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Cattle Urine as a Fertiliser: Micro-biochemical Changes in Fermenting Cattle Urine and Implications on Plant Nutrient Conservation

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Authors' contributions

This work was carried out in collaboration between all authors. Author GK participated in the design of the experiment and did laboratory analysis. Author JST originated the research, designed the experiment and drafted the manuscript. Authors MCRS and AAK did the statistical analysis and participated in the development of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to evaluate the microbial and biochemical changes in fermenting urine, a practice used by farmers in Sub-Saharan Africa before its application as a soil fertility input.
Methodology: Two 5-litre sterile plastic containers, with a closable ends were each filled with fresh urine to capacity. One container was closed and the other left open. The set-up was replicated three times. Twenty millitres of fresh urine was taken from the bulk collection for microbial and chemical analysis. Urine samples were also taken and analysed at 4-day fermentation intervals till 24 days.
Results: Fresh urine had pH=8.2 and contained *Aspergillus spp.* and *Escherichia coli*, with the latter being dominant. After 12 days of fermentation, *Penicillium spp.* and *Pseudomonas spp.*

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emerged and progressively increased, especially under the closed system. Whereas *Aspergillus spp.* counts increased in both systems, *E. coli* counts dropped dramatically and eventually disappeared at 16 days. The pH in the open system surged to 9.7, while that of the closed containers remained nearly stable (8.2). Organic N was not significantly ($p>0.05$) affected by closure of the containers. In the open system, Organic N concentration dropped up to 72%. However, NH_4-N concentration increased steadily in the closed system until day 24; but dropped dramatically in the open system. Nitrate concentration increased slightly up to day 8, and thereafter, declined sharply by 97% in the open system. Similarly, in the closed system, this N species dwindled progressively but not to extinction.

Conclusion: There is a shift in microbial communities in urine from *Aspergillus spp.* and *Escherichia coli* in fresh urine to *Penicillium spp.* and *Pseudomonas spp.* 12 days after the onset of fermentation. Nitrate-N is favoured by the open system, while the ammonium-N increased more in the closed system.

Keywords: Ammonium; Aspergillus; Escherichia coli; nitrate; Penicillium; Pseudomonas.

1. INTRODUCTION

Despite the overwhelming decline in soil fertility in sub-Saharan Africa, resources that are easily accessed by resource poor farmers such as cattle urine are heavily under-utilised. Moreover, the bulk of farming households in the region largely practice crop-livestock farming, with cattle as the dominant component. Cattle urine is a major residue on farms in the region which is renowned for richness in plant available nutrients, superseding the faecal manure which is widely advocated for crop production [1,2,3].

In Uganda, human and cattle urine is utilised sporadically largely for pest control [4,5,6] and rarely for soil fertility management (Tenywa, 2004 unpublished). Most farmers subject urine to fermentation for 14-21 days prior to use, with hardly any research to justify the practice. Tenywa (2004, unpublished) found no agronomic value in urine fermentation, as long as the material was applied at least 10 cm away from the plant in a moist soil. Nevertheless, the issue is still contestable in terms of sanitisation against high potential pathogenic microorganisms. It is imperative that the microbial and biochemical changes in fermenting urine are investigated in order to pave way for designing interventions that can conserve nutrient profiles as well as accelerate the fermentation and sanitisation processes. One of the entry-point in this direction is by understanding the biochemical processes occurring during urine fermentation under a variety of oxygenation conditions. This study was, therefore, conducted to characterise the microbial communities existent fermentation, as well as the N species as a basis for informing designers of strategies for speedy fermentation and nutrient conservation.

2. MATERIALS AND METHODS

A laboratory study was conducted at Makerere University in Uganda, four times during 2005. Cattle urine (30 litres) used was collected from 15 Friesian (Bos taurus) dairy cattle of the Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) herd, raised in a semi-intensive system. Six portions of 5 litres each were aseptically dispensed into sterile transparent plastic jars. Three jars, as replicates, were sealed immediately with their lids, while the rest were left open throughout the study period. The six jars were kept in a locker under room temperature (25-27℃) for 24 days. From each jar, 20-millitre samples were taken at 4 day intervals until the end of the experiment (24 days). The samples were used for sequential analysis for microbes, pH, Organic N, and ammonium and nitrate N using procedures [7].

