Paper ISSN 0189-0514
Online ISSN 1427-4410
J. Anim. Prod. Res. (2022) 34(1):96-105

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NUTRIENT COMPOSITION, *IN VITRO* GAS AND METHANE PRODUCTION OF *Pleurotus* SPECIES DEGRADED GRASSES FOR THEIR USE IN RUMINANT DIETS

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ABSTRACT

Pleurotus-treated substrates are nutritionally improved feed that can serve as supplementary improved dry season feed for ruminants. This current study therefore treated 4 grasses (*Brachiaria decubens-BD, Panicum maximum var. local-PM and var. ntcisii- PMMN, and Pennisetum purpureum-PP)* collected during the dry season with three Pleurotus species (*P. ostreatus* -PO, *P. florida* – PF and *P. sajor-caju*- PS) at room temperature for 20 days. After this the dried treated grasses were chemically analyzed for nutrient composition and for *in vitro* studies (*in vitro* gas and methane gas production). All of the Pleurotus species improved the grasses' nutrient composition in terms of increased crude protein and decreased neutral detergent and acid detergent fibre values (67.74 – 136.36 g/kg DM, 730.66 - 678.87 g/kg DM, and 555.38 – 510.34 g/kg DM – BD), (51.20 – 78.28 g/kg DM, 731.34 - 717.23 g/kg DM, and 549.52 – 518.01 g/kg DM – PP), (40.29 – 89.93 g/kg DM, 789.63 – 754.28 g/kg DM, and 572.33 – 542.76 g/kg DM – PMMN), and (18.30 – 36.61 g/kg DM, 825.30 -777.54 g/kg DM, and 597.08 – 550.11 g/kg DM – PM); and improved the *in vitro* gas production of only 3 grasses (PMMN > BD >and PM) with the highest effect produced by PO which was complemented with negligible low methane gas production and percentage methane in total gas production. The lowest methane production in PF-treated grasses can be attributed to PF inability to improve the grasses' *in vitro* gas production. It can be concluded that PO was capable of improving the nutritive value of most of the grasses except PP for inclusion in ruminant feeding.

Keywords: *Dry season, Chemical composition, Fibre content, Pleurotus species, Grasses, Nutritive value.*

INTRODUCTION

In Nigeria's farming systems, ruminant animals (cattle, sheep, and goats) mostly depend on browses and natural pastures as the major source of feed throughout the year (Brum *et al.,* 2008). During the dry season, most of these forages are affected negatively by the harsh weather, thus leading to their scarcity. However, grasses such as *Cynodon* spp, *Pennisetum* spp, *Panicum* spp, and *Brachiaria* spp, etc. are able to survive due to their hardiness, persistence, and tendency to tolerate heat stress. Although they are available, they are usually low in quality in that they are extremely lignified and fibrous; low in protein content (around 2%); low in vitamins and minerals; which subsequently affects intake and digestibility (Minson, 2012) that influences animal production, performance, and health. This is evidenced by the poor performance of the animals in terms of reduction in growth rate, draught ability, production, and reproduction (Shelton, 2004). There is therefore the need to improve these grasses to meet animal nutrient requirements. One of the available methods of improvement

adopts the technique of degradation of lignin; a component that encapsulates and protects other polysaccharides (e.g. cellulose and hemicellulose) needed as a source of energy by animals from enzyme hydrolysis (Abdel-Hamid *et al*., 2013). Several methods (chemical, physical, physicochemical, and biological (bacteria, yeast, and fungi)) are able to achieve lignin degradation, of which fungal delignification has more pronounced benefits because they are cheap to obtain, require less capital, and are safe both to the environment, human and the animals (Sharma Arora, 2015). Among these fungi (brown rot, soft rot, and white rot), white rot fungi- WRF have greater potential in achieving lignin degradation with less effect on other polysaccharides (Guillén *et al*., 2005). The improvement is usually coupled with high fungal biomass deposition, a source of additional protein that aids in increasing the CP content of treated substrates. *Pleurotus* spp (WRF) has been mostly researched upon as it has no pathogenicity effect when consumed by ruminants and it is perceived to be capable of upgrading crop residues and agricultural by-products as reported by several researchers ((Tuyen *et al*., 2013; Nayan *et al*., 2018; Niu *et al*., 2018), although these fungi have not been tested with matured grasses as possible improved supplementary diets in ruminant feeding. In addition, the fungi also produce different outcomes even when used under the same growth condition but with different substrates. This current research, therefore, investigated the effect of three *Pleurotus* spp. (*P. ostreatus*, *P. florida*, *P. sajor-caju*) on the nutrient composition, *in vitro* gas, and methane gas production as well as the percentage methane in total gas production of four grasses (*Brachiaria decumbens* - BD, *Panicum maximum* var. ntchisi – PMMN, *Panicum maximum* var. local – PM, *Pennisetum purpureum* – PP).

