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Full Length Research Paper

Time related total lactic acid bacteria population diversity and dominance in cowpea-fortified fermented cereal-weaning food

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The total lactic acid bacterial community involved in the spontaneous fermentation of malted cowpea fortified cereal weaning food was investigated by phenotypically and cultivation independent method. A total of 74 out of the isolated 178 strains were *Lactobacillus plantarum,* 32 were *Pediococcus acidilactici* and over 60% were *Lactobacillus* strains. *L. plantarum* and *P. acidilactici* strains were the dominant strains during the fermentation process and were identified in all the three cereals used. Separation of amplified V3 region from 16S rDNA by denaturing gradient gel electrophoresis (DGGE) using species, specific primer was carried out. The results show that the total lactic acid bacterial species richness (R) decreased with increase in fermentation time, while the species diversity and dominance indices were not significantly (P < 0.05) affected by fermentation time but rather by cereal-type. Comparison of 16S rDNA sequences of pure culture isolates with those in Genbank database revealed that, the dominating lactic acid bacteria were *L. plantarum* and *Pediococcus* species.

Key words: Lactic acid bacteria, communities' diversity, fortified weaning foods, polymerase chain reaction amplification-denaturing gradient gel electrophoresis (DGGE).

INTRODUCTION

Fermented cereal-legume gruel/beverages are prepared by soaking cereals or legumes in water, wet-milling and then fermenting them. Fermentation is mainly by a complex microflora, which develops spontaneously with lactic acid bacteria generally overcoming the other flora. Lactic acid bacteria are a heterogeneous family of mainly low G + C gram positive, anaerobic, non-sporulating and acid tolerant bacteria that are generally regarded as safe (GRAS) for use in food and food products (Gancel et al., 1997). They contribute to rapid acidification of food products and also to flavor, texture and nutrition (Liu et al., 2005).

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Abbreviations: PCR, Polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis; **MRS,** de Man, Rogosa and Sharpe medium; **EDTA,** ethylene diamine tetraacetic acid; **GRAS,** generally regarded as safe; **LAB,** lactic acid bacteria.

The population dynamics of microbial food ecosystem have been studied mainly through microbiological analysis (Giraffa, 2004). In recent years, culture independent methods have been developed to circumvent the limitation of conventional cultivation techniques for the analysis of microbial communities in fermented foods (Ercolini, 2004). Thus, denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction amplification (PCR)-amplified 16S-rRNA fragments is frequently used as a relatively rapid and reliable cultivation independent method to study the biodiversity and population dynamics of microbial communities (Ercolini, 2004). PCR-DGGE has the potential to characterize and monitor the microbial population involved in fermentation processes (Muyzer and Smalla, 1998) and has been successfully applied to study the lactic acid bacteria composition and population dynamics of sourdough ecosystem (Meroth et al., 2003; Miambi et al., 2003; Gatto and Torriani, 2004; Randazzo et al., 2005). Also, PCR-DGGE has been used to monitor the diversity and dynamics of lactic acid bacteria (LAB) population in fresh sausages (Cocolin et al., 2004), cassava starch (Omar et al., 2000; Ampe et al., 2001)

and Pozol (Ampe et al., 1999a). However, there is little or no information on traditionally prepared weaning foods especially in tropical Africa.

The objectives of this study were to characterize lactic acid bacteria that occur naturally in spontaneously-fermented cereal-cowpea weaning blends formulated using culture dependent methods. The dynamic and diversity of the LAB during the 72 h fermentation time was also monitored using the same method. We believe that, more information about the diversity of LAB present in traditionally formulated African weaning foods should help in improving the microbiological quality, nutritional value and safety of the formulated blends/foods.

MATERIALS AND METHODS

Sample collection

Maize (DMR-ESR-Y) and cowpea(Ife-brown) used were collected from the Institute of Agricultural Research and Training (I. A. R. & T.), Moor plantation,Ibadan,Nigeria, while sorghum (KSV-15) and millet 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 cereals and cowpea was as previously described (Wakil et al., 2008). The method involved steeping, malting and dry milling. The cereal- legume blends were formulated in ratios 70:30 (cereal-cowpea) (Malleshi et al, 1989) 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 ± 2°C.