2.1 Microbial Evaluation

Using the Dilution Spread Plate Technique [8], 25 millitres of the broth culture from each of the six fermentation jars was aseptically taken and mixed thoroughly with 225 millitres of sterile saline peptone solution, to a 10-1 dilution in a polyethylene stomacher bag. The contents were thoroughly shaken for approximately five minutes. Then, three aliquots of 0.1 millitre making 10-2 dilution were aseptically pipetted and each inoculated (by spreading the inoculum evenly on the medium) on two different Plate Count Agar (PCA Oxoid, UK medium). Another set was plated on Saboraud Dextrose Agar (SDA, Merk, Germany medium). The plates were inverted and incubated at 25-27℃ for 24 hours for PCA and 3 weeks for SDA. The exercise was repeated at 4 day intervals till day 24. Bacterial

and fungal forming units (cfus) were enumerated with a colony counter and the total was computed using the procedure [8,9].

2.2 Isolation and Identification of Bacteria and Fungi

A loop-full of bacterial cells (picked from colonies) from PCA media were characterised morphologically under a light compound microscope. Furthermore, the cells were subjected to Gram stain, catalase reaction using hydrogen peroxide, Voges Proskauer (VP) and Methyl red (MR) [10]. Similarly, fungal colonies appearing on SDA were characterised based on morphological characteristics. Hyphae were stained using lactophenol cotton-blue [10]. The set up was then observed microscopically using a light microscope (Olympus CK, Olympus Optical Co. Ltd., Tokyo, Japan) at a magnification of 40 x 100.

2.3 Nitrogen Analysis

Urine samples from both types of containers were analysed for pH at the initiation and termination of the study using a glass electrode. Organic N was analysed using the Kjeldahl technique [AOAC, 1995.] and nitrate N by the procedure outlined in [AOAC, 1995].

2.4 Data Analysis

The data were subjected to analysis of variance (ANOVA) at 5% probability level, using GenStat Discovery version software [11]. Significant means were separated using Fisher's Protected Least Significant Differences (LSD) at 5% level. Relationships between various organisms and chemical parameters under different oxygenation conditions were computed using Canonical Correlation Analysis (CCorA) and Principal Component Analysis (PCA). Diversity of the organisms was determined using the Shannon Diversity Index [12]. Significant differences in microbial diversity were tested using the Boot trap p [13].

3. RESULTS

3.1 Microbial Composition and pH

The microorganisms identified in the fermenting cattle urine, both in the open and closed systems, were *Aspergillus*, Escherichia, *Penicillium* and Pseudomonas (Table 1). Initially, only *Aspergillus* and *E. coli* were prominently detectable, but their counts diminished drastically to nil by the 16-24 days of fermentation. Contrastingly, *Penicillium* and Pseudomonas genera were insignificant at the onset of the study, but increased considerably beyond day-16. Significant population differences ($p<0.05$) were registered for Escherichia and Pseudomonas under the open system, and *Aspergillus*, Escherichia and Pseudomonas under the closed setup.

3.2 Nitrogen Forms in Urine

The concentration of Organic N became reduced significantly during fermentation (Fig. 1, Table 2); however, the decline was more drastic ($p=0.03$) under the open than the closed system. In contrast, the concentrations of ammonium and nitrate were rather low initially, although that of the former rose, especially under closed fermentation. Nitrate concentrations were particularly low in the closed system.

3.3 Relationships between Microbial Diversity and N Forms

The concentration of Organic N was positively correlated with *E. coli* in both open and closed systems (Table 3). On the other hand, the correlations were negative for *Penicillium* and *Pseudomonas* under both fermentation conditions, respectively. Ammonium N was only significantly related to *E. coli*. In contrast, significant correlations emerged between nitrate and *Escherichia* and *Penicillium* under the closed fermentation condition (Table 3). Furthermore, pH was positively correlated with *Penicillium*, but negatively with *E. coli.*

Based on Canonical Correlation Analysis (Fig. 2), Organic N and ammonium were closely associated in the upper positive quarter, whereas *Pseudomonas* was located on the negative side. In contrast, *Penicillium, pH and Aspergillus* occurred together in the lower negative quarter.

