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Dynamics and Diversity of Bacterial Communities of Fermented Weaning Foods via Denaturing Gradient Gel Electrophoresis PCR-DGGE

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Abstract: Different cereal-legume weaning blends were formulated and subjected to spontaneous fermentation for a period of 72 h. The analysis of the DGGE pattern, of the fermented blends, obtained with bacterial primer targeting the 16S rDNA genes clearly demonstrated that there was a major shift in the community structure within the first 24 h. The species richness R for total bacterial community varied from a low value 6.50 for maize-based blends to a higher value 9.50 for sorghum-based 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 sample type. Statistical analysis showed a significance difference $p < 0.05$ in the total bacterial diversity within samples with increase in fermentation time but with no significant difference in the diversity among the different fermented samples.

Key words: Dynamics, diversity, bacterial communities, fermented weaning foods, PCR-DGGE

INTRODUCTION

Weaning foods in most developing countries are usually thin cereal or root gruel that is called by different names depending on the type of cereal or the country. In Nigeria, the usual first weaning food is called pap, *akamu*, *ogi* or *koko* and is made from maize *Zea mays*, guinea corn *Sorghum* sp. or millet *Pennisetum americanum* (Odunfa and Oyewole, 1999). Legumes are rarely used for weaning and are introduced much later after 6 months of age because of the problems of indigestibility, flatulence and diarrhoea associated with their use (King *et al.*, 1985; Uwaegbute and Nnanyelugo, 1987). In developing countries, fermented foods are produced primarily at the household and village level where they find wide consumer acceptance (Steinkraus, 2002). Traditional fermentation processes used in the production of these foods are uncontrolled and are dependent on microorganisms from the environment or the fermentation substrate for initiation of the fermentation processes (FAO/UN, 2004). Many indigenous cereal fermentations involve the combined action of bacteria, yeast and fungi. Some microflora may participate in parallel while others may participate in a sequential manner with a changing dominant flora during the course of the fermentation. However, the soaking of the grains in excess water allows the selection of desirable microorganisms such as Lactic acid bacteria, yeasts and moulds (Sefa-Dedeh *et al.*, 1999; Sefa-Dedeh and Cornelius, 2000).

The microorganisms contributing to the characteristic properties of the food during the course of the fermentation process should be known in order to allow control of the process by selection of the appropriate technological conditions and by using defined cultures (Meroth *et al.*, 2003). As such the

definition of a microbial starter to standardize the quality of a fermented food has been repeatedly claimed to be of primary importance (Sanni, 1993).

The microbial ecology of natural fermentation of plant materials in tropical countries has been exclusively studied using cultivation-based techniques (Hounhouigan *et al.*, 1993; Johansson *et al.*, 1995; Nuraida *et al.*, 1995; Brauman *et al.*, 1996; Hamad *et al.*, 1997; Leisner *et al.*, 1999). However, this strategy to describe a microbial community by the sole culture-dependent approach was shown in other environment to be biased as only small fractions of species are recovered (Ward *et al.*, 1990; Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). This was also found to be true for fermented foods where most microorganisms were thought to be culturable (Ampe *et al.*, 1999). Hence, previous findings using cultivation techniques cannot be used to assess microbial diversity in fermented foods, as basic ecological parameters such as biodiversity indexes cannot be calculated. Thus, in order to study interactions between microorganisms it is important to study the ecosystem without dissociating it.

One-culture independent method for studying the diversity of microbial communities is the analysis of PCR products, generated with primers homologous to relatively conserved regions in the genome, by using denaturing gradient gel electrophoresis DGGE or temperature gradient gel electrophoresis TGGE (Muyzer and Smalla, 1998; Muyzer, 1999). These approaches allow separation of DNA molecules that differ by single bases (Myers *et al.*, 1987) and hence have the potential to provide information about variations in target genes in a bacterial population.

Most of the molecular techniques for the detection, identification and classification of bacteria are based on the direct PCR amplification and analysis of nucleotide sequence of 16S rRNA. Both DGGE and TTGE are now methods of choice for environmental microbiologists and have been used to determine the genetic diversities of natural microbial communities such as the communities in biofilms (Muyzer *et al.*, 1993), soil (Felske *et al.*, 1996; Gelsomino *et al.*, 1999), ocean depths (Teske *et al.*, 1996), hot springs (Ferris *et al.*, 1996; Santegoeds *et al.*, 1996), lakes (Zwart *et al.*, 1998), a biodegraded wall painting (Rolleke *et al.*, 1996) and fermented foods (Ampe *et al.*, 1999; Coppola *et al.*, 2001; Cocolin *et al.*, 2001, 2004). The aim of this study was to use molecular approaches to describe the factor responsible for dynamics and diversity of the bacterial community during fermentation of cereal- based weaning food.