MATERIALS AND METHODS

Pleurotus spawn source

The pure spawn of *Pleurotus ostreatus*, *Pleurotus florida and Pleurotus sajor-caju* were purchased from the Plant Pathology Department of Forestry Research Institute of Nigeria (FRIN), Jericho road, Ibadan Oyo state, Nigeria

Preparation of forages and inoculation with Pleurotus species

B. decumbens, *P. purpureum*, and *P. maximum* var. ntchisi and var. local were harvested from the Demonstration plot of Pasture and Range Management Department, Federal University of Agriculture, Abeokuta FUNAAB Nigeria in November 2020 during the late dry season. The grasses were chopped to about 3- 5 cm and dried under open air for 5 - 7 days. About 10g of each grass was weighed into 9 (3 Pleurotus species x 3 replicates) cleaned and dried Erlenmeyer flasks (250 ml), the content was soaked with water for 24hrs and afterward drained to remove excess water. The flasks were immediately covered with cotton wool and foil paper and then autoclaved at 121^0C for 15mins, cooled, and then inoculated with each of the Pleurotus species spawn (2 % w/v), covered and incubated at room temperature in a dark room in the laboratory for 20 days. Alongside the flasks containing the Pleurotus species treated grasses, 2 types of control flasks were provided which are the intact (grasses with no autoclaving or fungal inoculation or moisture) and autoclaved (grasses autoclaved but no fungal mycelium). On the 21st day, the various flasks excluding the control flasks were autoclaved to terminate the fungal mycelia growth. The samples were dried at 55- 60^0C until a constant weight is achieved, mill (2 mm in size), and then kept in bags for chemical analyses and *in vitro* study.

Chemical analyses

Sub samples of the intact, autoclaved, and Pleurotus species treated grasses were oven dried at 100^0C for 24hrs for dry matter (DM) determination and ashed at 550^0C for 5hrs in the muffle furnace for Ash determination (AOAC, 1990) methods. Crude protein was determined following the procedure of Bradford (1976). Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin were assessed using the methods of Van Soest *et al*. (1991) and

Goering and Van Soest (1970) without using sodium sulphite and Dekalin.

***In vitro* gas and methane production**

This was carried out following the procedure of Menke and Steingass (1988). Rumen content was collected from a freshly slaughtered cow in Odo-eran abbatoir, Abeokuta, Ogun State. The content was strained using four layers of cheesecloth to obtain rumen fluid (RF). The RF was then mixed with buffer solution (9.8 $NaHCO_3$ + 9.33 $Na_2HPO_4.12H_2O$ + 0.5 NaCl + 0.6 KCl + 0.01 ($CaCl_2$ anhydrous) + 0.1 ($MgCl_2$ anhydrous)) in 1: 2 ratio of RF: buffer in order to obtain buffered inoculum (BI). The pH of the BI was adjusted to 7.0 ± 0.2 using 1M HCl. The bottles containing the BI were slightly closed and kept in a water bath at 39 ^{0}C until used to incubate the test grasses. The grasses weighing 0.2 g (n =3) for each selected post-incubation period (24h, 48h, and 72h) were weighed into 50 ml calibrated transparent syringes to which the tip end was locked with a four-way male slip-stop cock and were inoculated with 20 ml of the BI. The syringes were then kept in the incubator set at 39±1°C for 72h to determine gas production of the intact, autoclaved, and pre-treated grasses. Blank syringes that contain only BI were provided to aid in correcting the gas volume of the test grasses from the gas emitted from the BI. The gas was measured at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60, 63, 66, 69 and 72hr. At the end of the selected post-incubation period (n=3), 4ml of NaOH (10M) was introduced to the selected syringes (n=3) to estimate the methane gas production as reported by Fievez *et al* (2005).

