



Monitoring the effect of fortification on bacterial population dynamics in malted and fermented maize-based weaning foods using PCR-DGGE

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ABSTRACT

Objective: To evaluate the effect of fortification on the microbial population dynamics of naturally fermenting maize– legume weaning blends.

Methodology and results: Maize fortified with cowpea, and cowpea-groundnut blends were formulated and spontaneously fermented for 72h. Enumeration using culture- dependent method revealed that microbial cell counts increased drastically within the first 24h in maize-cowpea blends while it took longer (within the first 48h) in maize-cowpea-groundnut blends. The analysis of the Denaturing Gradient Gel Electrophoresis (DGGE) pattern of the fermented blends obtained with bacterial primers targeting the 16S rDNA genes clearly demonstrated that there was a major shift in the community structure within the first 24h. The species richness (R) for the total bacterial community varied from a low value (9) for maize-cowpea blends to a 15 for maize-cowpea -groundnut blends. The biodiversity index (H') as well as concentration of dominance (S) according to Shannon and Weaver and Simpson's index, respectively, varied significantly with the supplementation type with the maize-cowpea -groundnut sample maintaining high biodiversity throughout the fermentation period. Statistical analysis showed a significant difference ($P < 0.05$) in the total bacterial diversity within samples with increase in fermentation time as well as between the two formulations.

Conclusion and application of findings: Conclusively, the study showed that the shift in microbial dynamics is determined more by the supplement used and to a lesser extent by the length of fermentation period. Based on the results obtained in this work, it is recommended that fortification of maize gruel with cowpea – groundnut mixture will be preferred in order to enhance fermentation of the blend within a very short time.

Key words: Bacterial communities fermented weaning food, species richness, PCR-DGGE.

INTRODUCTION

There are several nutritional diseases in the developing world including kwashiorkor, the result of protein deficiencies, and marasmus, caused by a combination of protein and calorie deficiencies, that affect large numbers of children between the ages of 1 and 3 years (Steinkraus, 2002). Due to impaired immune systems, such children often

develop diarrhoea. Kwashiorkor is associated with tissue oedema, bloated abdomens, susceptibility to infection and changes in hair pigmentation (Steinkraus, 2002), and the disease can develop in children consuming sufficient calories if protein is deficient. Marasmus is the result of both calorie and protein deficiencies in children under 1 year



old, due to early cessation of breast-feeding and the attempted replacement with artificial milk, which has low protein contents. The principal symptoms of marasmus are growth retardation, muscle wasting and loss of subcutaneous fat. Both kwashiorkor and marasmus can result in mental retardation if the child survives.

Among the weaning foods prepared in Nigeria and some parts of West African countries is either ogi (akamu) which usually consist of bulky mono cereal foods from maize, sorghum or millet. These weaning foods have been reported by many workers to be inadequate in energy and nutrient density (Mosha & Svanberg, 1990, Lorri, 1993). These foods are eaten in large quantities and are expected to provide the bulk of the proteins needed for growth, but the quality of this protein is poor (Brown, 1991). In Nigeria, cowpea and soya beans are the most commonly used complementary foods; since both legumes have high nutrient content (Nnakwe, 1995). Protein quality is synergistically improved in cereals-legume blends because of the lysine contributed by the cowpea and methionine contributed by the cereal (Bressani, 1993).

The microbial ecology of natural fermentation of plant materials in tropical countries has been exclusively studied using cultivation-based techniques (Johansson *et al.*, 1995; Nuraida *et al.*, 1995; Brauman *et al.*, 1996; Hamad *et al.*, 1997; Leisner *et al.*, 1999). However, the cultivation technique cannot be used to assess microbial

diversity in fermented foods, as basic ecological parameters such as biodiversity indices cannot be calculated. Culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) of PCR-derived gene amplicons have been developed to assist in the study of the microbial biodiversity and population dynamics of complex ecosystems over space and/or time, including food (Ercolini, 2004; Giraffa, 2004). In a DGGE gel, the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of the numerically dominant ribotypes in the sample (Boon *et al.*, 2002).