MATERIALS AND METHODS

Sample Collection

The cereals; DMR-ESR-Y maize and cowpea Ife-Brown used was collected from the Institute of Agricultural Research and Training I. A. R. and T., Ibadan, Nigeria while KSV-15 sorghum and millet was obtained from the Institute of Agricultural Research I.A.R., Zaria, Nigeria in clean sterile polyethylene bags and kept in the refrigerator until use. The samples were collected in February and analyzed between May and August, 2006.

Sample Treatment, Formulation and Fermentation

The grains were weighed \approx 500 g into clean beakers, washed thoroughly with distilled water during which the floating seeds were discarded. The seeds were steeped in distilled water for 24 h at room temperature. The soaked grains were later germinated in a stainless tray lined with wet filter paper in an air-circulating incubator at 30°C for 24 and 48 h for legume and cereals, respectively. Germinated seeds were separated from non-germinated seeds. The rootlets of the germinated seeds were removed and were thoroughly washed with distilled water and oven dried at 60°C for 24 h. For the legumes, the germinated seeds were dehulled, washed and oven dried at 60°C for 36-48 h. The germinated and dried cereal and legume samples were separately milled, sieved to obtain a fine flour 90% passing through a 300 nm pore sieve, packed in sterile bags before storage at 4°C until use. The cereal- legume blends were formulated in ratios 70:30 cereal-cowpea (Malleshi *et al.*, 1989).

The different flour combinations were mixed with sterile milli Q water at a concentration of 30% w/v (Livingstone *et al.*, 1993). Spontaneous fermentation was allowed to proceed at 30°C and sampling done 24 h for 3 days.

DNA Extraction

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 by using 1% agarose-1 X TAE gel.

PCR Amplification

Different regions of the 16S rDNA of the total bacterial community was amplified with the universal primer (Muyzer *et al.*, 1993), while the total lactic acid bacteria community DNA was amplified with primers gc 338f and 518r spanning the V3 region of the 16S ribosomal DNA (Øvreas *et al.*, 1997) as described by Ampe *et al.* (1999). Aliquots 5 µL of the amplification products were analysed by electrophoresis in 1% agarose-1X TAE gels.

Denaturing Gradient Gel Electrophoresis Analysis

The PCR products were then analyzed by denaturing gradient gel electrophoresis DGGE using a Bio-Rad D code apparatus and the procedure first described by Muyzer *et al.* (1993). Electrophoresis was performed in a 6 and 8% wt/vol polyacrylamide gels with 1XTAE buffer diluted from 50 XTAE buffer 40 Mm Tris base, 20 Mm glacial acetic acid and 1 Mm EDTA for total bacterial community and total lactic acid bacteria community respectively. Two denaturant gradients were used for optimal separation of the products; one from 30 to 60% urea-formamide gradient for gc 338f-518r primer and the other universal primer from 40 to 60% urea-formamide gradient increasing in the direction of electrophoresis. Electrophoresis buffer 1XTAE was maintained at 60°C. The gels were electrophoresed to a constant voltage of 200 V for 5 h for total bacterial community, (Muyzer *et al.*, 1993) and for 10 min at 20 V and then 3 h at 200 V for lactic acid bacteria community (Ampe *et al.*, 1999). Gels were then stained with silver staining, 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 microbial populations as well as the similarities between the microbial communities of the different 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:

- 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)
- 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
- The Shannon- Weaver index of general diversity, H' Shannon and Weaver, 1963 was calculated with the following Eq.

$$H' = -\sum p_i \ln p_i$$

where, Pi is the importance probability of the bands in a track. H' was calculated on the basis of the bands in the gel tracks by using the intensities of the bands as judged by peak heights in the densitometric curves. Pi 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.

Using the same data, the Simpson index of dominance, S Simpson (1949) was calculated using the following function:

$$S = \sum p_i^2$$

- The Evenness of the bacterial species within the bacterial community was evaluated using Equitability indices. This includes
- Shannon-Weaver index of Equitability, J , which was calculated using the following equation:

$$J = H'/\ln R$$

where, R is the species richness.