Statistical analysis and calculation

One-way ANOVA was used to analyze the chemical composition data. All data sets for the *in vitro* study parameters measured for each grass were statistically analyzed separately using the completely randomized design procedure on Minitab 16 software. A $p < 0.05$ was considered to indicate statistical significance. Various means were separated using the Tukey *post-hoc* test. The percentage of methane in total gas production was calculated by dividing the methane gas (ml/g) by the gas produced (ml/g) x 100.

RESULTS AND DISCUSSION

The chemical compositions of the various Pleurotus spp-treated grass with the various controls (intact and autoclaved) are presented in Table 1. Most autoclaved grasses produced similar ($p > 0.05$) slightly lower CP and ash contents as well as similar ($p > 0.05$) slightly lower NDF and ADF contents than intact grasses. This is in support of the findings of Siah *et al*. (2014) when the effect of autoclaving was investigated on the nutrient composition of faba beans. The reduction in fibre content of the autoclaved grasses might indicate that the autoclaving was capable of slightly breaking some structural bonds while the loss in CP and ash might be that the soluble released after degradation of structural bonds is lost by volatilization, denaturing, etc. (El-Waziry *et al*., 2007; Udensi *et al*., 2010). When compared to the control and autoclaved grasses, Pleurotus species treatment of the grasses significantly ($P < 0.05$) increased the ash and CP content as well as decreased the NDF, and ADF contents with greater increasing and decreasing effects recorded by *P. ostreatus* treatment. The CP increase by the Pleurotus spp could be as a result of fungal biomass (mycelium/chitin) addition or the secretion of proteinous enzymes into the substrates during degradation while fibre reduction might be due to the fungus enzymatic activities potential in degrading structural carbohydrates (van Kuijk *et al*., 2015).

The obtained results were in agreement with the findings of Akinfemi (2010a), Tuyen *et al*. (2013); Niu *et al*. (2018), and Zuo *et al*. (2018). The increased CP values ranged from 67.74 – 136.36 g/kg DM (BD), 51.20 – 78.23 g/kg DM (PP), 40.29 – 89.93 g/kg DM (PMMN), and 18.30 – 36.61g/kg DM (PM). The reduced NDF values ranged from 730.66 – 678.87 g/kg DM (BD), 731.34 – 717.23 g/kg

DM (PP), 789.63 – 754.28 g/kg DM (PMMN), and 83.30 – 777.54 g/kg DM (PM) while the reduced ADF values ranged from 555.38 - 510.34 g/kg DM (BD), 549.52 – 518.01 g/kg DM (PP), 572.33 – 542.76 g/kg DM (PMMN), and 597.08 – 550.11 g/kg DM (PM). The differences obtained in the grasses nutrient composition can be influenced by several factors such as the degree of crystallinity of the grass structural carbohydrate, Pleurotus species used, and autoclaving effect etc. (Mahesh and Mohini, 2013; Siah *et al*., 2014; Zheng and Rehman, 2014). *P. ostreatus* treated grasses recorded the lowest NDF and ADF contents as well as the highest CP contents. The greater potential of *P. ostreatus* in improving the nutrient composition more than other species could be attributed to its high enzymatic activity during degradation and the release of more fungal biomass as additional source of protein (Maza *et al*., 2015).

Table 1: Chemical composition (g / kg DM) of intact, autoclaved (i.e. control) and 21 days Pleurotus treated grasses

