

Microbial Population Dynamics during Anaerobic Digestion of Guinea Grass (*Panicum maximum*)

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Received July 26, 2014; Revised November 04, 2014; Accepted November 26, 2014

Abstract The effect of rumen fluid on microbial population dynamics during anaerobic digestion of Guinea grass (*Panicum maximum*) at ambient condition with respect to time was investigated. A one stage batch-type mesophilic anaerobic digestion system was configured using rumen fluid (RF) as inoculum (AD_{RF}) and a low solid loading of approximately 7.0% total solid (TS). Physicochemical parameters such as process temperature (PTM_{RF}), process pH_{RF}, chemical oxygen demand (COD_{RF}) and volatile fatty acid (VFA_{RF}) were monitored with time. Selected indicator microbial populations were monitored by standard cultural enumerations based on metabolic capacity and oxygen sensitivity with respect to time. Furthermore, their respective growth rates and population proportions were determined. Result showed that the average PTM_{RF} increased from 27.5°C to 35.2°C while average process pH_{RF} ranged from 6.5 to 7.9 with time, respectively. The COD_{RF} decreased from 11,250.60 mg/L to 2,865.20 mg/L, while VFA_{RF} ranged from 1,080.00 mg/L to 4,800.33 mg/L with time, respectively. In terms of metabolic capacity, the populations of cellulolytic bacteria (ACB_{RF}), lactose fermenting bacteria (LFB_{RF}) and glucose fermenting bacteria (GFB_{RF}) ranged from 3.6 x 10⁴ MPN/ml to 2.9 x 10⁵ MPN/ml, 3.4 x 10⁴ MPN/ml to 2.9 x 10⁵ MPN/ml and 4.4 x 10⁴ MPN/ml to 4.6 x 10⁵ MPN/ml respectively with time. The populations of propionate oxidizing bacteria (POB_{RF}), ethanol oxidizing bacteria (EOB_{RF}) and acetate oxidizing methanogens (AOM_{RF}) ranged from 2.9 x 10⁴ MPN/ml to 2.4 x 10⁵ MPN/ml, 2.7 x 10⁴ MPN/ml to 2.1 x 10⁵ MPN/ml and 1.4 x 10⁴ MPN/ml to 2.1 x 10⁵ MPN/ml respectively with time. In terms of O₂-sensitivity, the populations of obligate anaerobic bacteria (OAB_{RF}) and facultative aerobic and anaerobic bacteria (FAAB_{RF}) ranged from 2.12 x 10⁵ CFU/ml to 4.53 x 10⁶ CFU/ml and 4.6 x 10⁵ CFU/ml to 4.74 x 10⁶ CFU/ml respectively with time. The population of GFB_{RF} had the highest growth rate of 0.057 day⁻¹ while the population of EOB_{RF} had the lowest growth rate of 0.021 day⁻¹. In terms of O₂-sensitivity, the population of FAAB_{RF} had the highest growth rate of 0.051 day⁻¹ compared to the population of OAB_{RF} with growth rate of 0.040 day⁻¹. The population of GFB_{RF} predominated (26.3%), while the population of AOM_{RF} were the minority (10.44%). In terms of O₂-sensitivity, the population of FAAB_{RF} predominated (56.73%) compared to the population of OAB_{RF} (43.23%). Rumen fluid significantly (p < 0.05) increased the microbial populations inside AD_{RF} with respect to time. Therefore, rumen fluid could be used to boost the microbial population in anaerobic digesters as this could enhance depolymerisation, obtain higher degradation rates of cellulosic (or lignocellulosic) substrates and thus higher energy (biogas/methane) benefits.

Keywords: Anaerobic digestion (AD), Guinea grass, Rumen fluid, microbial population dynamics

Cite This Article: Ogbonna C. B., Ibiene A. A., and Stanley H. O., "Microbial Population Dynamics during Anaerobic Digestion of Guinea Grass (*Panicum maximum*)."
Journal of Applied & Environmental Microbiology, vol. 2, no. 6 (2014): 294-302. doi: 10.12691/jaem-2-6-5.

1. Introduction

One of the most important agricultural crops in Nigeria is Guinea Grass (*Panicum maximum*). It is a native of Africa, widely distributed and readily available in the country. It has leaves that are fine and soft and contains good levels of protein (13-21%) and can be grown for uses such as; pasture, silage, hay, and rough grazing. Bio-energy, especially biogas production can provide a new prospect for grassland use in the country without affecting the balance of the ecosystem (Gerin *et al.*, 2008; Beatrice

et al., 2009; Ahn *et al.*, 2010; Uzodinma and Ofoefole, 2009). Anaerobic degradation of organic matter involves a series of microbial metabolic reactions such as hydrolysis, acidogenesis, acetogenesis and methanogenesis (Themelis and Ulloa, 2007; Schnurer and Jarvis, 2010) and it has the potential to provide useful products such as biofuel (e.g., biogas) and organic amendment for agricultural soil (Chanakya *et al.*, 2007; Schnurer and Jarvis, 2010). Anaerobic digestion of cellulosic (or lignocellulosic) materials is limited mainly by the rate of hydrolysis of the polymeric compounds (Labat and Garcia, 1986). These substrates are usually devoid of appropriate microflora and, to be efficiently fermented, require suitable

inoculation (e.g., from sludge or cow dung, etc.) for biogas production (Labat and Garcia, 1986; Lopes *et al.*, 2004; Forster-Carneiro *et al.*, 2007; Dong *et al.*, 2009; Uzodinma and Ofoefole, 2009).

The Potential applications of rumen microorganisms [See Allison and Leek, 1993] in AD systems for the conversion of lignocellulosic materials were investigated (Dalhoff *et al.*, 2003; Barnes & Keller 2003, 2004). For example, O'Sullivan *et al.*, (2006) studied cellulose solubilisation rates in batch reactors using rumen culture and MSW leachate as sources of inocula and microcrystalline cellulose as substrate. The authors obtained about 34-50% higher cellulose solubilisation rates when rumen culture was used as an inoculum source than with the MSW leachate. Furthermore, Hu and Yu (2005), studied AD of corn stover in batch and semi-continuous cultures and obtained a high VS conversion efficiency of 65-70% after 10 days of incubation at 25-40°C. In another such study by Yue *et al.*, (2007), a degradation efficiency of 52.3% was achieved during AD of aquatic plant such as *Canna indica L.* using rumen cultures in batch experiments. Total bacterial count was generally estimated to be 10^{10} bacteria/ml in stabilized anaerobic digesters. These results were obtained after addition of rumen fluid or biodigester juice to the enumeration media (Hobson and Shaw 1973; Iannotti *et al.* 1978; Ueki *et al.*, 1978). Due to the high anaerobic bacteria population inside the rumen of Cow [Aurora, 1983; Allison and Leek, 1993] and the abundance of rumen waste disposal from slaughterhouses in Nigeria, this study focused on the effect of rumen fluid on the population dynamics of the microbial community taking part in anaerobic digestion of the Guinea grass (*P. maximum*) at ambient condition with respect to time using selected indicator microbial groups.