Compared to traditional culturing, these methods, generally based on nucleic acids such as the 16S rRNA gene, are more effective at obtaining both a qualitative and a semi quantitative result of a microbial community without the need to isolate and culture its single components. However, it has previously been demonstrated that while these techniques are valuable, it would be prudent to use a combined system to overcome the bias of the "culture-(in) - dependent-only" approach (Ampe *et al.*, 1999). Therefore, culture-independent and cultivation methods should be applied in parallel for population dynamics and biodiversity studies.

The objective of the present study was to formulate cereal-legume blend, and to evaluate the effect of fortification on the microbial population dynamics and diversity of naturally fermenting maize-based weaning foods.

MATERIALS AND METHODS

Sample collection: Maize (DMR-ESR-Y) was obtained from the Institute of Agricultural Research and Training (I. A. R. & T.), Moor Plantation, Ibadan, Nigeria while cowpea(IAR/48) and groundnut (Ex-Dakar) were obtained from the Institute of Agricultural Research (I. A. R.), Zaria, Nigeria in clean sterile polyethylene bags and kept in the refrigerator until use.

Sample treatment, formulation and fermentation: The procedure for the treatment of the cereal, cowpea and groundnut was as previously described (Wakil *et al.*, 2008). The method involved steeping in sterile milli Q for 24h followed by malting in a stainless tray lined with wet filter paper in an air-circulating incubator at

30°C for 24h and 48h for legumes (cowpea and Groundnut) and maize respectively. The germinated grains were thoroughly washed, oven dried for 24h at 60°C while the legumes were dehulled, washed and oven dried at 60°C for 36-48h. The malted maize, cowpea and groundnut were dry milled separately followed by sieving through a 300nm pore sieve. The cereal-legume blends were formulated in ratios 70:30 (maize-cowpea) (Mallehi *et al.*, 1989) and 70:20:10 (maize-cowpea-groundnut), and were reconstituted with sterile milli Q water at a concentration of 30% (w/v) (Livingstone *et al.*, 1993). Spontaneous fermentation was allowed to proceed at $30 \pm 2^\circ\text{C}$



Microbiological analysis: The fermenting blend samples were subjected to microbiological analysis to monitor the dynamic changes in the populations responsible for maize–legume blends' fermentation for all the samples. Ten grammes (10g) of each blend were homogenized in 100ml, ¼ strength Ringer's solution. Serial dilution was effected with sterile milli Q water and 1ml of the appropriate dilutions was mixed with molten agar and pour–plated in duplicates on the following media: (1) Plate count agar (Oxoid) for estimation of total viable bacteria incubated for 24h at $30 \pm 2^{\circ} \text{C}$; (2) MRS agar (Oxoid) for total lactic acid bacteria (LAB) incubated at 35°C for 48h in anaerobic jars with Anaerogen (Oxoid, Basingstoke, Hampshire, England); (3) Violet Red Bile Agar (Difco) was used for the enumeration of total enterobacteria at $30 \pm 2^{\circ}\text{C}$ for 48h, and (4) Malt extract agar (oxoid) containing 0.5mg/l streptomycin sulphate (sigma) for yeasts and moulds, incubated at 30°C for 3 – 5 days. Microbiological counts were made after incubation and the results were calculated as means of two determinations. Counts were expressed as \log_{10} colony forming units (cfu) per gram of sample.

pH and titratable acidity: Potentiometric pH measurements were obtained with the pin electrode of a pH meter (Hanna Instrument HI 8521) inserted directly into the fermenting samples. In the determination of titratable acidity, 10 grams of sample was mixed in 100 ml of sterile milli Q water. The mixture was allowed to stand for 15 minutes and filtered with Whatman No. 4 filter paper. Ten (10) ml aliquots (triplicates) were pipetted and titrated against 0.1M NaOH to phenolphthalein end-point and the acidity was calculated as g lactic acid/100g sample (AOAC, 1980).