Microbiological analysis

Sampling of the naturally-fermenting blends was carried out at regular intervals (24 h for 3 days) to monitor the dynamic changes in the lactic acid bacterial population responsible for cereal-legume blends fermentation. Ten grammes (10 g) of each blend were homogenized in 100 ml, ¼ strength ringer solution. Serial dilution was carried out with sterile milli Q water and 1 ml of the appropriate dilutions was mixed with molten MRS agar (Oxoid), aseptically pour-plated in duplicates and incubated at 30 ± 2°C for 48 h in anaerobic jars with Anaerogen (Oxoid, Basingstoke, Hampshire, England). Counts were expressed as colony forming units (cfu) per gram.

pH and titratable acidity

Potentiometric pH measurements were obtained with the pin electrode of a pH meter (Hanna Instrument HI 8521) that was 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 min and filtered with Whatman No. 4 filter paper. 10 ml aliquots (triplicates) were pipetted and titrated against 0.1 M NaOH to phenolphthalein end-point and the acidity was calculated as gram lactic acid/100 g sample.

DNA extraction and PCR amplification

Total bacterial DNA was extracted from different fermented blends by the modified method described by Ampe et al. (1999a). The quality of the DNA extracts was routinely checked by using 1% agarose - 1 × TAE gel. Different regions of the 16S rDNA of the total lactic acid bacteria community DNA was amplified with primers gc 338 f and 518 r spanning the V3 region of the 16S ribosomal DNA (Øvreas et al., 1997) as described by Ampe et al. (1999a). Aliquots (5 µl) of the amplification products were analysed by electrophoresis in 1% agarose- 1× 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 8% (wt/vol) polyacrylamide gels with 1× TAE buffer diluted from 50 × TAE buffer (40 Mm Tris base, 20 Mm glacial acetic acid and 1 Mm EDTA) for total lactic acid bacteria community . The denaturant gradient used for optimal separation of the products was from 30 to 60% urea–formamide gradient increasing in the direction of electrophoresis. Electrophoresis buffer (1×TAE) was maintained at 60°C. The gels were electrophoresed to a constant voltage for 10 min at 20 V and then, 3 h at 200 V for lactic acid bacteria community (Ampe et al., 1999a). 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 lactic populations as well as the similarities between the lactic acid bacterial communities of the three 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; (1) the total numbers of bands in a gel track was first corrected for crowding and transformed into richness estimates R as described by Nubel et al. (1999). (2) The Shannon-Weaver index of general diversity, H' (Shannon and Weaver, 1963) was calculated with the following equation:

$$H' = -\sum piLnPi$$

Where, Pi 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. Pi was calculated as follows:

$$Pi = ni/N$$

Where, ni 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:

$$S = \sum pi^{2}$$

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

Table 1. Frequency of occurrence of viable lactic acid bacteria during fermentation of cereal-cowpea blends.

Microorganism	Number of isolate	Percentage of occurrence
L. plantarum	74	41.57
L. pentosaceus	3	1.69
L.fermentum	8	4.49
L. delbrueckii	14	7.87
L. brevis	6	3.37
L.amylovorous	2	1.12
L. species	13	7.30
P. acidilactici	32	17.98
P. pentosaceus	11	6.18
L.mesenteroides	10	5.62
Lactococcus species	2	1.12
Unidentified	3	1.69
Total	178	100

PCR and cloning of polymerase chain reaction amplification (PCR) products

Extraction of DNA from dominant lactic acid bacterial isolates was carried out by modified method of Ampe et al. (1999a). The purified DNA of the pure cultures was amplified with primers spanning the 200 bp V3 region of the 16 S rDNA (Øvreas et al., 1997). Amplification of the 16S rDNA was carried out in Eppendorf thermal cycler (Mastercycler Personal Model, Fisher Scientific, UK) and the PCR products were cleaned with a DNAce quick clean purification kit (Bioline), ligated in pCR 4-TOPO vector system and transformed into *Escherichia coli* top 10 cells as specified by the manufacturer (Invitrogen). Selection of transformants was done on Luria agar containing 50 mg/ml of ampicillin and 40 mg/ml X-Gal after incubation at 37°C for 18 to 24 h. The plates were then incubated at 4°C for 2 to 8 h to enhance color. Blue colonies mean no insertion, while white colonies are transformant or inserts. An efficient TOPO cloning reaction should produce hundreds of colonies. Between 5 and 10 transformants were randomly picked and amplified with primers M13F and M13R using programmable Eppendorf thermal cycler (Fisher Scientific, UK). The products were electrophoresed on a 1% agarose – 1×TAE gel and viewed by ethidium bromide staining using UV light.