Grasses	Nutrients	Intact	Control	*P. ostreatus*	*P. florida*	*P. sajor-caju*	SEM	p value
BD	DM(g/kg)	943.60^{b}	935.36^{c}	951.57^{a}	955.08^{a}	956.18^{a}	2.17	0.000
				g / kg DM				
	Ash	72.13^{c}	79.39^{c}	108.02^{b}	111.36^{b}	130.21^{a}	5.78	0.000
	CP	77.89^{c}	67.74^{c}	122.52^{a}	104.33^{b}	136.36^{a}	7.05	0.000
	NDF	730.66^{a}	718.43^{a}	678.87^{c}	693.01^{b}	684.50^{bc}	5.47	0.000
	ADF	555.38^{a}	546.84^{b}	510.34^{c}	542.15^{b}	515.80^{c}	4.77	0.000
PP	DM	907.88^{a}	904.79^{a}	887.99^{b}	877.89^{b}	900.72^{a}	3.17	0.000
	Ash	104.17^{b}	100.58^{b}	121.62^{a}	117.91^{a}	119.76^{a}	2.38	0.000
	CP	57.43^{bc}	51.20^{c}	78.23^{a}	69.32^{abc}	71.77^{ab}	3.07	0.006
	NDF	731.34^{a}	728.90^{a}	717.23^{b}	725.58^{ab}	722.04^{ab}	1.55	0.006
	ADF	549.52^{a}	541.60^{a}	518.01^{b}	523.28^{b}	523.42^{b}	3.36	0.000
PMMN	DM	934.71^{a}	930.91^{ab}	914.60^{c}	928.40^{ab}	922.54^{bc}	2.03	0.000
	Ash	89.04^{d}	84.54^{d}	105.88^{b}	114.64^{a}	97.71^{c}	2.97	0.000
	CP	43.76^{b}	40.29^{b}	89.93^{a}	74.95^{a}	80.24^{a}	5.52	0.000
	NDF	789.63^{a}	785.15^{a}	754.28^{c}	764.32^{b}	757.99^{bc}	3.90	0.000
	ADF	572.33^{a}	567.16^{b}	542.76^{e}	562.14^{c}	557.09^{d}	2.72	0.000
PM	DM	949.62	946.31	947.96	946.38	950.39	0.99	0.651
	Ash	61.92^{b}	54.44^{c}	66.34^{ab}	63.03^{ab}	68.89^{a}	1.40	0.000
	CP	18.30^{b}	27.52^{ab}	36.61^{a}	32.01^{a}	36.60^{a}	2.08	0.003
	NDF	825.30^{a}	821.44^{a}	777.54^{c}	809.35^{b}	782.79^{c}	5.29	0.000
	ADF	597.08^{a}	591.71^{a}	550.11^{c}	582.54^{b}	552.40^{c}	5.31	0.000

DM (dry matter), CP (Crude protein), NDF (neutral detergent fibre), ADF (Acid detergent fibre), ADL (Acid detergent lignin), HEM (hemicellulose), CEL (cellulose), TA (total antioxidant), SEM (Standard error of means)
^{a,b,c} Values within a *row with different superscripts are significantly different*

Figure 1 shows the *in vitro* gas production of the intact (untreated), autoclaved, and Pleurotus species treated grasses after 0h, 12h, 24h, 36h, 48h, 60h and 72h of incubation. The various grass treatment produced different total *in vitro* gas amount. These amounts were not comparable with the *in vitro* digestibility of energy feedstuff needed in ruminants feed (Cone *et al*., 1999) which indicates that the treated grasses can only be used as supplementary feed. The amounts were similar to the values (33 – 72 ml/g DM) reported by Kinfemi *et al*. (2009) for cowpea shells treated with Pleurotus specie but lower than the values

(160 – 270 ml/g OM) reported by Zuo *et al*. (2018) for corn stover treated with different fungal species. The variation might be due to differences in fungal species, strain and characteristics, length of treatment and the substrate used (Arora and Sharma, 2009). Autoclaving did not have the highest effect on the total gas production of BD, PMMN, and PM but increased the total gas production of treated PP. This might be attributed to the level of carbohydrate bond loosening in the grasses.

The obtained variation in responses of substrates to autoclaving was similar to the report of Tuyen *et al*. (2013) for fibrous agricultural products treated with different fungal species. When compared to the intact and autoclaved grasses, the Pleurotus species increased the *in vitro* gas production of almost all of the grasses in this order: PMMN > BD > PM but did not increase the *in vitro* gas production of PP. The obtained increased *in vitro* gas production in most of the treated grasses was in agreement with the reports of Okano *et al*. (2005) and Akinfemi (2010b); and Niu *et al*. (2018). Among the Pleurotus species, the highest *in vitro* gas production was recorded in *P. ostreatus* treated grasses. The high *in vitro* gas production might indicate the availability of more soluble carbohydrate in these treated grasses as gas production is seen as a measure of carbohydrates degradability (Cone and Becker, 2012; Zuo *et al*., 2018) or might be attributed to the species degrading potential (Niu *et al*., 2018). Also the variation in the grasses *in vitro* gas production could be attributed to the rate of chitin availability as high chitin tends to reduce feed degradability from which the gas is been released (Di Mario *et al*., 2008) or it might be related to the availability of high CP and less structural carbohydrate (NDF and ADF) in the treated grasses (Akinfemi, 2010a). The inability of all the Pleurotus species to improve PP *in vitro* gas production might be attributed to the grass nutrient contribution to gas production. Therefore the use of Pleurotus species treated *P. purpureum* in ruminant diets might not be the best approach. Also among the improved grasses, BD and PMMN seems promising more than PM due to their higher improvement through the treatment.