4. DISCUSSION

4.1 Cattle Urine Microbial Composition and pH

The presence of four microbial genera, *viz*. *E. coli* and *Pseudomonas* (bacteria), and *Aspergillus* and *Penicillium* (fungi) in urine under both closed and open systems (Table 1), demonstrates the competence of the four genera to thrive under both conditions, though at different levels. This contrasts directly with earlier findings [14] that cattle urine is free of

E. coli. However, there was limited diversity of organisms in the cattle urine possibly due to the restrictive nature of the dilution plate technique used, as no single medium or growth environment is conducive for all microbial species [8]. Furthermore, taxonomical resolution was inadequate since only *E. coli* and *Aspergillus niger* were successfully characterised to species level, based on morphological characteristics (shape, size, etc.), staining (Gram stain, methyl red, Voges Proskauer, lactophenol cotton blue), selective media (MacConkey agar), microscopic examination and biochemical (catalase reaction) attributes . Often these tests are not sufficiently rigorous and currently molecular methods that involve DNA or rRNA are preferred but costs for this study are often prohibitive.

Numerically, there were more bacteria counts than fungi, and indeed *E. coli* was the most abundant (100 folds) in the fresh urine (Table 1). In the current study, despite the urine pH ranging between 7.6 and 9.7, it contained $1.85x10^4$ of *E. coli* cfus, reflecting their tolerance to alkalinity. This directly contrasts with earlier reports which suggest that high pH due to accumulation of ammonia from urease hydrolysis of urea is toxic to non-alkalophilic microbes, especially bacteria [15,16,17]. Microbes which thrive under high pH and NH_4-N concentrations are often alkalophilic and tolerate ammonium toxicity. In this respect, many bacteria and fungi may be responsible for mediating ammonification, while a few microbes (nitrifiers), largely aerobic chemolithotrophs, participate in oxidising ammonium to nitrite and, subsequently to nitrate through the nitrification process [18,19]. Traditionally, the nitrifiers include *Nitrosomonas sp.* and *Nitrobacter sp,* however, these organisms were not detected in the measurements of this study.

The microbial habitation of the urine used in this study seems to present evidence of faecal contamination during urine collection as noted in earlier studies [20,21], although contamination due to the animal's urethral infection cannot be precluded [22]. All the organisms identified in the study possess facultative anaerobic characteristics as they occurred under both open and closed fermentation; though higher numbers were recorded in the former condition (Table 1). Other reports also allude to this behavior [23].

The early appearance of *Penicillium* and *Pseudomonas* organisms (8 and 20 days of the experiment) in the open and closed systems appears to be linked with their capacity to metabolise urea, since they reportedly possess urease which hydrolyses this substrate into ammonia and carbon dioxide [24,25]. It is proposed that ammonium is assimilated through glutamine synthatase (GS), glutamine-2 oxoglutarate amino transferase (GOGAT) pathway [26]. The production of glutamine, in turn leads various transferases to mediate synthesis of other amino acids, depending on cell or microbe requirements. Literature shows that, there are different types of GS, but most bacteria including *E. coli* possess GS 1 form encoded by *glnA* [27]. Progressively, the substrates could be depleted, along with a flux in microbial wastes. This observation seems to explain the gradual disappearance of *E. coli* and *Aspergillus* populations in the present study. Furthermore, it is likely that the dead microbial debris, together with some intermediate metabolites or metabolic byproducts of the two organisms provided substrates or stimulants for the *Pseudomonas* and *Penicillium* proliferation later. This phenomenon is comparable to co-metabolism in microbial succession [25].

The Canonical Correlation Analysis showed that *Penicillium* and *Aspergillus* co-occurred in the acid range (Fig. 2), reflecting the acidophilic nature of fungi compared to bacteria. Indeed, various fungi spp. are historically known to thrive better under acidic conditions than bacteria. This phenomenon is further attested to by the positive Pearson's Correlation between pH and *Penicillium* and the negative one for *E. coli* in both open and closed conditions (Table 3). *Pseudomonas* was diagonally opposite to *E. coli* which was associated with nitrate, confirming that the two organisms behave differently. The bulkier presence of *Pseudomonas spp,* particularly *P. denitrificans* under the closed than open fermentation system seems to be linked to its capacity to perform denitrification using its naturally endowed nitrate reductase enzyme [26,27,28,29]. In contrast, the association of NO_3^- with *E. coli* presupposes that the former is a substrate of the latter [19].