Sequencing analysis

Sequencing was performed using only the reverse (No. GC-clamp) primer for cloned PCR product/fragments. Sequencing was determined by the dideoxy chain termination method. The closest relatives of 16S rDNA sequences were determined by a search of the GenBank DNA database using the BLAST algorithm (Altschul et al., 1990). Identities of isolates were determined based on the highest score.

RESULTS

The total lactic acid bacteria count which was not detectable at the beginning of fermentation in maize-cowpea blend (MaC) was highest (3.61×10^{11} cfu/g) by 24 h of fermentation and decreased thereafter in all the samples till the end of fermentation time. A total number of 178 lactic acid bacterial cultures were isolated and identified phenotypically based on their morphological and biochemical characteristics and sugar fermentation pattern. Overall, 42% of the isolates were identified as *Lactobacillus plantarum*, 18% as *Pediococcus acidilactici*, 8% as *Lactobacillus delbrueckii*, 7% as *Lactobacillus* species, 6% as *Pediococcus pentosaceus* and *Leuconostoc mesenteroides*, 5% as *Lactobacillus fermentum,* 3% as *Lactobacillus brevis,* 2% as *Lactobacillus pentosaceus,* 1% as *Lactobacillus amylovorous* and *Lactococcus* species and about 2% as unidentified species (Table 1). The two amylolytic strains of *L. amylovorous* were isolated from maize-fortified weaning food.

From Table 2, the pH values for all the cereal-cowpea fortified blends decreased with increase fermentation time and then, increased by 72 h fermentation period. The lowest pH value of 3.55 was reached in maize-based blend (MaC) and 3.75 to 3.92 in fermented sorghum (SoC) and millet (MiC) based blends within 48 h fermentation time. On the contrary, the total titratable acidity (TTA) increased drastically within 24 h fermentation time and later decreased till the end of fermentation with the least value of 7.2 mg/g lactic acid observed in (MaC) maize-cowpea fortified blend by 72 h.

Effect of fermentation time on total lactic acid bacterial species richness

The richness index (R) was calculated for the entire DGGE pattern in Figure 1. R is the number of lactic acid bacterial bands on each gel track (Table 2). The lactic acid bacterial species richness (R) varied from the mean least value (16.25 ± 0.95) for (MiC) millet- based blend to the mean highest value (18.88 ± 1.61) for (MaC) maize-based blends. The total number of lactic acid bacterial

Table 2. Effect of fermentation time on total lactic acid bacterial species richness (R) and acidity of formulated fermented cereal-cowpea blends.

Samples	Fermentation time (h)	Average R	Mean pH	TTA(mg/g)
MaC mean	0	22	*5.60	*3.60
	24	21	3.65	11.70
	48	17.5	3.55	11.70
	72	15	3.95	7.20
		18.875 ± 1.61		
SoC mean	0	19	5.52	5.40
	24	18	3.80	18.90
	48	16	3.75	18.00
	72	16	3.79	16.20
		17.25 ± 0.75		
MiC mean	0	19	5.50	9.00
	24	15	4.05	21.60
	48	15	3.92	18.00
	72	16	4.08	17.10
		16.25 ± 0.95		

*Each value is a mean of duplicate determinations. MaC, Maize-cowpea blend; SoC, sorghum-cowpea blend; MiC, millet-cowpea blend.

species present in (MaC) maize-based blend was the highest, while the least number of species was found in (MiC) millet-based blend. The result also showed that, lactic acid bacterial species richness decreased with increase in fermentation time.

Effect of fermentation time on total lactic acid bacterial biodiversity and dominance indices

The biodiversity index (H') and the dominance index (S) for the total lactic acid bacterial communities for all the three cereal-cowpea blends exhibited similar trends with little or no difference in H' values (Figures 2 and 3). Maize-based blend (MaC) have the highest bacterial diversity ($2.44 < H' < 2.78$) associated with the least dominance index ($0.07 < S < 0.11$), while sorghum-based blend (SoC) has the lowest lactic acid bacterial diversity index ($2.45 < H' < 2.64$) associated with the highest lactic acid bacterial dominance index ($0.09 < S < 0.10$). The lactic acid bacterial species diversity (H') decreased from 0 to 72 h fermentation period in all the cereal-cowpea blends (Figure 2) but statistical analysis using 2-way analysis of variance (ANOVA) ($P < 0.05$) revealed that, fermentation time had no significant effect on the total lactic acid bacterial diversity of the different cereal-legume blends. Although, the lactic acid bacterial species diversity exhibited similar trends in the three fermented cereal-cowpea blends, statistical analysis however, showed that there was significant difference in the total lactic acid bacterial species diversity among the fermented samples.