2. Materials and Methods

2.1. Anaerobic Digestion Set-up

One-stage 500 litre – capacity anaerobic digesters (AD) were configured for batch-type mesophilic reactors with useful volumes of around 310.30 litres respectively. The anaerobic digester with rumen fluid inoculation (AD_{RF}) was the experimental set-up, while the anaerobic digester without rumen fluid inoculation (AD_{NRF}) was the control set-up. Anaerobic digestion of the feed was performed inside the biodigesters at ambient condition and a retention time of 105 days. Cow's rumen content was collected from an abattoir in Aluu community located in Rivers State (Nigeria). After collection, Rumen fluid was prepared and stored in an air-tight 60L – capacity gallon for a month as described by Budiyo *et al.*, (2009). Furthermore, Budiyo *et al.*, (2009) suggested that keeping the rumen fluid for such a period of time does not affect the microbial community inside the fluid. Matured guinea grass was harvested within Abuja Campus (University of Port Harcourt, Nigeria), chopped into smaller pieces, minced and weighed with a weighing balance. Samples were immediately taken to the laboratory for determination of water content (WC) and total solid (TS) content of the grass using the USEPA (2001) method 1684. The feed inside both AD systems (AD_{RF} and AD_{NRF}) was prepared as presented in Table 1 using the formula below (Yadvika, *et al.*, 2004);

$$\text{Solid content (\%)} = \frac{\text{Mass of dry grass} \times 100}{\text{Mass of dry grass} + \text{Volume of H}_2\text{O}}$$

Table 1. Composition of the Feed for Anaerobic digestion

System	Substrate	TS (Kg)	WC (Kg)	WS* (Kg)	RF* (L)	WA* (L)	Total (L)	%TS
AD _{RF}	<i>P. maximum</i>	21.72	18.28	40.00	50.00	220.30	310.30	~7.00
AD _{NRF}	<i>P. maximum</i>	21.72	18.28	40.00	0.00	270.30	310.30	~7.00

TS = Total solid, WC = Water content, WS = Wet solid, RF = Rumen fluid, WA = Water, AD_{RF} = Anaerobic digester with rumen fluid inoculation, AD_{NRF} = Anaerobic digester without rumen fluid inoculation, * = Feed components.

2.2 Sample Collection and Determination of Physicochemical Parameters

To monitor the anaerobic digestion (AD) process, digester slurry samples were collected anaerobically at two-week intervals from the anaerobic digesters into air tight sterile sample bottles by not allowing any head space in the bottles and transported to the laboratory in a PVC Box for physicochemical and microbiological analyses. However, the last sample was collected at three-weeks interval (i.e., day 0, day 14, day 28, day 42, day 56, day 70, day 84 and day 105, respectively). Process temperature (PTM) and pH were measured using water thermometer (SCT-lilliput, Scichem Tech.) and a general purpose pH meter (SCT-lilliput, Scichem Tech.), respectively. Chemical oxygen demand (COD) and total volatile fatty acid (VFA) were determined using the titrimetric methods described by Reaffirmed (2006) and Buchauer (1998), respectively.

2.3. Enumeration of Selected Microbial Population

Immediately after collection and homogenization of biodigester slurry samples, the samples were serially diluted (anaerobically) from 10^{-1} to 10^{-6} using the reduced mineral solution of Ralph (2011) as described by Labat and Garcia, (1986) and Claudia *et al.* (2009) respectively. Bacteria populations were evaluated by counting selected groups based on metabolic capacity and oxygen sensitivity, respectively. The metabolic groups selected included the populations of cellulolytic bacteria (ACB), lactose fermentative bacteria (LFB), glucose fermentative bacteria (GFB), propionate oxidizing bacteria (POB), ethanol oxidizing bacteria (EOB) and acetate oxidizing methanogens (AOM), respectively. They were enumerated anaerobically by the Most Probable Number (MPN) technique (n=3) described by Labat and Garcia, (1986) and Claudia *et al.* (2009) respectively and incubated at 35°C with incubation times depending on the metabolic group (Diaz *et al.*, 2002). The MPN results were interpreted with appropriate tables from Oblinger and Koberger (1975) and reported as most probable number per millimetre (MPN/ml) of the digester slurry sample. The growth of POB, EOB and AOM was respectively reported as positive tube(s) in biogas production (Claudia

et al., 2009). The growth of GFB and LFB was respectively evaluated for acidification (i.e., change in medium colour from blue to yellow) and gas production, while the growth of cellulolytic bacteria (ACB) was evaluated for yellow or brown pigmentation (or deposits) and or gas production (Claudia *et al.*, 2009).

The oxygen-sensitive groups selected included obligate anaerobic bacteria (OAB) and facultative aerobic and anaerobic bacteria (FAAB) populations, respectively. They were enumerated by the pour plate count method described by Bryant (1972) and incubated at 35°C for 72 and 48 hours, respectively. Their growth was reported as colony forming unit per millimetre (CFU/ml) of digester slurry sample. After enumeration, the growth rate and the proportion of all selected microbial populations were estimated from their respective growth curves using the formulae stated in Dubey and Maheshwari (2008);

$$\text{Growth Rate } (K_r) = \frac{2.302 (\text{Log}_{10} N_2 - \text{Log}_{10} N_1)}{T_2 - T_1}$$

$$\text{Proportion } (\%) = \frac{\text{Cumulative Population of Individual Metabolic Groups} \times 100}{\text{Total Cumulative Population of all Selected Metabolic groups}}$$

$$\text{Proportion } (\%) = \frac{\text{Cumulative Population of Individual Oxygen - Sensitive Groups} \times 100}{\text{Total Cumulative Population of all Selected Metabolic groups}}$$

Where;

T_1 = Initial time, T_2 = Final time, N_1 = Cell number at T_1 , N_2 = Cell number at T_2

2.4. Statistical Analysis

Within the Microsoft Excel (2013) environment, the two-factor analysis of variance (2-Way ANOVA) was used to determine if there was a significant difference between the population of microbes inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and the population of microbes inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) system.