The more pronounced presence of NO^-_3-N under the open system, in part demonstrates the presence of greater aerobiosis therein than in the closed system. Heterotrophic nitrification is largely attributed to fungi such as *Aspergillus flavus* first reported as a nitrifying bacteria in 1954 [30].

Table 1. Progressive change in microbial populations and diversity in cattle urine fermenting conditions

Time (days)	Semi-aerobic					Anaerobic				
	Asper	***E.sch (x10^{2})cfu millitre^{-1})***	***Pen.*** ……	***Pseudo*** ……	**pH (H_2O)**	***Asper.*** ……	***E.sch*** ……	***Pen (x10^{2})cfu millitre^{-1}***	***Pseud*** …..	**pH(H_2O)**
0	2.80	707.0	0	0	8.2	3.2	691.5	0	0	8.2
4	4.7	584.1	0	0	9.0	0.7	590.2	0	0	7.9
8	2.6	9.8	3.8	0	9.2	0	9.4	0	0	7.6
12	1.7	7.4	0.7	0	9.6	0	4.0	0	0	8.3
16	1.3	0.9	2.0	0	9.7	0	0	1.0	5	8.3
20	0.3	0	13.0	29.0	9.7	0	0	2.6	20.33	8.2
24	0.3	0	4.7	4.33	9.7	0	0	1.0	4.33	8.2
LSD (0.05)	ns	12.4	ns	5.41		0.77	13.5	ns	8.07	
CV (%)		2.1		17.6		33.1	1.7		31.3	

Asper. = Aspergillus, Pen. = Penicillium, Esch=Escherichia and Pseudo. = Pseudomonas. cfu = colony forming units (equivalent to viable spores), ns = not significant at p<0.05

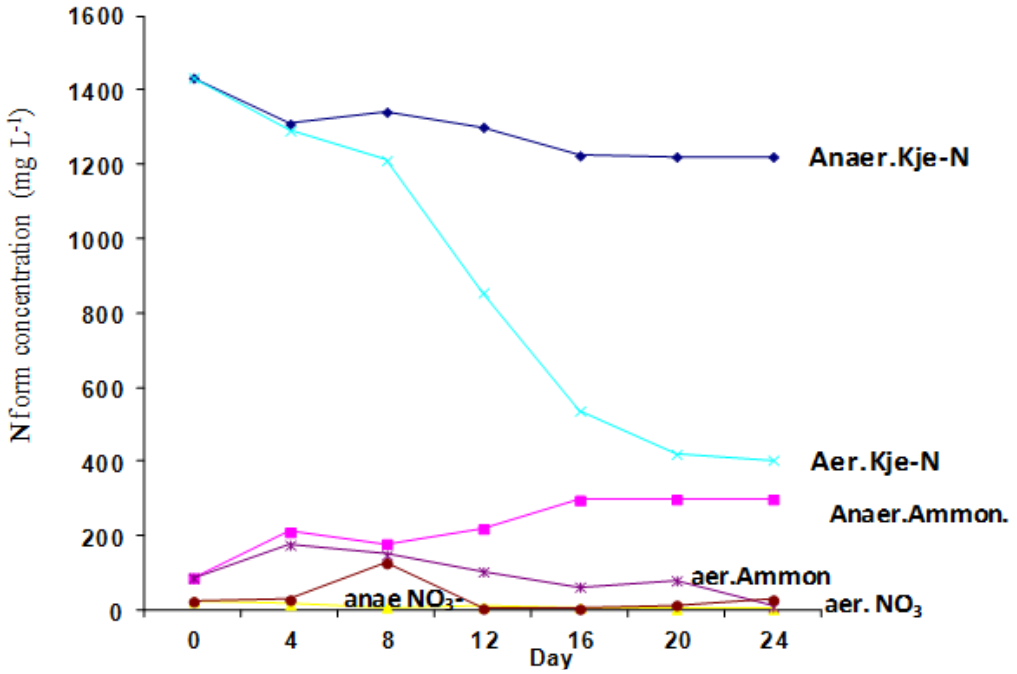

Fig. 1. Nitrogen forms concentration during aerobic (Aer) and (Anaer) conditions of cattle urine fermentation