Conversely, the Simpson's dominance indices in the total lactic acid bacterial communities in all the fermented cereal-cowpea blends increased with increase in fermentation time with little variation in the (SoC) sorghum-based blends (Figure 3). The indices showed that, maize-based blends (MaC) have the least dominant LAB species within the first 48 h fermentation time, while sorghum- based blends (SoC) had the highest indices within the same period. Statistical analysis using 2-way ANOVA ($P < 0.05$) however revealed that, both sample type and fermentation time had no significant effect on the lactic acid bacterial dominant species. Hence, the total lactic acid bacterial species diversity and dominance indices were not significantly affected by the fermentation time.

The identification of the cloned sequences was obtained by a BLAST search of the GenBank database. Isolates SW1 and SW3 which were conventionally identified as *L. plantarum* and *L. pentosus* had their closest relative by sequence comparison as *L. plantarum* (Accession no. AL935260 and AL935261). Isolate SW2; conventionally identified as *L. fermentum* had its closest relative as *P. pentosaceus,* while SW4 (*L. brevis*) had its closest relative as *Lactobacillus* species. Isolates SW5 and SW6, which were conventionally identified as *L. mesenteroides* and *P. acidilactici,* respectively, had their closest relative as *P.acidilactici.* Isolate SW7 (*P. pentosaceus*) had its closest relative as *P. pentosaceus.* The identity of the remaining 3 LAB isolates has no closeness or relativity with lactic acid bacteria.