- Simpson index of equitability, E , which was calculated using the following equation.

$$E = D/R$$

where, D is the Simpson index of diversity and is calculated as follows:

$$D = 1/\sum p_i^2$$

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

RESULTS

Bacterial Community

The fingerprints of the total bacterial community obtained from the composite gel consisting of bands of the DGGE profile of total DNA extracted from fermented maize-legume blends 1A-series, fermented sorghum-legume blends 4A-series and millet-legume blends 6A-series contained many visible bands (Fig. 1). The number of visible bands increased with fermentation time, while the profile intensity decreased with time. There was a major shift between 0 and 24 h, but only minor differences were observed between samples taken at the same time from all the different fermented formulated blends. Generally, there were some prominent bands, which were common to all the different samples at the various fermentation times, with more visible bands at 0 h.

In Fig. 2, the comparative analysis of the DGGE pattern for the total bacterial community profile for all the three cereal-legume blend samples resulted in 3 distinct clusters. Cluster 1 consisted of day 1 to 3 day of millet-based samples 6A11-6A31 with 89% similarity. Cluster II contained day 1 to 3 day of fermented sorghum based samples 4A11-4A31 and 2 and 3 days of fermented maize-legume blend samples, while cluster III comprised of unfermented blends of all the three samples 6A01, 4A01 and 1A01 and day 1 of fermented maize-based blend 1A11 with 84% similarity. Here, time seems to have little or no effect on the bacterial community profile. Rather, the profile seemed to be influenced by the sample type/formulation.

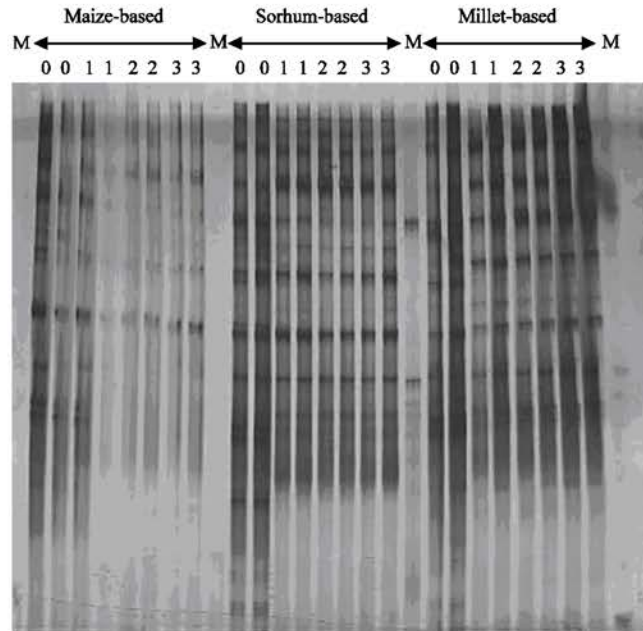


Fig. 1: DGGE analysis of PCR-amplified 16S rDNA fragment of total community profile of fermented cereal-legume blends. 0:- unfermented blend 0 h, 1:- 24 h fermented blend, 2:- 48 h fermented blend and 3:- 72 h fermented blend

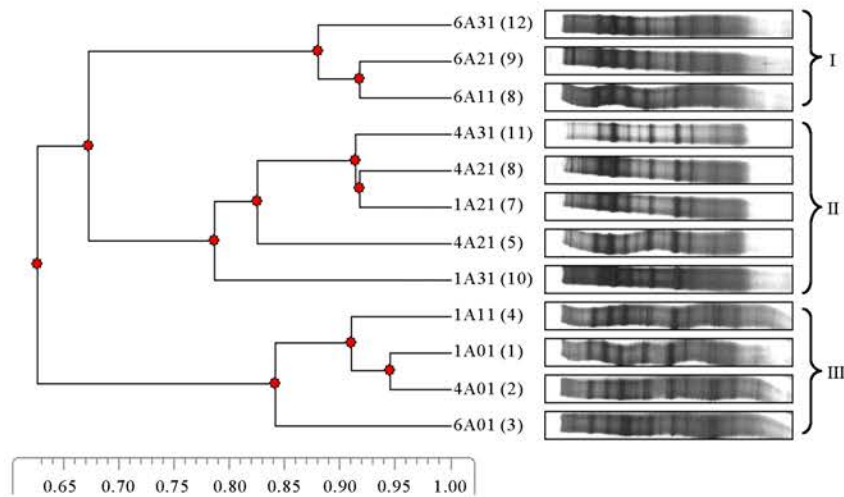


Fig. 2: UPGMA dendrogram of comparative analysis of the DGGE pattern of amplified 16S rDNA from fermented cereal-legume blends for the total bacterial community

Effect of Fermentation Time on Total Bacterial Species Richness

Duplicate DGGE pattern was used for the generation of the 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

Table 1: Species richness R, Biodiversity index H' and Dominance Index S calculated from composite DGGE profile for total bacterial community for fermented cereal-legume blends DGGE gel in Fig. 1