DNA extraction and PCR amplification: Total bacterial DNA was extracted from different fermented blends by the modified method described by Ampe *et al.* (1999). The quality of the DNA extracts was routinely checked using 1% agarose - 1 X TAE gel. Different regions of the 16S rDNA of the total bacterial community were amplified with the universal primer (Muyzer *et al.*, 1993). Aliquots (5µl) of the amplification products were analysed by electrophoresis in 1% agarose – 1X TAE gels.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis: The PCR products were analyzed by DGGE using a Bio-Rad D code apparatus as first described by Muyzer *et al.* (1993). Electrophoresis was performed in 6% (w/v) polyacrylamide gels with 1XTAE buffer diluted from 50 XTAE buffer (40Mm Tris base, 20Mm glacial

acetic acid, and 1Mm EDTA) for total bacterial community. The denaturant gradient used for optimal separation of the product was from 40 to 60% urea–formamide gradient increasing in the direction of electrophoresis. Electrophoresis buffer (1XTAE) was maintained at 60°C . The gel was electrophoresed to a constant voltage of 200V for 5h for total bacterial community (Muyzer *et al.*, 1993). Gel was then silver stained, scanned and analyzed with the Quantity One software package (Bio-Rad, Richmond, California).

Analysis of the DGGE patterns: The richness, diversity and dominance indices within the bacterial populations as well as the similarities between the total bacterial communities of the formulated blends were calculated from DGGE profiles. Scanned gels were analyzed with Quantity one software package (Bio Rad,) Richmond, Calif.) using the strategy proposed by Eichner *et al.* (1999). The patterns were analyzed as follows;

(i). The total number of bands in a gel track was first corrected for crowding and transformed into richness estimates 'R' as described by Nubel *et al.* (1999).

(ii). After bands were assigned to the gel tracks and the corresponding bands in independent tracks were matched, Unweighted paired group mean arithmetic (UPGMA) was used to calculate the corresponding dendrograms using Phoretix ID advanced analysis package.

(iii). The Shannon- Weaver index of general diversity, H' (Shannon & Weaver, 1963) was calculated with the following equation: $H' = - \sum p_i \ln p_i$

Where p_i is the importance probability of the bands in a track. H' was calculated based on the bands in the gel tracks by using the intensities of the bands as judged by peak heights in the densitometric curves. p_i was calculated as follows: $p_i = n_i/N$

Where n_i is the height of the peak i (or volume) and N is the sum of all peak heights in the densitometric curve.

(iv) Using the same data, the Simpson index of dominance, S (Simpson, 1949) was calculated using the following function: $S = \sum p_i^2$

The results given are the means of two independent determinations performed after independent DNA extractions, PCR amplifications, and DGGE separations.



RESULTS

The results obtained using the culture-dependent method with four different culture media during the 72h fermentation time shows a general drastic increase in cell counts within the first 24h for cowpea – and 48h for cowpea-groundnut fortified blends (Fig 1). The total microbial density was highest at 24h with total viable count of $\log_{10}11.85$ in maize-cowpea blend while the highest count of $\log_{10} 11.49$ was recorded in maize-cowpea -groundnut blend by 48h of fermentation. The number of aerobic bacterial count increased within the first 24h of fermentation in both blends. The lactic acid bacteria count on MRS agar ranged from 0 to $\log_{10}11.56$ in maize-cowpea blend, and from $\log_{10}8.65$ to $\log_{10}11.03$ in maize-cowpea-groundnut blend within 24 and 48h, respectively. No culturable yeast counts were observed on MEA before active fermentation in both blends. No enterobacterium was observed after 24h on VRBGA medium. In both blends, the pH decreased within the first 48h while the total titratable acidity (TTA) increased within 24h and thereafter the pH increases with a decrease in TTA till the end of fermentation period.