3. Result and Discussion

3.1. Physicochemical Analysis

The average process temperature (PTM_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 27.5°C to 35.2°C with time while the average process temperature (PTM_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 27.5°C to 34.4°C with time. The average process pH (pH_{RF} and pH_{NRF}) inside AD_{RF} and AD_{NRF} ranged from 7.9 to 7.4 and 8.1 to 7.3 respectively with time. The COD_{RF} inside AD_{RF} decreased from 11,250.60 mg/L to 2,865.20 mg/L while the COD_{NRF} inside AD_{NRF} decreased from 11,250.20 mg/L to 4,538.90 mg/L with time. The concentration of volatile fatty acid (VFA_{RF}) inside AD_{RF} increased from 1,080.00 mg/L to 4,800.33 mg/L while the concentration of volatile fatty

acid (VFA_{NRF}) inside AD_{NRF} increased from 729.34 mg/L to 4,632.04 mg/L with time.

3.2. Microbiological Analysis

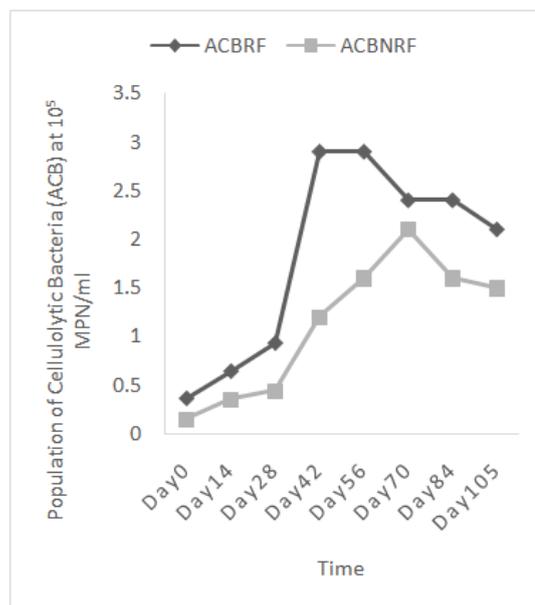


Figure 1. Population dynamics of cellulolytic bacteria (ACB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of cellulolytic bacteria (ACB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*

Generally, the indicator bacteria populations enumerated during anaerobic digestion of *Panicum maximum* increased with respect to time as shown in Figure 1 to Figure 8. The population of cellulolytic bacteria (ACB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 3.6×10^4 MPN/ml at day 0 to 6.4×10^4 MPN/ml and 9.3×10^4 MPN/ml at day 14 and day 28, respectively. The population peaked at 2.9×10^5 MPN/ml on day 42 and day 56 respectively. However, at day 70, day 84 and day 105, this population decreased slightly to 2.4×10^5 MPN/ml and 2.1×10^5 MPN/ml respectively (Figure 1). Likewise, the population of cellulolytic bacteria (ACB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 1.5×10^4 MPN/ml at day 0 to 3.5×10^4 MPN/ml, 4.4×10^4 MPN/ml, 1.2×10^5 MPN/ml and 1.6×10^5 MPN/ml at day 14, day 28, day 42 and day 56, respectively. The population peaked at 2.1×10^5 MPN/ml on day 70 however, at day 84 and day 105, this population decreased slightly to 1.6×10^5 MPN/ml and 1.5×10^5 MPN/ml respectively (Figure 1). The periods when the populations of CB_{RF} and CB_{NRF} increased, peaked and then decreased during anaerobic digestion of the grass (*Panicum maximum*) indicated that cellulose hydrolysis (or cellulolytic activity) may have increased, peaked and then decreased at those times inside AD_{RF} and AD_{NRF} , respectively (Claudia *et al.*, 2009; Labat and Garcia, 1986).

The population of lactose fermenting (acidogenic) bacteria (LFB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 3.4×10^4 MPN/ml at day 0 to 7.5×10^4 MPN/ml, 1.2×10^5 MPN/ml and 2.1×10^5 MPN/ml at day 14, day 28 and day 42 respectively. The population peaked at 2.9×10^5 MPN/ml

on days 56 and 70, respectively. However at day 84 and day 105, this population decreased to 2.4×10^5 MPN/ml and 2.1×10^5 MPN/ml respectively (Figure 2). Likewise, population of lactose fermenting (acidogenic) bacteria (LFB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 1.6×10^4 MPN/ml at day 0 to 3.6×10^4 MPN/ml, 6.4×10^4 MPN/ml, 1.2×10^5 MPN/ml and 1.5×10^5 MPN/ml at day 14, day 28, day 42 and day 56 respectively. The population peaked at 1.6×10^5 MPN/ml on day 70 however, this population decreased to 1.5×10^5 MPN/ml and 1.2×10^5 MPN/ml at day 84 and day 105 respectively (Figure 2).

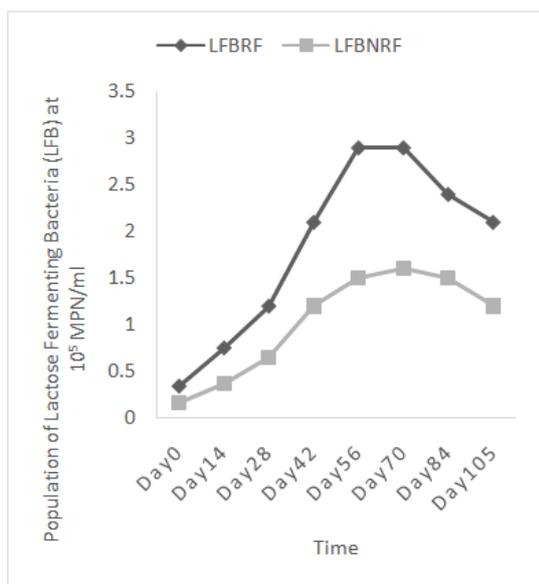


Figure 2. Population dynamics of lactose fermentative bacteria (LFB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of lactose fermentative bacteria (LFB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*.