DISCUSSION

Fermentation was observed to decrease the pH and

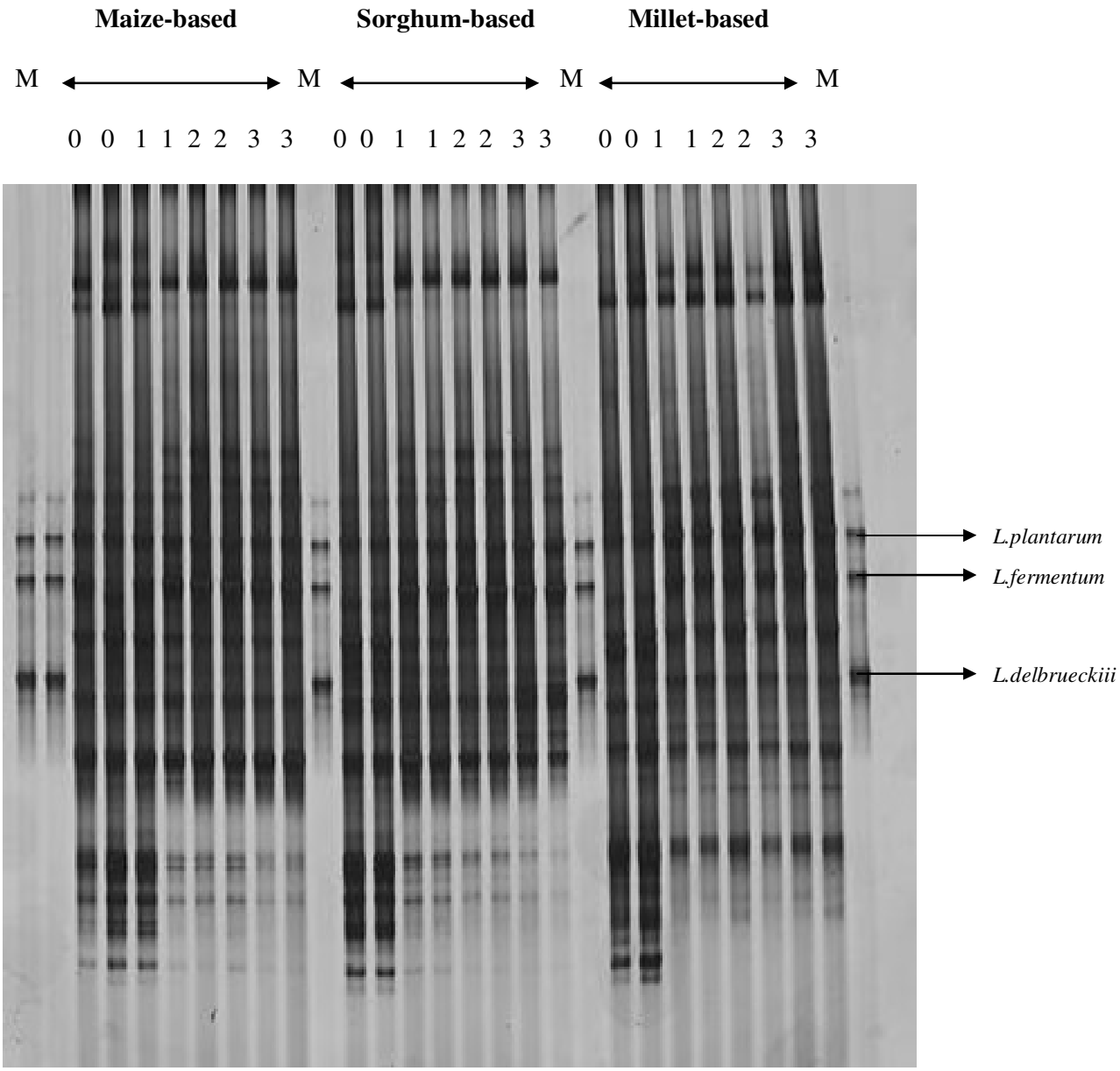

Figure 1. DGGE analysis of PCR- amplified 16S rDNA fragment for total Lactic acid bacterial community from cereal-legume weaning blends. 0, unfermented blends; 1, 24 h fermented blend; 2, 48 h fermented blend; 3, 72 h fermented blend.

increase the titratable acidity of the fermented formulated weaning blends. The decrease in pH and increase in titratable acidity up to 48 h and the further increase and decrease at 72 h in pH and titratable acidity, respectively, may be attributed to inhibition of the growth of the microbial population at pH below 4.3. The observed increase in titratable acidity could be due to the dominance of the environment by lactic acid bacteria which degrade carbohydrates resulting in acidification. These observations are in agreement with earlier studies by Nout et al. (1989) and Ariahu et al. (1999). High titratable acidity has been reported to reduce incidence of diarrhoea in infants consuming fermented cereal porridge (Mensah et al., 1990). The cowpea-fortified blends will then have two important attributes, such as antimicrobial properties and high protein content.

Phenotypic characters have frequently been used for bacterial characterization and are the basis for numerical taxonomy (Vandamme et al., 1996). The traditional plating results shows that, the total culturable LAB populations in the fermented formulated weaning blends consisted of *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* strains. Previous studies had shown that, naturally-fermented cereal-based African foods are dominated by *L. plantarum, L. fermentum, L. reuteri, L. mesenteroides, P. pentosaceus* and *L. lactis* strains

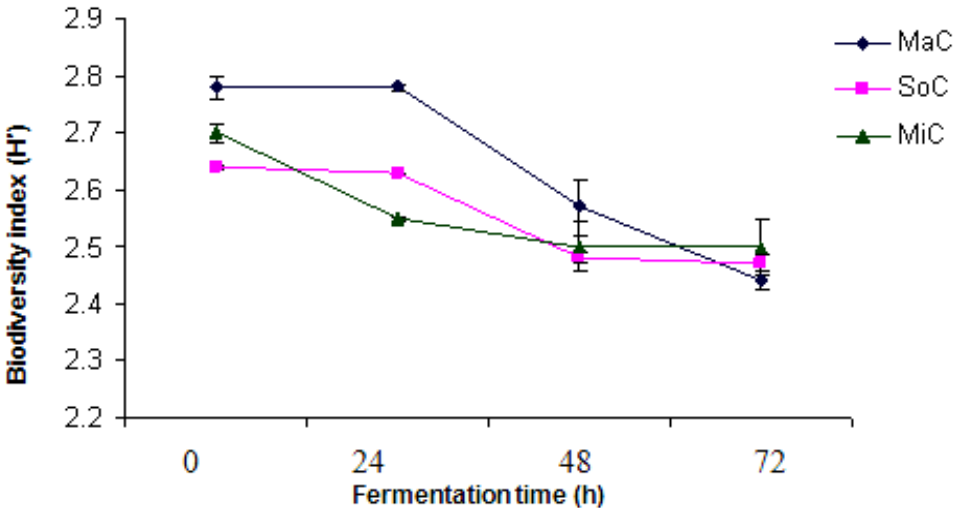

Figure 2. Shannon-Weaver biodiversity index (H') for total lactic acid bacterial community profile for fermented cereal-legume blends. MaC, Maize-based blend, SoC, sorghum-based blend, MiC, millet-based blend.