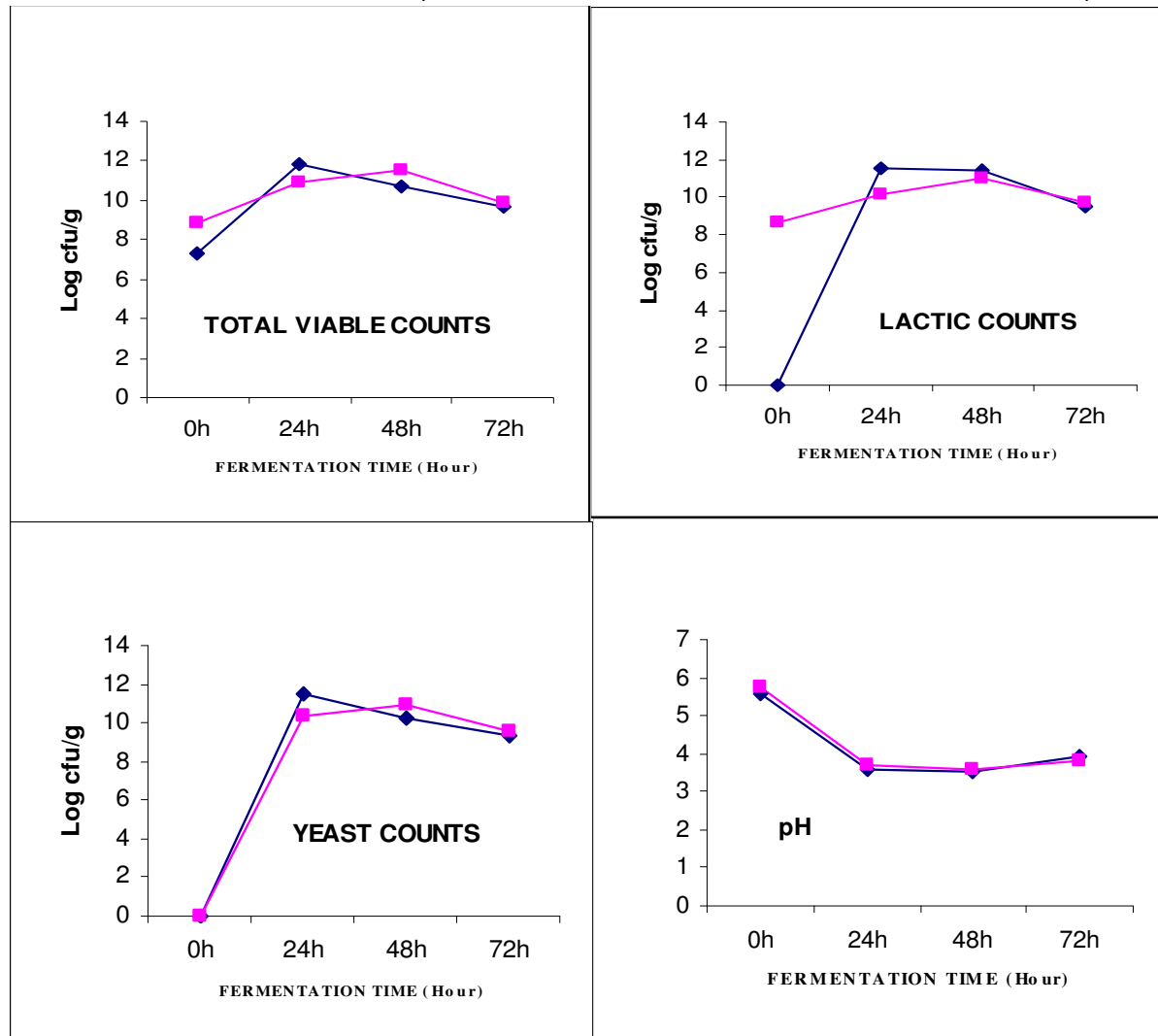


Figure 1: Microbial counts during fermentation of malted maize-legume blends using culture-dependent method: - ■ - Maize-cowpea-Groundnut blend ; - ◆ - Maize-cowpea blend.

The total bacterial community profile monitored by Denaturing Gradient Gel Electrophoresis (DGGE) was as shown in figure 2. There was a shift in total bacterial composition, as some bands were becoming dominant

or recessive and new bands being formed. The analysis of the DGGE-based pattern of the total bacterial community profile in maize-cowpea blends and maize-cowpea-groundnut blend resulted in two

distinct clusters (Fig. 3). Cluster 1 consisted of maize-cowpea-groundnut blend from day 0 – 3 of the fermentation period with 77% similarity. Cluster 2 consisted of days 0 – 3 of maize-cowpea blend with similarity of 81%. Time seemed to have little or no effect on the diversity of the microbial community profile

of the fermented samples based on the 16S rDNA gene. Furthermore, there was no replicate variability (difference) in duplicate samples of the treated blends at most time intervals except on day 1 of maize-cowpea blend (1A11 & 1A12 on fig. 3) with 81% similarity.