Table 2. Nitrogen forms and pH in cattle urine under two fermentation conditions

	Aerobic				Anaerobic				Level of significance
Chemical changes	**Min**	**Max**	**Mean**	**SD ±**	**Min**	**Max**	**Mean**	**SD ±**	***p*-value**
Organic N–(mg L^{-1})	404.3	1433.3	878.9	436.0	1221	1443	1292.9	78.9	0.031
Ammonium-N (mg L^{-1})	9	175.7	95.3	55.1	87.0	299.0	227.1	78.9	0.016
Nitrate-N (mg L^{-1})	3.6	126.67	32.4	42.8	2.67	23.33	9.86	7.3	0.137
pH(H_2O)	8.2	9.7	9.3	0.6	7.6	8.3	8.1	0.3	0.002

Table 3. Pearson's correlations between microbial diversity and N- forms in cattle urine under two fermentation conditions

Microbial diversity and N form relationships	**Correlation coefficient**			
	Aerobic		**Anaerobic**	
	r	***p* value**	**r**	***p* value**
Organic –N *vs E. coli*	0.991	0.000	0.935	0.007
Organic N *vs Penicillium*	-0.84	0.024	-0.87	0.012
Organic N *vs Pseudomonas*	-0.76	0.048	-0.84	0.024
NH_4^+ - N *vs E. coli*	0.721	0.088	-0.94	0.003
NO3-N *vs E. coli*	0.136	0.783	0.926	0.007
NO3-N *vs Penicillium*	-0.10	0.84	-0.84	0.024
pH *vs Penicillium*	0.804	0.034	0.804	0.034
pH *vs E. coli*	-0.972	0.000	-0.972	0.000

