid [32, 38-41], may cause a depression in voluntary intake of dry matter in animals fed hay [32, 37, 38, 39, 42]. The depression was usually less than that observed when silage was fed, although the amount of lactic acid added was similar to the quantities which would be ingested from feeding silage. However, Rooke [43] also suggested that the lactic acid may have a direct effect on palatibility, since sour taste has been associated with reduced palatability. Any direct effects of lactic acid on silage DM intake may be more important in the short term than the long term and related to a negative feedback [44]. Therefore, it may be the presence of high concentration of lactic acid as a result of rapid fermentation of the silage that was responsible for low acceptability of the silage.

CONCLUSION

The combination of the physicochemically different *L. plantarum* was effective in rapidly decreasing the NDF and ADF of the silage while the CP of the silage was also increased. But it was observed that ensiling for more than 45 days led decrease in the CP for the inoculated silage. We, therefore, suggest that in order to maintain good protein quality of the silage, silage inoculated with combination of these two *L. plantarum* should not be fermented up to 45 day before feeding out to animals.

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