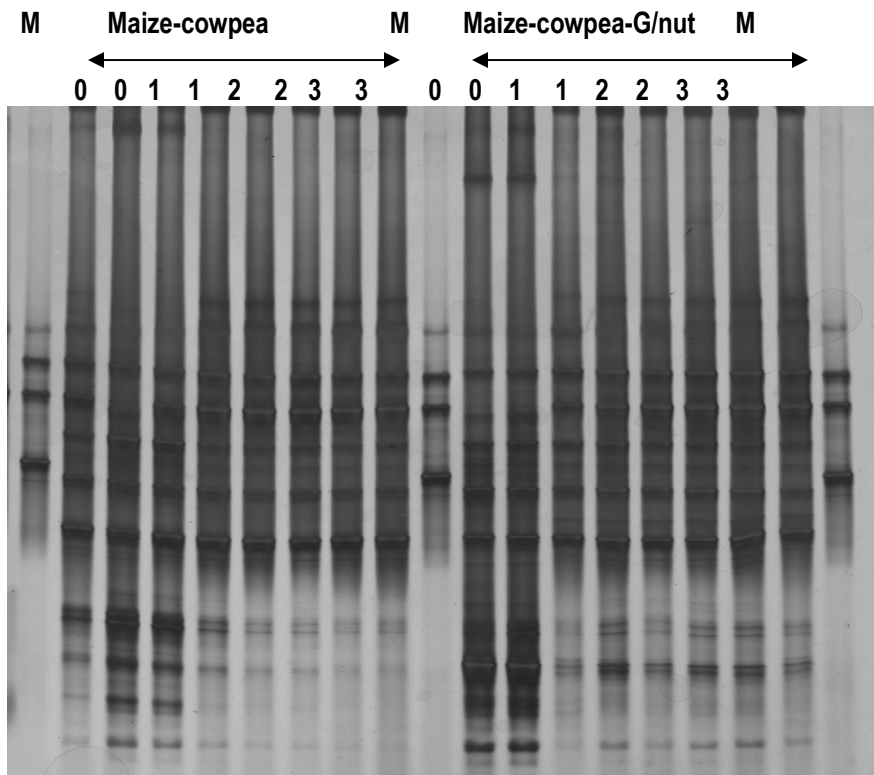


Figure 2: DGGE analysis of PCR- amplified 16S rDNA fragment for total bacterial community from cereal-legume weaning blends. 0:- unfermented blends, 1:- 24h fermented blend, 2:- 48h fermented blend and 3:- 72h fermented blend.

Duplicate DGGE pattern was used to generate species richness since each band in DGGE is likely to be derived from one phylogenetically distinct population; an estimation of species number was then based on the total number of bands in the profile. The richness index R was calculated for all the DGGE patterns in Fig.2. R equals to the number of bacterial bands on each gel track (Table 1). The species richness R

varied from relatively low value (9) for maize – cowpea blend to high value of 15 for maize–cowpea-groundnut blend. While the bacterial species richness decreases with increase in fermentation time throughout the sampling period for the cowpea-fortified blend, it increases after 48h of fermentation time for cowpea-groundnut fortified blend.