Samples	Fermentation Time (h)	Mean R	Mean H'	Mean S
1A	0	9	*1.830±0.09	0.185±0.025
	24	7	1.690±0.25	0.220±0.06
	48	5	1.325±0.095	0.300±0.02
	72	5	1.270±0.06	0.335±0.035
4A	0	12	2.220±0.10	0.125±0.015
	24	9	2.080±0.065	0.130±0.01
	48	8	1.890±0.095	0.180±0.03
	72	9	1.990±0.02	0.150±0.00
6A	0	8	1.860±0.045	0.175±0.005
	24	8	1.850±0.09	0.175±0.025
	48	10	2.090±0.035	0.135±0.005
	72	10	1.990±0.01	0.170±0.00

*Each value is a mean of duplicate determinations

for all the DGGE patterns in Fig. 1. R equals to the number of bacterial bands on each gel track Table 1. The species richness R varied from relatively low value 5 for maize-based fermented blend to higher value 12 for sorghum-based blend. While the bacterial species richness decreases with increase in fermentation time for maize- and sorghum-based blends, it increases with increase in fermentation time for millet-based blend where R varies between 8 and 10.

Effect of Fermentation Time on Total Bacterial Biodiversity and Dominance Indices

The analysis of the DGGE patterns for the estimation of the biodiversity and dominance indices derived from the work of Shannon and Weaver (1963) and the work of Simpson (1949), respectively was carried out on the total bacterial community for the fermented cereal-legume blends (Table 1).

The biodiversity index H' as well as concentration of dominance S varied significantly with the sample type. Sorghum - based blend appeared to have the highest microbial diversity $1.99 < H < 2.22$ associated with the lowest concentration of dominance $0.125 < S < 0.18$. Conversely, maize-based blend exhibited the lowest diversity indexes $1.27 < H < 1.83$ associated with highest concentration of dominance $0.185 < S < 0.335$.

The diversity in the total bacterial communities in all the fermented samples exhibited similar trends, but statistical analysis using 2 way ANOVA $p > 0.05$ revealed that there was significant difference in the bacterial diversity within samples as fermentation time increased. Statistical analysis also showed that there was no significant difference in the bacterial species diversity indices H' among the different fermented samples.

Although, the Simpson's dominance index S showed that maize-based blends has the highest dominance indices i.e., more dominant species than other fermented cereal-based samples, but statistical analysis ANOVA, $p < 0.05$ revealed that there was no significant difference in the dominant species among the fermented samples. Conversely, fermentation time had significant effect on the dominant species present in the total bacterial communities of all the three fermented cereal-based samples. Therefore, the total bacterial diversity and dominance in all the fermented cereal-based samples were significantly affected by the fermentation time and not by the sample type.

Effect of Fermentation Time on Diversity and Dominance Equitability Indices

The diversity equitability J and dominance equitability E indices values calculated from the DGGE gel profiles (Fig. 1) of the total bacterial community for the fermented cereal legume blends is as shown in Table 2. The equitability index showed that the bacterial species community in

Table 2: Dominance diversity D, Shannon-Weaver index of equitability J and Simpson index of equitability E calculated from composite DGGE pattern for total bacterial community for fermented cerea-legume blends

Samples	Fermentation Time (h)	Mean D	Mean J	Mean E
1A	0	*5.505±0.745	0.833±0.041	0.612±0.083
	24	4.910±1.34	0.868±0.065	0.688±0.093
	48	3.350±0.22	0.823±0.059	0.669±0.045
	72	3.015±0.315	0.789±0.037	0.604±0.063
4A	0	8.115±0.975	0.893±0.040	0.676±0.081
	24	7.735±0.595	0.970±0.004	0.909±0.017
	48	5.715±0.955	0.906±0.046	0.714±0.119
	72	6.670±0.0	0.931±0.016	0.787±0.046
6A	0	5.720±0.16	0.892±0.0216	0.715±0.02
	24	5.835±0.835	0.889±0.043	0.729±0.104
	48	7.415±0.275	0.906±0.015	0.742±0.027
	72	5.880±0.0	0.864±0.004	0.588±0.0

*Each value is a mean of duplicate determinations

sorghum-based sample was highest in terms of community diversity and dominance evenness at most of the fermentation times, while maize-based blends had the least indices at most sampling time.

Generally, fermentation causes a comparative reduction in species evenness in all the fermented samples, apart from 24 h when there was an increase in the equitability indices of the bacterial species in maize-and sorghum-based samples. In contrast, the evenness increases till 48 h fermentation period in millet- based blend and later decreased by 72 h.

