Flora dynamics of *Lactococcus lactis* from *nyarmie* –a traditional Ghanaian fermented milk product.

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ABSTRACT

Aims: To ascertain the diversity of biotypes among *Lactococcus lactis* isolates isolated during the 'spontaneous' fermentation of *nyarmie*, and from this select strains with appropriate characteristics for use as starter cultures.

Methods and Results: Strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, isolated from *nyarmie,* at different stages of fermentation were grouped by profiles of assimilation and Pulse Field Gel Electrophoresis (PFGE). PFGE patterns were compared visually and by cluster analysis. By visual comparison with a reference strain, seven PFGE patterns were obtained for 72 strains of the two *Lactococcus* subspecies with PFGE type 1 occurring throughout fermentation. Cluster analysis suggested 59 genotypes were present and the relationship of these was made with assimilation profiles and stage of fermentation at which they were isolated.

Conclusions: Different biotypes of *L. lactis* strains were present at the various stages of *nyarmie* fermentation. The preparation of *nyarmie* may play a major role in selecting dominant strains from the natural flora as was the case with *L. lactis* PFGE type 1.

Significance and Impact of Study: The selection of biotypes of *L. lactis* as starter cultures will play a very important role in producing final products with individual characteristics.

Key works: *Nyarmie*, *Lactococcus lactis,* traditional fermentation, PFGE, sub-species typing

INTRODUCTION

Strains of *Lactococcus, Lactobacillus*, *Leuconostoc, Streptococcus, Pediococcus* and *Enterococcus* have been identified in various African fermented milks. The importance of these strains to the dairy industry has led to a continual search for new ones (Jarvis and Jarvis 1981). In an initial investigation into the microbiology of *nyarmie*, a naturally fermented milk product, we have shown that a complex microflora is present including *L. lactis* subsp. *lactis, Leuconostoc mesenteroides, Streptococcus thermophilus*, an unknown *Strep.* species, *Lactobacillus delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis, Lb. delbrueckii* subsp. *delbrueckii, Lb. helveticus*, *Lb. fermentum* and *Saccharomyces cerevisiae*, depending on producer and occasion of production (Obodai and Dodd 2006.). Changes in the populations of lactic acid bacteria (LAB) also occurred during the fermentation process. The possibility of choosing biotypes with different biotechnological characteristics from within a species could improve the level of control over a fermentation process, thereby producing end products which have individual characteristics (Hayford 1998). Therefore to provide control over a fermentation, it is important to understand the exact composition of the microflora both at the species and the subspecies levels (Giraffa and Neviani 1999.).

An important component of the flora was *L. lactis* which was isolated throughout the fermentation process. Several methods, based on phenotypic characterization, have been used for the differentiation of strains of the two subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, which generally include biochemical tests. Genotypically, these two subspecies have been differentiated by using rRNA oligonucleotide probes (Le Bourgeois et al. 1992; Salama et al. 1993) and also PFGE combined with controlled restriction by rare-cutting endonucleases (Leong-Morgenthaler et al. 1990; Tanskanen et al. 1990; Tornai-Lehoczki and Dlauchy 1996).

Some strains however, that were phenotypically identified as *L. lactis* subsp. *lactis* appeared genotypically as *L. lactis* subsp. *cremoris* and vice versa (Wouters et al. 2002). This is an indication that the standard tests for phenotypic characterization can result in ambiguity especially with those strains isolated from natural and hostile niches (Corroler et al. 1998).

The objective of the present study was to reveal the diversity of biotypes among *L. lactis* isolates collected during the 'spontaneous' fermentation of *nyarmie*, and from this select strains with appropriate characteristics for use as starter cultures.

MATERIALS AND METHODS

Isolation of *nyarmie* microflora

The isolation of the *Lactococcus* isolates from *nyarmie* has been described previously (Obodai and Dodd 2006). Colonies from Rogosa, M17 and MRS agars were randomly selected as putative *Lactococcus* isolates, purified and then stored at -20°C in broth containing 15% glycerol. Strain notation was based on processor source (A and B), fermentation time (3=24h, 4=48h) and individual isolate number eg. A3-9 is Processor A, at 24h fermentation and isolate 9.

Strain identification and phenotypic characterization

Gram positive, non-motile, catalase negative, non spore forming cocci were speciated using API 20 Strep (BioMérieux SA, Marcy-L'Etoile, France) and identification made using the APILAB PLUS personal computer identification programme version 3.2.2 (BioMérieux). Confirmation of identification to the genus level was supported using Lancefield grouping serotyping (Strep test kit; Oxoid X3981).