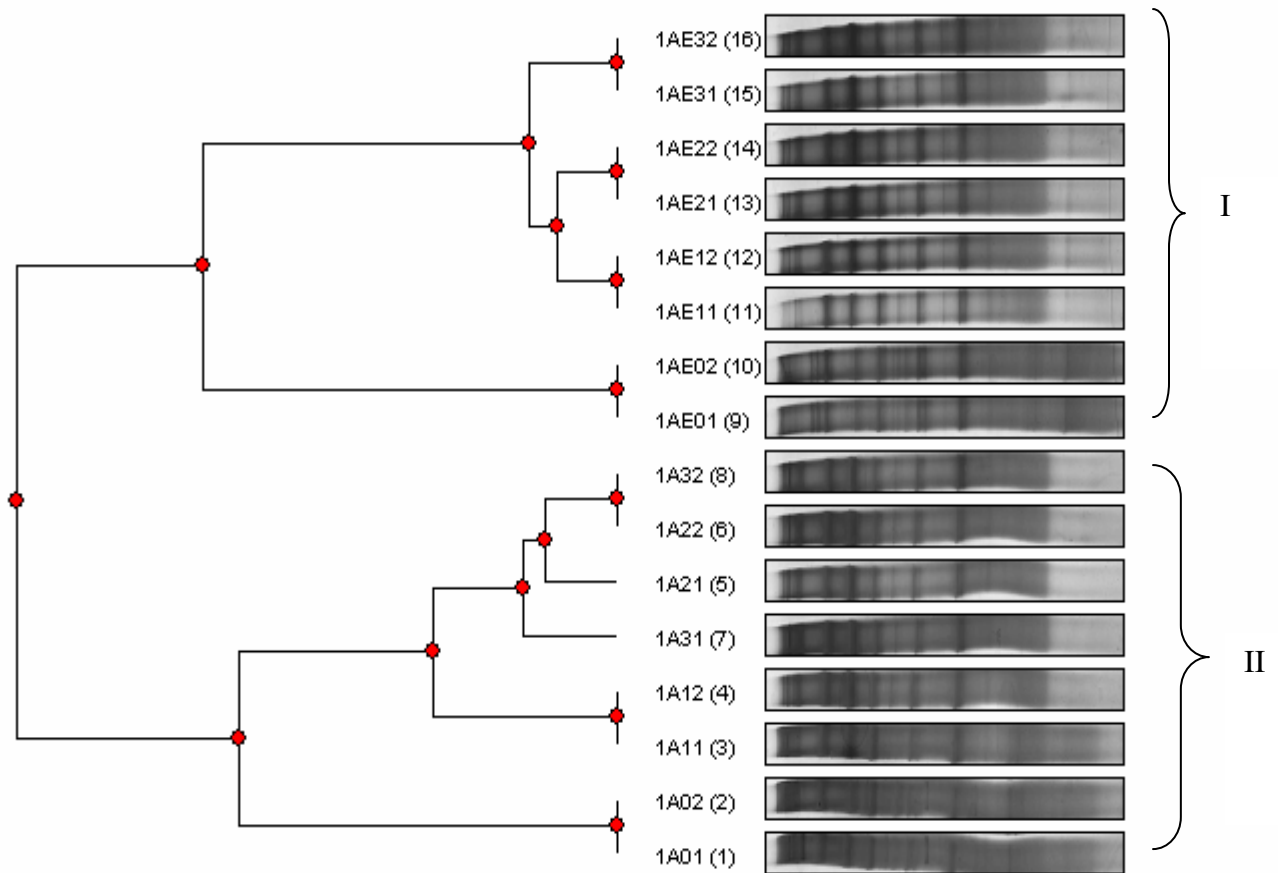


Figure 3: UPGMA dendrogram for the total bacterial community derived from analysis of the DGGE pattern of amplified 16S rDNA from malted and fermented maize-fortified blends.

Table 1: Species Richness (R), Biodiversity Index (H'), and Dominance Index (S) calculated from DGGE pattern for total bacterial community for fortified fermented maize-based blends.

Samples	Fermentation time (Hour)	Average R	Average H'	Average S
Maize-cowpea blend	0	11	*2.20	0.12
	24	9.5	2.08	0.13
	48	9	1.99	0.15
	72	9	1.83	0.185
	Mean		9.625 ± 0.95	2.025 ± 0.16
Maize-cowpea-groundnut blend	0	15	2.495	0.095
	24	12	2.22	0.125
	48	12	2.09	0.132
	72	13	2.44	0.110
	Mean		13 ± 1.41	2.31 ± 0.19

*Each value is a mean of two determinations.