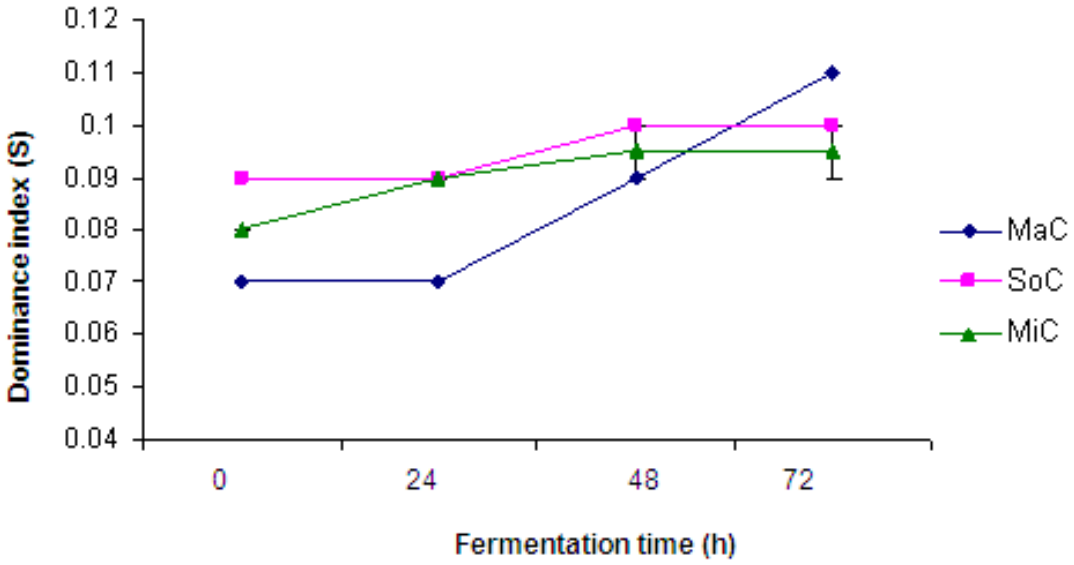

Figure 3. Simpson dominance index (S) for total lactic acid bacterial community profile for fermented cereal-legume blends. MaC, maize-based blend; SoC, sorghum-based blend; MiC, millet-based blend.

(Oyewole, 1995; Rombouts and Nout, 1995). According to Sharpe (1979), the ability to produce acid from carbohydrate is used routinely to differentiate and identify species of *Lactobacillus* as well as other lactic acid bacteria. The sugar fermentation patterns of the LAB isolates in this study conform to the finding.

The majority of the 178 LAB isolates characterized in this study were found to be *L. plantarum* (74) and *P. acidilactici* (32). These two species have been isolated previously from a variety of food products and environmental sources and were reported as the most dominant species during fermentation of sorghum-based infant -weaning foods (Nout, 1991; Olsen et al., 1995). Furthermore, a great species diversity was observed since other species of LAB were also identified; *L. fermentum, L. brevis, L. amylovorous, L. pentosaceus, L. delbrueckii, P. pentosaceus, L.mesenteroides, Lactobacillus* species, *Lactococcus species* and yet to be identified species. Although, the natural habitat of lactococci is milk, *L. lactis* subspecies *Lactis* has been isolated previously from plants, vegetables and cereals as obtained in the current study (De Vuyst and Vandamme 1994; Salama et al., 1995). Also, *L. fermentum* has received less interest despite its

occurrence in a large number of fermentations of starchy products (Agati et al., 1998; Brauman et al., 1996).