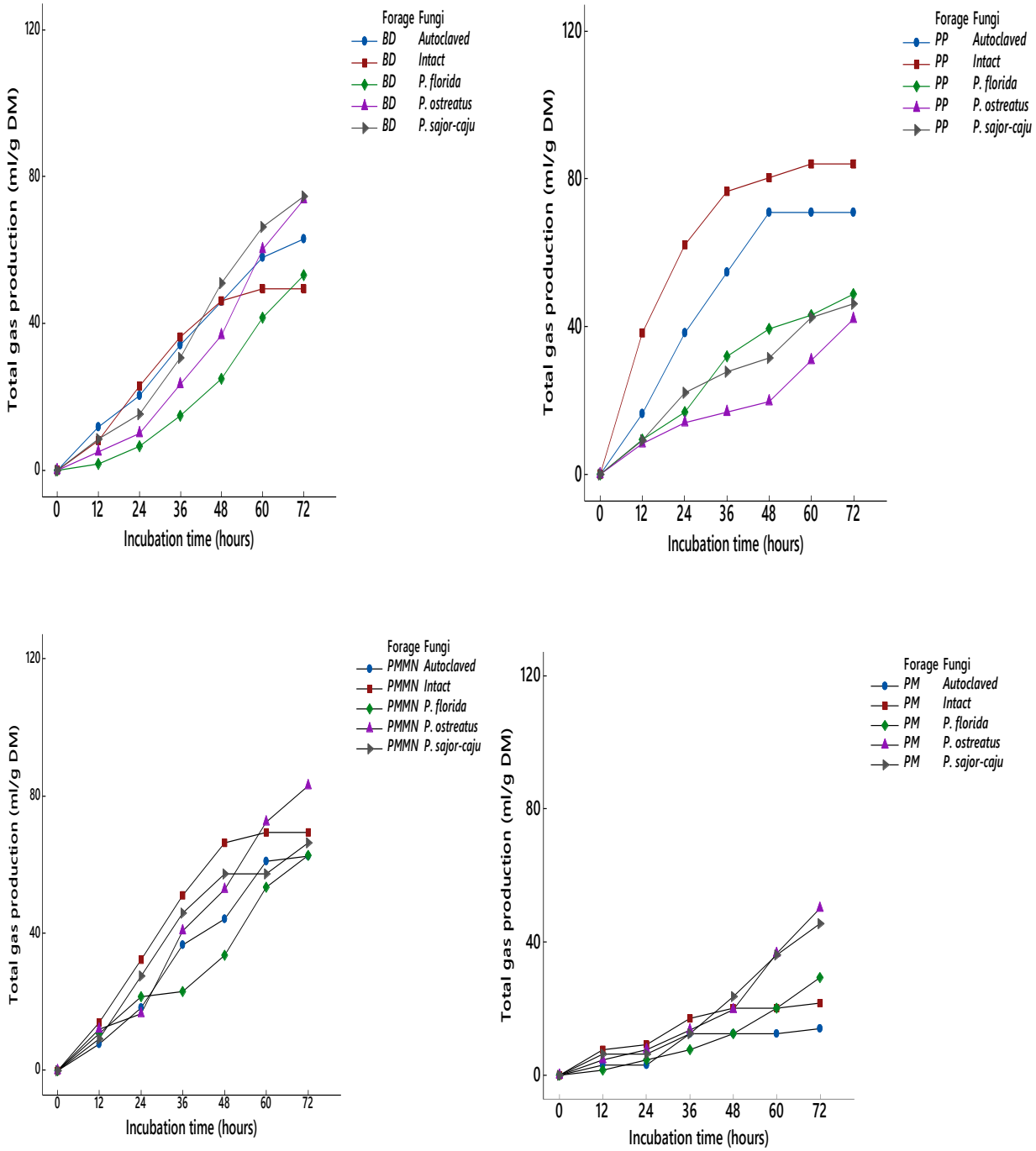


Figure 1: *Total gas production (ml / g OM) of intact, autoclaved (i.e. control) and Pleurotus treated grasses after 72h of incubation in rumen fluid. BD: B. decumbens; PP: P. purpureum; PMMN: P. maximum var. ntchisi; PM: P. maximum*