The population of glucose fermenting (acidogenic) bacteria (GFB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 4.4×10^4 MPN/ml at day 0 to 9.3×10^4 MPN/ml and 1.6×10^5 MPN/ml at day 14 and day 28 respectively. The population peaked at 4.6×10^5 MPN/ml (on day 42 and day 56, respectively). However, this population decreased to 2.9×10^5 MPN/ml and 2.4×10^5 MPN/ml at day 70, day 84 and day 105 respectively (Figure 3). Likewise, the population of glucose fermenting (acidogenic) bacteria (GFB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 2.6×10^4 MPN/ml at day 0 to 4.4×10^4 MPN/ml, 7.5×10^4 MPN/ml, 1.6×10^5 MPN/ml and 2.1×10^5 MPN/ml at day 14, day 28, day 42 and day 56 respectively. The population peaked at 2.4×10^5 MPN/ml on day 70 however, this population decreased to 2.1×10^5 MPN/ml at day 84 and day 105 respectively (Figure 3). The periods when the respective populations of LFB_{RF} and GFB_{RF} and LFB_{NRF} and GFB_{NRF} increased, peaked and then decreased during anaerobic digestion of the grass (*Panicum maximum*) indicated that fermentation (or acidogenic activity) may have increased, peaked and then decreased at those times inside AD_{RF} and AD_{NRF}, respectively (Claudia *et al.*, 2009; Labat and Garcia, 1986). This is because fermentation of

sugars in anaerobic digestion processes have been associated with acidogenesis (Ike *et al.*, 2010; Schnurer and Jarvis, 2010; Chanakya and Sreesha, 2012; Ljupka, 2010; Yassar, 2011).

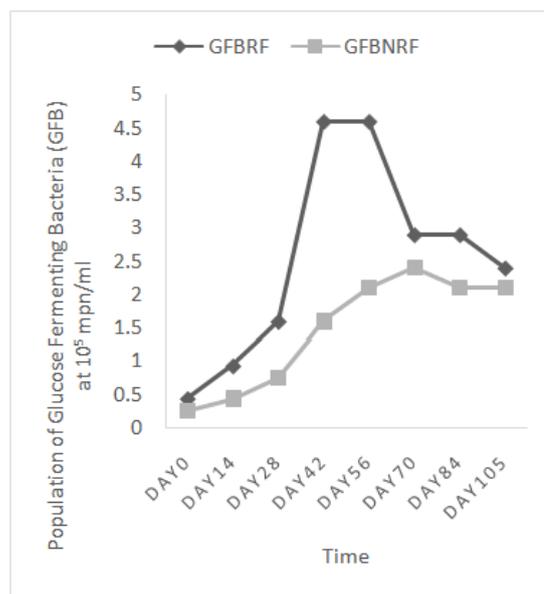


Figure 3. Population dynamics of glucose fermentative bacteria (GFB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of glucose fermentative bacteria (GFB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*.

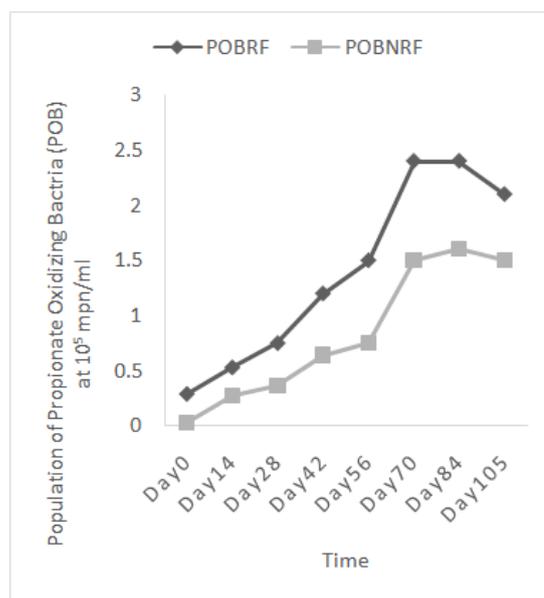


Figure 4. Population dynamics of propionate oxidizing bacteria (POB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of propionate oxidizing bacteria (POB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*.

The population of propionate oxidizing (acetogenic) bacteria (POB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 2.9×10^4 MPN/ml at day 0 to 5.3×10^4 MPN/ml, 7.5×10^4 MPN/ml, 1.2×10^5 MPN/ml and 1.5×10^5 MPN/ml at day 14, day 28, day 42 and day 56 respectively. The population peaked at 2.4×10^5 MPN/ml on day 70 and day 84 respectively however, this population decreased to 2.1

$\times 10^5$ MPN/ml at day 105 (Figure 4). Likewise, the population of propionate oxidizing (acetogenic) bacteria (POB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 3.0×10^3 MPN/ml at day 0 to 2.7×10^4 MPN/ml, 3.6×10^4 MPN/ml, 6.4×10^4 MPN/ml, 7.5×10^4 MPN/ml and 1.5×10^5 MPN/ml at day 14, day 28, day 42, day 56 and day 70 respectively. The population peaked at 1.6×10^5 MPN/ml on day 84 however, it decreased to 1.5×10^5 MPN/ml at day 105 (Figure 4).

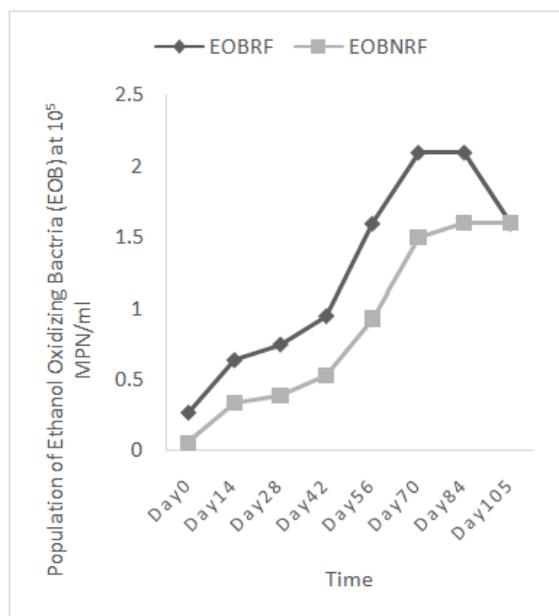


Figure 5. Population dynamics of ethanol oxidizing bacteria (EOB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of ethanol oxidizing bacteria (EOB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*