It has been stated that polyphasic approaches combining morphological, biochemical and molecular data are important for the accurate classification of LAB (Klein et al., 1998). This is because, if isolation is the sole approach used to study the ecology of a fermented food, then, it is obvious that a great number of strains have to be isolated and characterized. This would make the study labor-intensive and time consuming, especially when the number of samples is high (Ampe et al., 1999a). In this study, a molecular approach to monitor the dynamic changes in the lactics 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 (Lerman et al., 1984).

There was a shift in LAB communities' composition at different time intervals and the shifts in community composition occurred in different patterns in all the three cereal-based weaning samples. 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 because of increased fermentation time. This observation was similar to the findings reported by 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)which was also found to coincide with the development of *L. fermentum.* Furthermore, Ampe and Miambi (2000) reported differences between the microbial communities developed during indigenous maize fermentation for the production of *ogi, poto-poto* and *pozol* and reported that, the differences are likely to be due to the differences in the processing methods and not fermentation time.

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. (1999a) and Ercolini et al. (2003).

In addition, despite the differences observed between the DGGE fingerprints of the different cereal- legume blends, some bands were present in most blends. Three of these widely distributed bands could be assigned to LAB species, by comparison of the migration distance of their PCR amplicons in DGGE gels with those of reference strains (Figure 1) as suggested by Meroth et al. (2003) and which is also in accordance with the findings of Ampe and Miambi (2000) and Walter et al. (2001). These bands correspond to *L. plantaraum*, *L. fermentum* and *L. delbrueckii*. Thus, in the development of a specific starter for cereal-based fermented foods, one should consider that several species (or at least three) are needed to mimic natural conditions. 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 (5.60 to 3.60) which resulted in increased acidity that allows only acid-sensitive microorganisms to develop. Some workers (Steinkraus et al., 1983; Nout et al., 1989; Ampe et al., 1999b) have reported the dominance of acid-tolerant microflora in cereal fermentation.

Analysis of the DGGE profile using lactic acid bacterial species-specific primers resulted in the microbial community with little or no difference in the biodiversity and dominance indexes of the blends. However, the total lactic acid bacterial species diversity among the fermented blends was significantly different. In addition, it was observed that the total lactic acid bacterial species diversity and dominance indices were not significantly affected by the fermentation time and cereal type. This might be as a result of species- specific primer used for the amplification.

The selection of primer set for partial 16S rDNA sequence amplification is also very crucial in the analysis of bacterial diversity in the natural environment as some bacterial primers are known to co-amplify non-bacterial sequences in the environment (Lopez et al., 2003). It has also been shown that, the PCR amplification of 16S rDNA fragments can be biased by several parameters (Reysenbach et al., 1992; Suzuki and Giovannoni, 1996). Different primer sets have been designed and used for investigating lactic acid bacterial diversity using different molecular techniques with varying degrees of success. These include primer set P_1- P_2 (Klijn et al., 1991, Cocolin et al., 2004), HDA1^{GC}- HDA2 primer set (Tannock et al., 1999), Lac 1- Lac 2^{GC} primer set (Walter et al., 2000), Ec 338f^{GC}- Ec 518r (Ampe et al., 1999b; Omar and Ampe, 2000), primer set WBAC 1- WBAC 2 ^{GC} and WLAB 1- WLAB 2^{GC} (Lopez et al., 2003). The choice of primer set used is determined by researcher's choice and available data on the reliability of the selected primer at the time of the research and the environment to be studied. The gc 338f-518r primer set used for amplifying lactic acid bacterial in this study was found to be comparatively more sensitive and reliable (Ampe et al., 1999b). It amplified V3 variable region generating 210 bp sized PCR product.

PCR-DGGE has the advantage of allowing study of the species diversity of predominant members of the eco-

system, which can be achieved simply and economically by a single PCR. Sequencing of bands is laborious and inaccurate identification may occur due to the use of short sequences in combination with sequencing failures (Meroth et al., 2003). Hence, the lactic acid bacteria were further identified by sequencing few dominant strains so as to confirm their real identity. Identifications obtained by a BLAST search of the GenBank database with V3 region sequences correlated with those obtained with traditional plating, revealing that, the two dominant species involved with the cereal-legume fermentation were *L.plantarum* and *P. acidilactici*.

The conclusion could be drawn from the predominance of *L. plantarum* and *P. acidilactici* in a fermenting cereal-cowpea fortified system using the conventional system. The LAB population diversity as shown by the Shannon-Weaver index is also affected by the type of cereal utilized in the preparation.

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