Table 3 shows the methane gas production of intact, autoclaved and Pleurotus species treated grasses at 24h, 48h, and 72h of incubation. All autoclaved grasses produced lesser methane gas than intact grasses. This indicates lower structural carbohydrates in the autoclaved grasses than intact grasses as highly structural feeds produce high methane gas (Kim *et al*., 2013). Among the grasses, the autoclaving process only reduced the methane gas production of PM, this could be due to the reduced IVGP of the grass. When compared to intact grasses, Pleurotus species treatment reduced the methane gas production of BD, PP, and PMMN but increased the methane gas production of PM. The reduced methane gas production for some of the treated grasses was not in agreement with the findings of Kinfemi *et al*. (2009) for cowpea shells treated with fungal species. The increased methane gas production in treated PM was in support of the findings of Tuyen *et al*. (2013) for fungal-treated oil palm fronds. The differences in methane gas production could be linked to the total gas production of the grasses or a shift in the kind of volatile fatty acids (acetic, propionic, butyric) produced by the grasses (Kamalak *et al*., 2002). Acetic and butyric production does have a positive relationship with methane production while propionic does not (Kim *et al*., 2013). Among the Pleurotus species treated grasses at 72h of incubation, *P. florida* treated BD, PMMN, and PM recorded the least methane gas production and this can be linked to its inability to improve the grasses nutrient degradability which is seen in the reduced IVGP of these grasses. On the other hand, the Pleurotus species produced similar and reduced methane gas production in PP. The reduced methane gas production is also attributable to the inability of all the species to improve its IVGP.

Table 3: Methane gas production (ml / g DM) of intact, autoclaved (i.e. control) and Pleurotus species treated grasses after 24, 48 and 72 hours of incubation

Grasses	Incubation time (h)	Intact	Autoclaved	*P. ostreatus*	*P. florida*	*P. sajor-caju*	SEM	p value
				Methane gas production (ml / g DM)				
BD	24	1.09^{a}	0.92^{a}	0.25^{b}	0.15^{b}	0.27^{b}	0.11	0.000
	48	2.93^{a}	2.76^{a}	1.73^{ab}	0.65^{b}	2.25^{a}	0.24	0.001
	72	6.60^{a}	6.29^{ab}	4.93^{b}	2.65^{c}	5.73^{ab}	0.57	0.000
PP	24	3.75^{a}	2.32^{b}	0.27^{c}	0.56^{c}	1.02^{c}	0.47	0.000
	48	10.84^{a}	9.71^{a}	1.30^{b}	2.74^{b}	2.78^{b}	1.16	0.000
	72	14.26^{a}	11.09^{b}	3.94^{c}	4.30^{c}	4.85^{c}	1.22	0.000
PMMN	24	1.44^{a}	0.54^{c}	0.52^{c}	0.73^{bc}	1.08^{ab}	0.18	0.000
	48	10.15^{a}	5.90^{b}	5.52^{b}	3.47^{c}	6.50^{b}	0.60	0.000
	72	14.07^{a}	11.47^{bc}	13.56^{ab}	9.41^{c}	10.97^{c}	0.56	0.000
PM	24	0.16	0.10	0.09	0.10	0.06	0.02	0.307
	48	0.34	0.29	0.32	0.28	0.27	0.08	0.695
	72	0.47^{b}	0.40^{b}	2.37^{a}	0.96^{b}	2.45^{a}	0.30	0.000

SEM (Standard error of means),
^{a,b,c} Values within a *row with different superscripts are significantly different*