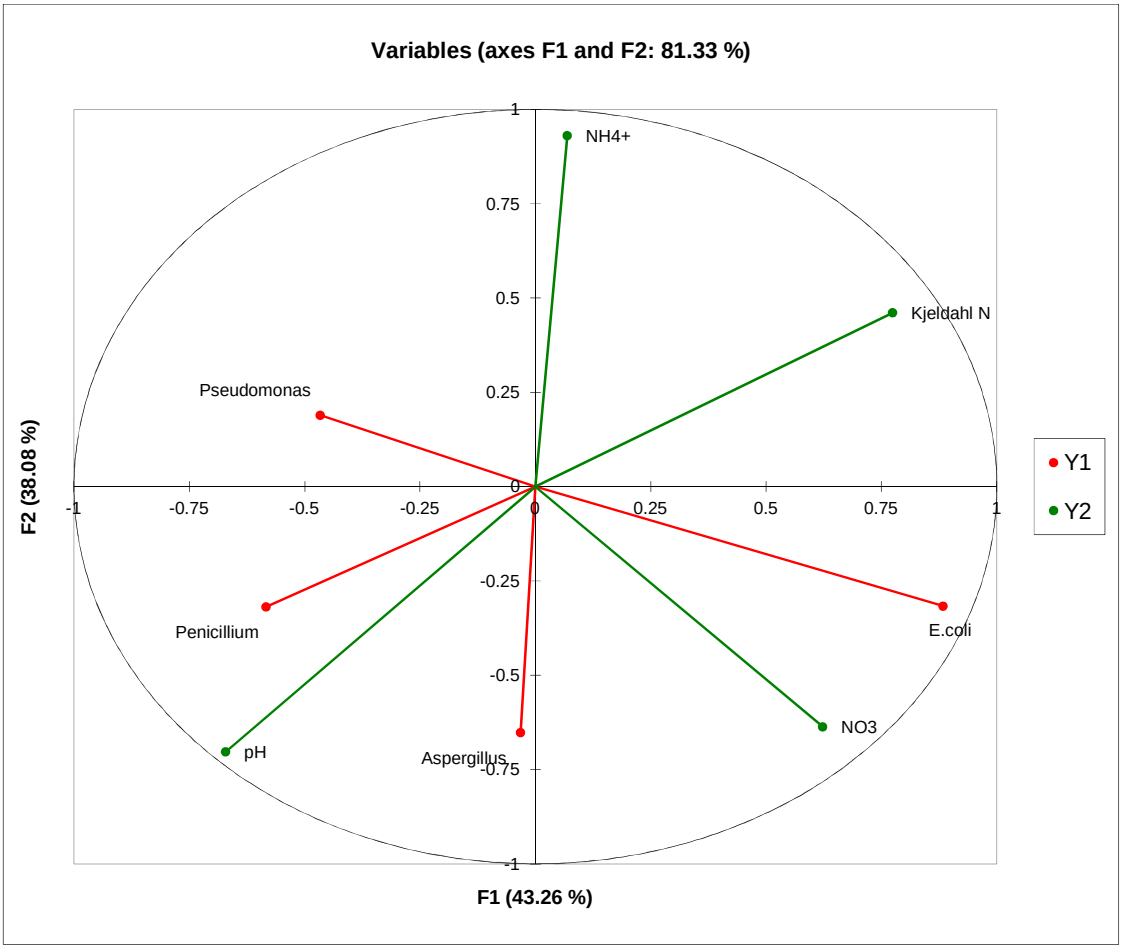

Fig. 2. Interaction between microbial spp and N forms using canonical correlation analysis.
Y1 and Y2 are correlations between input variables and canonical variables

In the present study, *Aspergillus niger* and *Aspergillus fumigans* were identified and these were likely involved in the nitrification process.

Other nitrifiers reported often limited to acidic environment are *Penicillium sp* and *Cephalosporium sp*. [19].

4.2 Nitrogen Forms in Cattle Urine under Fermentation

The rapid decline in Organic N nitrogen noted under the open (partially aerobic) than under the closed system suggests occurrence of intense ammonification as Organic N segregated together with ammonia in Canonical Correlation Analysis (Fig. 2 above). None of the microbes identified in the study segregated with the two forms of N, suggesting that the process was either chemical or spontaneous volatilisation of ammonia on exposure to air [31]. On the other hand, the presence of nitrate implied the occurrence NH_4^+ nitrification, which is largely a microbially mediated process. Surprisingly, the NH_4^+-N levels declined progressively under both open and closed environments, suggesting that the closed system was not leak proof (Fig. 2 above).

4.3 Relationships between Microbial Populations and N Forms

The strong positive correlation (r = 0.99) between Organic N–N and *E. coli* counts, under both fermentation conditions (Table 3) underscores the critical role played by this form of N as a substrate for the microbes. Actively growing bacteria require N, P and C for cellular amino acids, nucleic acid (DNA and RNA content) synthesis and the tricarboxylic acid cycle (TCA)

for energy production. Contrastingly, the correlation was not significant for mineral N forms (NH_4^+ and NO_3^-) forms under the open conditions. It is possible that the organisms lacked the enzyme cascade necessary to assimilate mineral N or that the enzymes are susceptible to alkaline conditions that characterise ammonification [24]. However, under the closed condition, there was a negative microbial correlation with NH_4^+-N (r = - 0.941), again pointing to the lethal nature of ammonia-laden conditions. Indeed, the relationship was inverse (strong and positive, r = 0.93) under the open system (Table 3). Under oxygen deficient conditions (closed system), ammonia accumulated likely to toxic levels, yet the nitrate that trickled in stimulated growth of *E. coli*.

Pseudomonas spp. (bacteria) which utilises a variety of N substrates behaved contrary to *E. coli,* thus presenting a negative correlation with Kjedahl (Table 3). Also, *Pseudomonas* had non-significant relationships with mineral N (ammonium and nitrate) under both fermentation conditions. These observations could be attributed to the fact that *Pseudomonas* survive on a range of substrates; some are N_2 fixing organisms, while others (*Pseudomonas stutzeri* and *Pseudomonas denitrificans*) participate in denitrification [26,27]. Similarly, *Penicillium* spp. (fungi) which were detected later in fermentation process under both conditions, were negatively correlated to Organic and NO_3^-N, but only under anaerobic conditions (Table 3). Generally, fungi prefer slightly acidic media, and urine in this study was strongly alkaline. Similar correlation evidences were well illustrated in the Canonical Correlation Analysis outputs (Fig. 2).

5. CONCLUSION

Four genera of microbes, namely, Aspregillus and Penicillium spp (fungi), *Pseudomonas* and *E.* coli (bacteria) exist in cattle urine under fermentation. Irrespective of aerobic condition to which cattle urine fermentation is subjected, *Aspergillus* and *E. coli* dominate in fresh urine, but these progressively disappeared by 16 days of fermentation. This observation is critical as *E. coli* is an indicator of the likely presence of gut-based pathogens. *Penicillium* and *Pseudomonas spp.* appear later as secondary colonisers, and both bear similar final colony counts. The closed fermentation system reduces urine N losses during fermentation by up to 97%, when compared with the open system.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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