The population of ethanol oxidizing bacteria (EOB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 2.7×10^4 MPN/ml at day 0 to 6.4×10^4 MPN/ml, 7.5×10^4 MPN/ml, 9.5×10^4 MPN/ml and 1.6×10^5 MPN/ml at day 14, day 28, day 42 and day 56 respectively. The population peaked at 2.1×10^5 MPN/ml on day 70 and day 84 respectively however, at day 105, it decreased to 1.6×10^5 MPN/ml (Figure 5). Likewise, the population of ethanol oxidizing bacteria (EOB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 6.0×10^4 MPN/ml at day 0 to 3.4×10^4 MPN/ml, 3.9×10^4 MPN/ml, 5.3×10^4 MPN/ml, 9.3×10^4 MPN/ml and 1.5×10^5 MPN/ml at day 14, day 28, day 42, day 56 and 70 respectively. This population peaked at 1.6×10^5 MPN/ml on day 84 and day 105 respectively (Figure 5). The periods when the respective populations of POB_{RF} and EOB_{RF} and POB_{NRF} and EOB_{NRF} increased, peaked and then decreased during the anaerobic digestion process indicated that anaerobic oxidation (or acetogenic activity) may have increased, peaked and then decreased at those times inside AD_{RF} and AD_{NRF} , respectively (Claudia *et al.*, 2009; Labat and Garcia, 1986). This is because anaerobic oxidation of short chain fatty acids (e.g., butyrate, propionate, etc.) and alcohols (e.g., ethanol, propanol, etc.) in an anaerobic digestion process have been associated with acetogenesis (Claudia *et al.*, 2009; Schnurer and

Jarvis, 2010; Chanakya and Sreesha, 2012; Ljupka, 2010; Yassar, 2011).

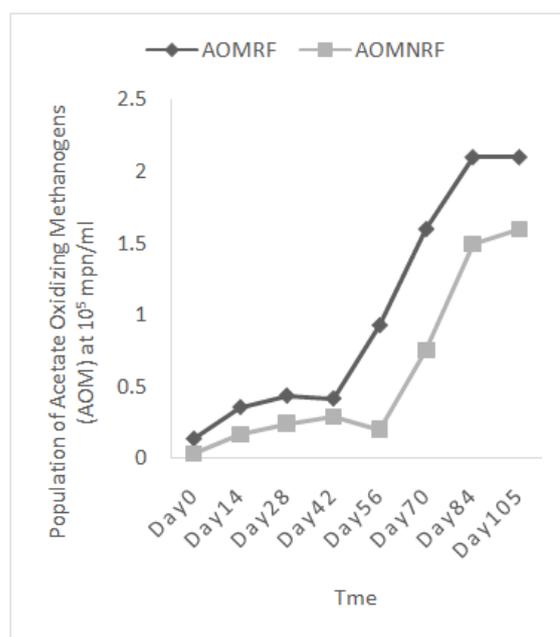


Figure 6. Population dynamics of acetate oxidizing methanogens (AOM_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of acetate oxidizing methanogens (AOM_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*

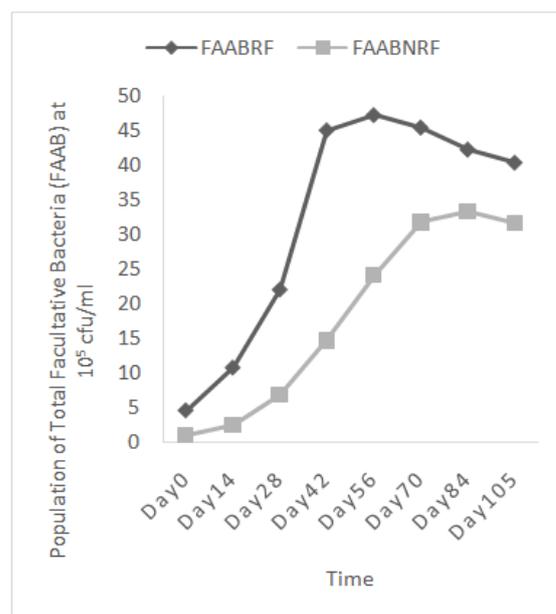


Figure 7. Population dynamics of facultative aerobic and anaerobic bacteria ($FAAB_{RF}$) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of facultative aerobic and anaerobic bacteria ($FAAB_{NRF}$) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*

The population of acetate oxidizing methanogens (AOM_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 1.4×10^4 MPN/ml at day 0 to 3.6×10^4 MPN/ml and 4.4×10^4 MPN/ml at day 14 and day 28 respectively. Their population decreased slightly to 4.2×10^4 MPN/ml at day 42. However, at day 56, day 70, day 84 and day 105, the population increased again to 9.3×10^4 MPN/ml, 1.6×10^5 MPN/ml

and 2.1×10^5 MPN/ml respectively (Figure 6). The population of acetate oxidizing methanogens (AOM_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 3.0×10^3 MPN/ml at day 0 to 1.6×10^4 MPN/ml, 2.4×10^4 MPN/ml and 2.9×10^4 MPN/ml at day 14, day 28 and day 42 respectively. Their population decreased slightly to 2.0×10^4 MPN/ml at day 56 and then increasing again to 7.5×10^4 MPN/ml, 1.5×10^5 MPN/ml and 1.6×10^5 MPN/ml at day 70, day 84 and day 105 respectively (Figure 6). This implied that acetotrophic methanogenesis (or acetate oxidation by acetotrophic methanogens) may have increased from day 0 to day 28 and day 42 inside AD_{RF} and AD_{NRF} respectively, but dropped slightly at around day 42 (inside AD_{RF}) and day 56 (inside AD_{NRF}) and then increased again at around day 56 and day 70 to day 105 inside AD_{RF} and AD_{NRF} respectively. (Schnurer and Jarvis, 2010; Chanakya and Sreesha, 2012; Ljupka, 2010; Yassar, 2011).