The proportion of CH_4 in total gas production after 72h of incubation of the intact, autoclaved, and Pleurotus species treated forages is presented in Figure 2. A similar percentage of methane in total gas was recorded for most of the autoclaved grasses and intact grasses except for BD. This is in agreement with the findings of Tuyen *et al*. (2013) for fibrous agricultural by-products treated with different fungal species. Pleurotus species-treated grasses recorded a lesser percentage of methane in IVGP than the intact and autoclaved grasses. This indicates that Pleurotus species were capable of improving

the *in vitro* degradability of these grasses with negligible reduced methane production, which is a good attribute of improved ruminant feed as the feed energy is not lost in the form of methane. Among the Pleurotus species, *P. florida* led to a lesser percentage of methane in IVGP for all of the treated grasses, followed by *P. ostreatus* and lastly by *P. sajor-caju*. It can be deduced from this study that the Pleurotus specie which produced less methane gas recorded less percentage methane in total gas production. This is similar to the observation of Kinfemi *et al*. (2009) for cowpea shells treated with different fungal species but not in agreement with the findings of Tuyen *et al*. (2013) where the *P. ostreatus* treated oil palm fronds and rice straw produced similar percentage methane gas to the control. The variations can be attributed to fungal specie used, substrate nutrient composition, growth condition, and length of treatment. Therefore the use of *P. florida* to improve the nutritive quality of these grasses is discouraged as methane gas reduction is due to its inability to improve the nutrient degradability of these grasses, so *P. ostreatus* is mostly preferred.

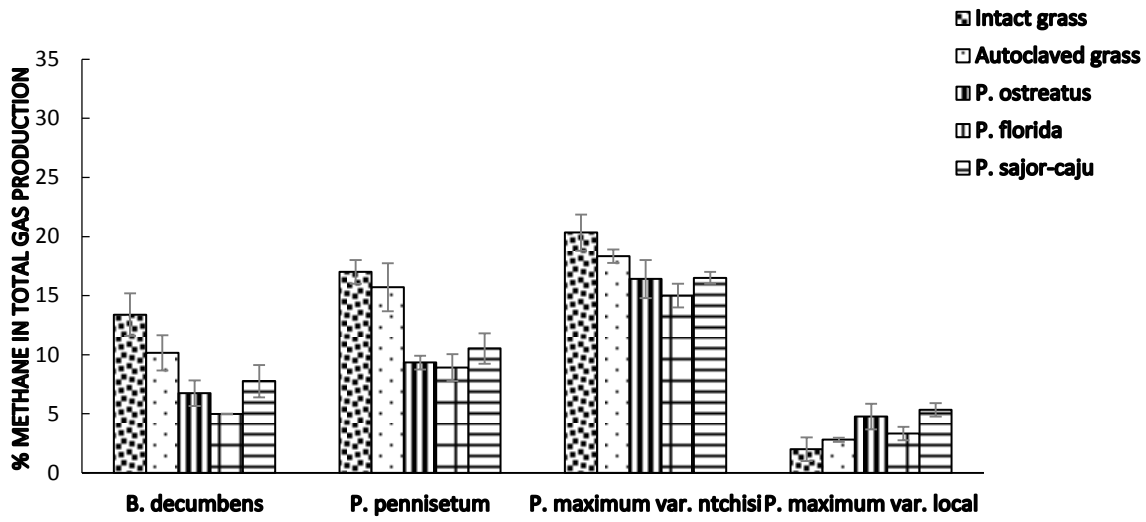


Figure 2: *The percentage of methane in total gas produced by intact, autoclaved, and Pleurotus species-treated grasses incubated for 72h*

CONCLUSION

P. ostreatus have a greater ability to improve the nutrient composition of all the treated grasses than *P. sajor-caju* and *P. florida*. All of the Pleurotus species were unable to improve the *in vitro* gas production of *P. purpureum*, but were able to improve that of all other treated grasses in this order: PMMN > BD > PM with the highest *in vitro* gas production in *P. ostreatus* treated grasses. The high *in vitro* gas production was coupled with negligible reduced methane gas and percentage methane in total gas in *P. ostreatus* and *P. sajor-caju* treated *Panicum maximum* var. ntchisi and *B. decumbens* respectively when compared to the intact grasses. Therefore, based on the outcome of this study, *P. ostreatus* or *P. sajor-caju* treated *Panicum maximum* var. ntchisi and *B. decumbens* can be recommended as supplementary improved feed in ruminants' feeding program during the dry season.

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