DISCUSSION

Bacteriological culturing of fermentation samples resulted in a more complex flora than did DGGE analysis. PCR- DGGE detects 90 to 99% of the most numerous species of a community without discriminating living from dead cells in a non- cultivable state (Meroth *et al.*, 2003). A molecular approach to monitor the diversity and dynamic changes in the main populations involved in fermentation of cereal-legume weaning blends was used. This approach exploited the potential of PCR to amplify, with suitable primers, regions conserved within the domain *Eubacteria*, as well as the discriminatory power of DGGE to differentiate DNA molecules on the basis of differences in their sequences (Muyzer, 1999).

There was a shift in total bacterial communities' composition at different time intervals and the shifts in community composition occurred in different patterns in all the three cereal-based weaning samples (Fig. 1). This result suggests that the shifts in microbial population some bands becoming dominant or recessive and new bands being formed might have been due to cereal type and not necessarily as a result of increased fermentation time. This observation was similar to the findings reported by Ben Omar and Ampe (2000), that the shift in the microbial community structure of fermented maize during pozol production coincide with the sampling point whole, centre or periphery m. Furthermore, Ampe and Miambi (2000) reported differences between the microbial communities developed during indigenous maize fermentation for the production of ogi, pote-pote and pozol and reported that the differences are likely to be due to the differences in the processing methods and not fermentation time.

DGGE fingerprinting also allows comparison of total bacterial communities of the different samples through pattern analysis. An increase in number of bands was observed in the DGGE profile for total bacterial communities indicating an increase in total bacterial species, an observation similar to that of Santegoeds *et al.* (1996). The highest number of bands with high band intensities was observed in millet-based blends, implying an increased number of predominating species of the microbial communities. This might be due to the high protein quality and mineral contents of pearl millet as reported by Khetarpaul and Chauhan (1989), FAO (1995) and Makokha *et al.* (2002).

Thus, the use of the molecular method by direct analysis of DNA shows that fermentation resulted in considerable reduction in species evenness with time in all the cereal samples. This means that a specific group of organisms which are likely to be lactic acid bacteria was predominant, thus conforming to the traditional phenotypic result which revealed the dominance of lactic acid bacteria with increased fermentation time. However, the observed LAB DGGE band intensities did not correlate with LAB concentration obtained by plating on MRS agar because the intensities increased with fermentation time. This result confirmed the unsuitability of MRS medium for the cultivation or growing of lactic acid bacteria, an observation similar to that of Ampe *et al.* (1999) and Ercolini *et al.* (2003).

As one of the important questions in the study of spontaneous fermentation is to determine whether it is the substrate, or the food matrix which 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. Hence, the effect of fermentation time on the total bacterial communities was assessed using unweighted pair group means with arithmetic dendrogram. For the evaluation of the effect of fermentation time on total bacterial communities, UPGMA dendrograms was constructed using composite samples [replicate samples of the different cereals Maize, Sorghum and Millet pooled together]. Results show a significant difference $p < 0.05$ between the total microbial communities of maize-sorghum- and millet- based blends. The observed differences between the microbial communities are likely to be due to the differences in the cereal-type substrate since each cluster belongs to a specific cereal-type while the third cluster suggested that there was no difference in the microbial community of unfermented cereal-blends irrespective of their type.

Microflora from the Sorghum-based blends exhibited higher richness and biodiversity indexes than those from maize-and millet-based blends. Furthermore, the biodiversity index H' known to combine the relative abundance of species and the total species richness, thus reflecting the distribution of the dominance amongst the major species present in the samples studied. Therefore, the high biodiversity index value of sorghum-based blend revealed that a high number of different species are involved in the fermentation of the sample, an observation similar to those of Ampe and Miambi (2000). Fermentation was also found to have resulted in considerable reduction in species evenness in all the cereal samples. This may be due to the considerable reduction in pH 6.0-3.6, which resulted in increased acidity that allows only acid- sensitive microorganisms to develop. The dominance of acid-tolerant microflora in cereal fermentation has been reported by some researchers Steinkraus *et al.* (1983), Nout *et al.* (1989), Ariahu *et al.* (1999), Nuraida *et al.* (1995), Ampe *et al.* (1999) and Ampe and Miambi, (2000). Thus, the profiles obtained by DGGE agreed with the results obtained by traditional methods that the LAB population was the largest population during fermentation.

CONCLUSION

This study demonstrated that the ecology of fermented weaning cereals could better be studied and the diversity could be well-determined using PCR-DGGE analysis. The results also confirmed previous findings that the microbial dynamics and diversity were not determined by fermentation time as shown by traditional plating rather by substrate/cereal type.

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