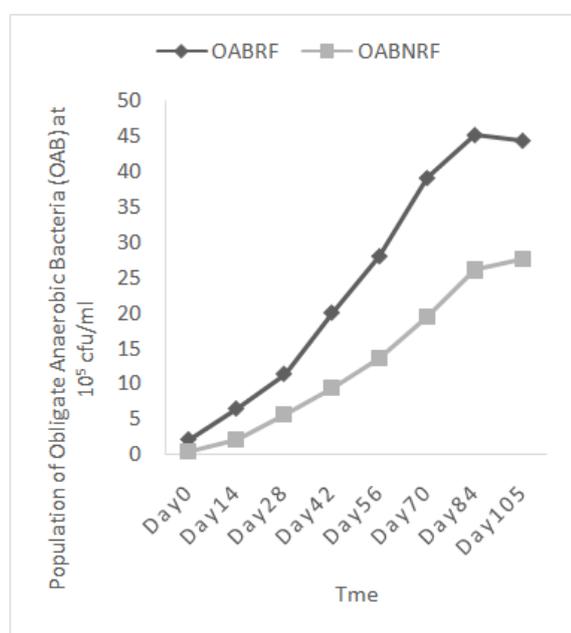


Figure 8. Population dynamics of obligate anaerobic bacteria (OAB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and population dynamics of obligate anaerobic bacteria (OAB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) during anaerobic digestion of *Panicum maximum*

The population of facultative aerobic and anaerobic bacteria (FAAB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 4.6×10^5 CFU/ml at day 0 to 1.08×10^6 CFU/ml, 2.21×10^6 CFU/ml and 4.52×10^6 CFU/ml at day 14, day 28 and day 42 respectively. The population peaked at 4.74×10^6 CFU/ml on day 56 however, on day 70, day 84 and day 105, this population decreased to 4.56×10^6 CFU/ml, 4.24×10^6 CFU/ml and 4.05×10^6 CFU/ml respectively (Figure 7). Likewise, the population of facultative aerobic and anaerobic bacteria (FAAB_{NRF}) inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) increased from 9.7×10^4 CFU/ml at day 0 to 2.37×10^5 CFU/ml, 6.85×10^5 CFU/ml, 1.47×10^6 CFU/ml, 2.42×10^6 CFU/ml and 3.19×10^6 CFU/ml at day 14, day 28, day 42, day 56 and day 70 respectively. The population peaked at 3.34×10^6 CFU/ml on day 84 and then decreased slightly to 3.18×10^6 CFU/ml at day 105 (Figure 7). The population of obligate anaerobic bacteria

(OAB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) increased from 2.12×10^5 CFU/ml at day 0 to 6.5×10^5 CFU/ml, 1.14×10^6 CFU/ml, 2.01×10^6 CFU/ml, 2.81×10^6 CFU/ml and 3.92×10^6 CFU/ml at day 14, day 28, day 42, day 56, and day 70 respectively. The population peaked at 4.53×10^6 CFU/ml on day 84 and then decreased slightly to 4.45×10^6 CFU/ml at day 105 (Figure 8). Likewise, the population of obligate anaerobic bacteria (OAB_{NRF}) inside the anaerobic digester with rumen fluid inoculation (AD_{NRF}) increased from 4.6×10^4 CFU/ml at day 0 to 2.12×10^5 CFU/ml, 5.6×10^5 CFU/ml, 9.45×10^5 CFU/ml, 1.36×10^6 CFU/ml, 1.95×10^6 CFU/ml, 2.61×10^6 CFU/ml and 2.76×10^6 CFU/ml at day 14, day 28, day 42, day 56, day 70, day 84 and day 105 respectively (Figure 8).

The pattern of growth exhibited by these indicator microbial populations with respect to time inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and the anaerobic digester without rumen fluid inoculation (AD_{NRF}) tend to obey the sigmoid function (S curve) as generally occurred in batch culture. Their respective populations increased (about 10-fold) with respect to time (see Figure 1 to Figure 8). This Observation is in line with the result of other authors who also reported significant increase in the population of various bacteria and archaea groups based on metabolic capacity and oxygen sensitivity during anaerobic digestion of Municipal solid waste (MSW), Sugar beets and Cassava peels respectively (Claudia *et al.*, 2009; Labat and Garcia, 1986; Cuzin *et al.*, 1992). Furthermore, statistical analysis (one – way ANOVA) showed that there was a significantly ($P < 0.05$) difference between the microbial population inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and the microbial population inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) with respect to time.

This was most likely due to the addition of rumen fluid inside the AD_{RF} system. Some author(s) have also shown that rumen fluid inoculation may be capable of positively affecting the microbial population in an anaerobic digestion system (Dalhoff *et al.*, 2003; Barnes & Keller 2003, 2004; Hu and Yu, 2005; O'Sullivan *et al.*, 2006; Yue *et al.*, 2007; Jagadabhi *et al.*, 2010). This is because rumen fluid contains high population of bacteria that are specialized in biogas production through anaerobic digestion of lignocellulosic substrates such as grasses (Allison and Leek, 1993; Barnes and Keller 2003). Rumen fluid may also contain some growth factors which may have been beneficial to these bacteria populations with time (Hobson and Shaw 1973; Iannotti *et al.* 1978; Ueki *et al.*, 1978). These may have been some of the reasons why the bacteria and archaea populations inside the AD_{RF} system were higher than the bacteria and archaea populations inside the AD_{NRF} system at any given period during anaerobic digestion of the guinea grass (Figure 1 to Figure 8).

In the anaerobic digester with rumen fluid inoculation (AD_{RF}), the population of cellulolytic bacteria (ACB_{RF}), lactose fermenting bacteria (LFB_{RF}), glucose fermenting bacteria (GFB_{RF}), propionate oxidizing bacteria (POB_{RF}), ethanol oxidizing bacteria (EOB_{RF}), acetate oxidizing methanogens (AOM_{RF}), facultative aerobic and anaerobic bacteria (FAAB_{RF}) and obligate anaerobic bacteria (OAB_{RF}) had growth rates of 0.054 day^{-1} , 0.038 day^{-1} , 0.057 day^{-1} , 0.030 day^{-1} , 0.021 day^{-1} , 0.028 day^{-1} , 0.051

day⁻¹ and 0.040 day⁻¹ respectively. In the anaerobic digester without rumen fluid inoculation (AD_{NRF}), the population of cellulolytic bacteria (ACB_{NRF}), lactose fermenting bacteria (LFB_{NRF}), glucose fermenting bacteria (GFB_{NRF}), propionate oxidizing bacteria (POB_{NRF}), ethanol oxidizing bacteria (EOB_{NRF}), acetate oxidizing methanogens (AOM_{NRF}), facultative aerobic and anaerobic bacteria (FAAB_{NRF}) and obligate anaerobic bacteria (OAB_{NRF}) had growth rates of 0.044 day⁻¹, 0.043 day⁻¹, 0.046 day⁻¹, 0.031 day⁻¹, 0.022 day⁻¹, 0.028 day⁻¹, 0.056 day⁻¹ and 0.053 day⁻¹ respectively (Figure 9).