Effect of fermentation time on total bacterial diversity and dominance indices: The Biodiversity index (H') as well as concentration of dominance (S) varied significantly with the type of supplement used. Maize –cowpea-groundnut blend had higher microbial diversity ($2.09 < H < 2.50$) associated with lower concentration of dominance ($0.095 < S < 0.135$). Conversely, maize – cowpea blend exhibited low

DISCUSSION

The results of the culture-dependent (plating) method, which was mainly characterized by an initial increase in total bacteria count, LAB population and yeast count was similar to those reported previously (Wakil *et al.*, 2004; Michodjehoun-Mestres *et al.*, 2005). Apart from the flora present on the surface of the grains, microbial flora may have also established during milling and malting processes, thus explaining the higher initial culturable count in the formulated weaning blends. The total disappearance of Enterobacteriaceae by 24h fermentation time agrees with the observations of Usha and Chandra (1997), Wakil *et al.* (2004) and Michodjehoun-Mestres *et al.* (2005). The observed decrease that culminated in total disappearance of the enterics population was parallel to pH decrease, which is excepted in accordance with the death kinetic of Enterobacteriaceae due to low pH (<4.5) (Nout *et al.*, 1989; Hounhonigan *et al.* 1993).

Fermentation was observed to decrease the pH and increase the titratable acidity of the formulated weaning blends. Such a decrease in pH and increase in acidity due to microbial activity has been well documented in cereals, millets and cereal-pulse mixtures fermented with endogenous grain microflora or with pure cultures (Soni & Sandhu 1989; Achi 1990; Khetarpaul & Chauhan 1991, 1992). The observed increase in titratable acidity could be due to dominance by lactic acid bacteria that degrade carbohydrates resulting in acidification. These observations are in agreement with earlier studies by Nout *et al.* (1989) and Ariaahu *et al.* (1999).

One of the important questions in the study of spontaneous fermentation is to determine whether it is the substrate, or the food matrix that is driving the process, or whether external conditions such as pH, oxygen, and water activity are more important in the selection of the active microflora. The effect of type(s)

CONCLUSION

In conclusion, it is apparent that the dynamics and diversity of the total microbial population of malted and

diversity indexes ($1.83 < H < 2.20$) associated with high concentration of dominance ($0.12 < S < 0.185$). However, statistical analysis using 2 way ANOVA showed a significant difference ($P < 0.05$) in the total bacterial diversity within samples with increase in fermentation time as well as between the two fortified fermented samples.

of supplement used in fortification on the total bacterial communities was assessed using unweighted pair group means with arithmetic (dendrogram). Results show a significant difference ($P < 0.05$) between the total microbial communities of maize- cowpea and maize-cowpea-groundnut fortified blends, resulting in 2 distinct clusters. The observed differences between the microbial communities are partly due to the differences in the supplement-type (substrate) and not necessarily due to differences in length of fermentation time. Omar and Ampe (2000) have reported that the shift in the microbial community structure of fermented maize during pozol production coincided with the sampling point (whole, centre or periphery). Further, Ampe and Miambi (2000) reported that the differences between the microbial communities established during indigenous maize fermentation (for the production of Ogi, poto-poto and pozol) are likely to be due to differences in processing methods rather than fermentation period. Furthermore, the succession of micro organisms during cocoa bean fermentation has been reported to be the actual reflections of the environmental factors (temperature, pH, and oxygen tension) (Camu *et al.*, 2007).

Microflora from the maize-cowpea-groundnut blends exhibited higher richness and biodiversity indices than those from maize-cowpea blends. The biodiversity index (H') combines the relative abundance of species and the total species richness, thus it reflects the distribution of the dominance amongst the major species present in a sample. Therefore, the high biodiversity index value of cowpea-groundnut fortified blend indicated that a high number of different species are involved in the fermentation of the sample, as observed by Ampe and Miambi (2000) and Santegoed *et al.* (1996).

fermented maize –legume blends are highly affected by the type of supplement used for the fortification. Based



on these results we recommend that fortification of maize gruel with cowpea –groundnut mixture will be preferred in order to enhance fermentation of the blend within a very short time.

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