This result suggested that the bio-degraders inside the anaerobic digesters (AD_{RF} and AD_{NRF}) were slow growers (see Figure 9). Nevertheless, the cellulolytic bacteria populations (i.e., ACB_{RF} and ACB_{NRF}) and the sugar

fermenting (acidogenic) bacteria populations (i.e., LFB_{RF} and GFB_{RF} and LFB_{NRF} and GFB_{NRF}) grew at a faster pace compared to the propionate and ethanol oxidizing (acetogenic) bacteria populations (i.e., POB_{RF} and EOB_{RF} and POB_{NRF} and EOB_{NRF}) and the acetate oxidizing methanogenic populations (i.e., AOM_{RF} and AOM_{NRF}) inside the respective anaerobic digesters (AD_{RF} and AD_{NRF}) as observed in Figure 9. This was predicted because of the metabolic relationships among these microbial populations in anaerobic digestion food chains. Usually, hydrolysis followed by acidogenesis must first occur to some degree before acetogenesis and methanogenesis (Schnurer and Jarvis, 2010; Chanakya and Sreesha, 2012; Ljupka, 2010; Yassar, 2011).

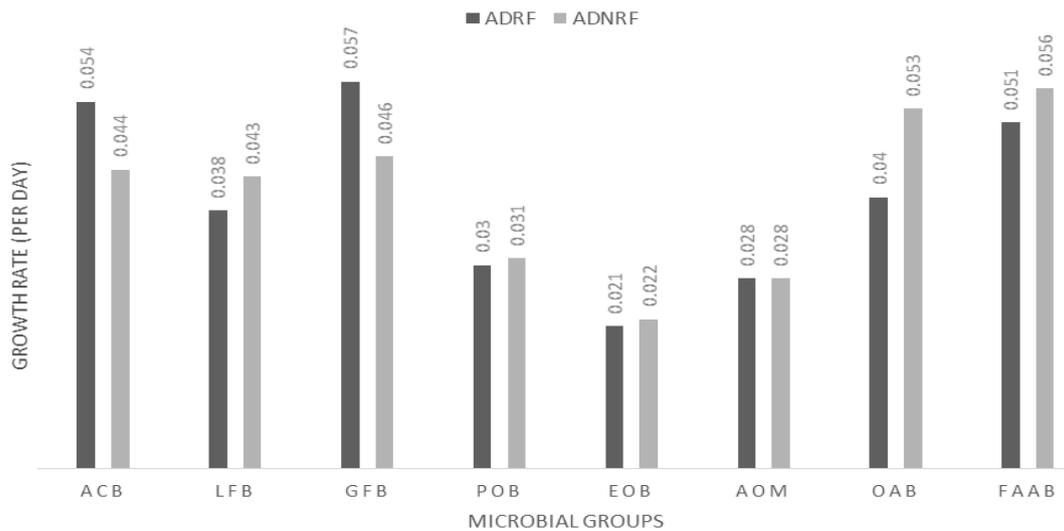


Figure 9. Population growth rates of selected indicator bacteria groups based on metabolic capacity and oxygen sensitivity inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and the anaerobic digester without rumen fluid inoculation (AD_{NRF}) respectively

Generally, methanogens are usually the slowest growers, while the acidogens are usually the fastest growers compared to other bacteria groups in most anaerobic digestion food chains (Schnurer and Jarvis, 2010). The reason why the population of ethanol oxidizing bacteria (EOB_{RF} and EOB_{NRF}) groups inside both anaerobic digesters (AD_{RF} and AD_{NRF}) may have had the slowest growth rate as observed in Figure 9, may be linked to the metabolic pathways by which the acetogens generated the acetate which was converted to biogas by the acetotrophic methanogens (Ljupka, 2010). It may have been that the propionate oxidation pathway was favoured (than the ethanol oxidation pathway) during Acetogenesis. In terms of O₂-sensitivity, the population of facultative bacteria groups (FAAB_{RF} and FAAB_{NRF}) grew at a faster pace compared to the population of obligate anaerobic bacteria (OAB_{RF} and OAB_{NRF}) inside the anaerobic digesters (AD_{RF} and the AD_{NRF}) as observed in Figure 9. This was predicted because obligate anaerobes are generally slower growers compared to facultative aerobes and anaerobes respectively (Schnurer and Jarvis, 2010; Labat and Garcia, 1986; Claudia *et al.*, 2009).

Inside the anaerobic digester with rumen fluid inoculation (AD_{RF}), the population of sugar fermenting acidogens (LFB_{RF} and GFB_{RF}) predominated with a proportion of 45.26%. This was followed by the population of propionate and alcohol oxidizing acetogens (POB_{RF} and EOB_{RF}) with a proportion of 25.41%. Next, was the population of cellulolytic bacteria (ACB_{RF}) with a

proportion of 18.89% and the population of acetate oxidizing methanogens (AOM_{RF}) were the minority with a proportion of 10.44% (Figure 10). Inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}), the population of sugar fermenting acidogens (LFB_{NRF} and GFB_{NRF}) predominated with a proportion of 42.16%. This was followed by the population of propionate and alcohol oxidizing acetogens (POB_{NRF} and EOB_{NRF}) with a proportion of 28.82%. The population of cellulolytic bacteria (ACB_{NRF}) followed with a proportion of 18.93%, while the population of acetate oxidizing methanogens (AOM_{NRF}) were the minority with a proportion of 10.0% (Figure 10). This result is in line with the result of other studies (Labat and Garcia, 1986; Claudia *et al.*, 2009; Schnurer and Jarvis, 2010) which suggested that the population of the acidogens and the methanogens may be the majority and the minority populations in most anaerobic digestion (AD) systems, respectively. This may be linked to their growth rates during the AD process as suggested by Figure 9 above (Schnurer and Jarvis, 2010).

In terms of O₂-sensitivity, the population of facultative aerobic and anaerobic bacteria (FAAB_{RF}) inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) predominated with a proportion of 56.73% compared with the population of obligate anaerobic bacteria (OAB_{RF}) which had a proportion of 43.23%. Inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}), the population of facultative aerobic and anaerobic bacteria (FAAB_{NRF}) predominated with a proportion of 58.34%

compared with the population of obligate anaerobic bacteria (OAB_{RF}) which had a proportion of 41.66% (Figure 10). This may also be linked to their respective growth rates as discussed earlier (Figure 9). It is very important to monitor the proportion (%) of important microbial indicator groups during anaerobic digestion processes (Claudia *et al.*, 2009; Labat and Garcia, 1986; Cuzin *et al.*, 1992). This is because for example, in most AD systems treating cellulosic or lignocellulosic substrates (e.g., matured grass) that are not easily biodegradable, hydrolysis is usually the rate limiting

phase especially when the proportion (%) of hydrolytic bacteria population that would attack the biopolymer in the initial substrate was not high enough to accelerate hydrolysis (Susan, 1995; Schnurer and Jarvis, 2010). However, if the substrate was easily (or readily) biodegradable, methanogenesis may become the rate limiting phase of the AD process especially when the proportion of methanogens that would convert acetic acid, hydrogen and carbon dioxide into biogas was insufficient at the time (Kim *et al.*, 2008; Azeem *et al.*, Schnurer and Jarvis, 2010; Susan, 1995).

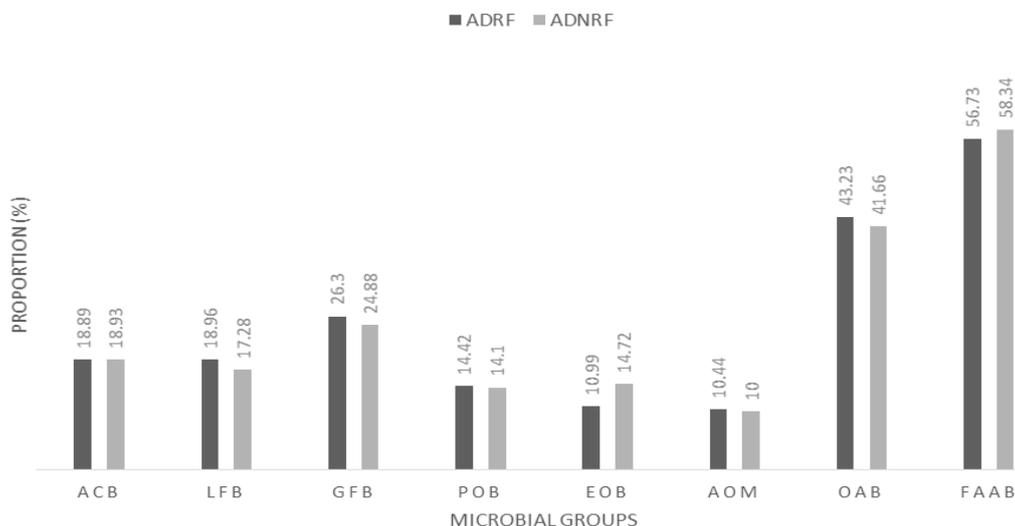


Figure 10. Proportion (%) of selected indicator microbial populations inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) and the anaerobic digester without rumen fluid inoculation (AD_{NRF}) respectively

4. Conclusion

Anaerobic digestion process is said to be divided into four (4) stages (or phases) namely, Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis (Schnurer and Jarvis, 2010; Chanakya and Sreesha, 2012). However, in reality, these phases may be difficult to distinguish. Therefore, if the populations of some metabolically important microbial indicators were monitored with respect to time (as was done in this study), it may be possible to use their population dynamics to at least indicate (or monitor) the occurrence or progress of these processes to some degree (Claudia *et al.*, 2009; Labat and Garcia, 1986; Ike *et al.*, 2010; Cuzin *et al.*, 1992). Furthermore, analysis of various bacterial groups during anaerobic digestion (AD) process could indicate bad running of the process. Two or three specific counts could possibly explain process failure, but cannot contribute to optimization of the biogas yield. However, the possibility of increasing the performance of an AD process by controlling its microflora appears possible. Our result suggested that rumen fluid significantly ($P < 0.05$) increased the microbial population inside the anaerobic digester with rumen fluid inoculation (AD_{RF}) compared to the microbial population inside the anaerobic digester without rumen fluid inoculation (AD_{NRF}) with time. Therefore, rumen fluid could be used to boost the microbial population inside anaerobic digesters which could enhance depolymerisation, obtain higher degradation rates of lignocellulosic substrates and thus higher energy (biogas/methane) benefits.

Nomenclature

- AD:** anaerobic digestion
- RF:** rumen fluid
- AD_{RF}:** anaerobic digester with RF inoculation
- AD_{NRF}:** anaerobic digester without RF inoculation
- ACB_{RF}:** cellulolytic bacteria inside AD_{RF}
- ACB_{NRF}:** cellulolytic bacteria inside AD_{NRF}
- LFB_{RF}:** lactose fermenting bacteria in AD_{RF}
- LFB_{NRF}:** lactose fermenting bacteria in AD_{NRF}
- GFB_{RF}:** glucose fermenting bacteria in AD_{RF}
- GFB_{NRF}:** glucose fermenting bacteria in AD_{NRF}
- POB_{RF}:** propionate oxidizing bacteria in AD_{RF}
- POB_{NRF}:** propionate oxidizing bacteria in AD_{NRF}
- EOB_{RF}:** ethanol oxidizing bacteria in AD_{RF}
- EOB_{NRF}:** ethanol oxidizing bacteria in AD_{NRF}
- AOM_{RF}:** acetate oxidizing methanogens in AD_{RF}
- AOM_{NRF}:** acetate oxidizing methanogens in AD_{NRF}
- FAAB_{RF}:** facultative aerobic and anaerobic bacteria in AD_{RF}
- FAAB_{NRF}:** facultative aerobic and anaerobic bacteria in AD_{NRF}
- OAB_{RF}:** obligate anaerobic bacteria inside AD_{RF}
- OAB_{NRF}:** obligate anaerobic bacteria inside AD_{NRF}
- MPN:** most probable number
- CFU:** colony forming unit
- ML:** millimetre
- TS:** total solid
- WS:** wet solid
- WA:** water

PTM_{RF}: process temperature in AD_{RF}
PTM_{NRF}: process temperature in AD_{NRF}
pH_{RF}: process pH inside AD_{RF}
pH_{NRF}: process pH inside AD_{NRF}
COD_{RF}: chemical oxygen demand in AD_{RF}
COD_{NRF}: chemical oxygen demand in AD_{NRF}
VFA_{RF}: volatile fatty acid inside AD_{RF}
VFA_{NRF}: volatile fatty acid inside AD_{NRF}

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