Genomic DNA preparation

Preparation of genomic DNA *in situ* in agarose blocks was modified from Moore & Datta and Tanskanen et al. (Moore and Datta 1994; Tanskanen et al. 1990). *Lactococcus* strains were inoculated in 10 ml of M17 broth and grown at 30°C overnight under gentle agitation (100 rpm). The culture (1 ml) was centrifuged at 13,000 x *g* for 1 min. The pellet was washed twice by re-suspension in TN buffer (2 ml; 10 mM Tris-HCl; Sigma-Aldrich Co, Gillingham, UK), and 1M NaCl (pH 7.6; Fisher Scientific) at 4°C. The cells were then re-suspended in 250 µl TN buffer to ensure a bacterial suspension absorbance of 1.7 at 600 nm. The suspension was mixed with 250 µl of 1% (w/v) Pulsed-field certified agarose (BIO-RAD) solution in TN buffer maintained at 60°C in a water bath. The mixture (300 µl) was dispensed into reusable plug moulds (Bio-Rad Labs, Hercules CA) and allowed to set for 20 min at room temperature. Plugs were incubated in 3 ml of lysis buffer (6 mM Tris-HCl, 0.1 mM EDTA (Fisher Scientific), 1% N-lauroyl sarcosine sodium salt (Sigma), 10 g l^{-1} lysozyme (Sigma) pH 7.6) at 37°C for 16 h. Lysis buffer was removed and the plugs washed three times with TE buffer (3 ml; 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at room temperature for 30 min, then incubated in proteinase-K (Sigma) buffer solution (0.5 M EDTA, 1% N-lauroyl sarcosine sodium salt, proteinase-K 1 g l^{-1}, pH 9) at 55°C under gentle agitation (100 rpm) for 24 h and washed three times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) at room temperature for 30 min. Finally the plugs were rinsed three times with TE buffer (10 mM Tris-HCl, 50 mM EDTA, pH 8.0) at room temperature for 30 min and stored in fresh TE buffer or 0.5 M EDTA at 4°C for long term storage.

Digestion of the plugs with *Sma*I

Plugs were placed in a sterile petri dish, sliced to about 2 mm thickness, rinsed in 500 µl sterilised deionised water (SDW) for 15 min, removed then rinsed in 1 x buffer A (100µl;

Boehringer Mannheim GmbH, Germany) for 15 min. Plugs were then transferred to tubes containing100 µl of buffer A and 4 µl *Sma*I (Boehringer 656348) for 4 h at 25°C then immersed in 0.5 x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1.25 mM EDTA, pH 8.3) at 4°C until use.

Gel electrophoresis

Electrophoresis of the restriction digests was performed in a Bio-Rad Contour-Clamped Homogenous Electric Field (CHEF) DRII electrophoresis cell. This was done through 1% (w/v) agarose gel in 0.5 x TBE buffer at 14°C. A 50-1000kb DNA ladder (Sigma-Aldrich Co.) was used as a molecular size marker. To improve the discrimination of the *Sma*I fragments, digests were electrophoresed using three different sets of pulse times. The selected conditions were pulse time ramped from 4 s to 45 s for 16 h cycle. The gel was stained in 10 µl ethidium bromide (10 mg ml^{-1}) in 200ml distilled water for 1 h and the DNA bands visualised using a UV transilluminator with 313 n m emission and photographed using a FujiFilm Imaging system (Amersham Pharmacia Biotech, USA). Fragment sizes were determined by their mobilities using a plot of log molecular size of marker (50-1000kb) and the distance migrated by the marker bands included in the same gel as a standard curve (Lim et al. 1994). The estimated final genomic size of a strain was obtained by the sum of all the individual fragment sizes.

Analysis and comparison of PFGE fingerprints

Analysis of the densitometric traces of band profiles of PFGE fingerprint patterns was performed with the analysis software package GelCompar II, version 4.5 (Applied Maths, Kortrjik, Belgium). Levels of similarity of the band profiles were calculated based on Dice association

coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992).

Screening for bacteriocin-producing *Lactococcus* strains.

Bacteriocin production was investigated using two known bacteriocin-sensitive strains *Lactobacillus casei* DSM 20011 (Guyonnet et al. 2000) and *Lb. sakei* DSM 20017 (Chumchalova 2004). Measurement of the zones of inhibition was carried out using both 'spot on lawn' and agar well diffusion assays (Olsen et al. 1995; Schillinger and Lücke 1989). All the test isolates were inoculated into M17 broth and the sensitive strains in MRS broth and incubated aerobically at 30°C for 24 h. For the 'spot on lawn' method, cultures of isolates (10 µl) were spotted on M17 agar and incubated overnight at 30°C; MRS broth was used as a control. Plates were then overlaid with soft MRS agar (10 ml 0.7%) containing approximately $1x10^6$ cells of the bacteriocin indicator strain *Lb. casei* DSM 20011 or *Lb. sakei* DSM 20017 and incubated overnight at 30°C and the diameters of zones of inhibition measured. For the agar well diffusion assay, test solution (100 µl of culture) was dispensed into a 10 mm diameter well cut into M17 plates. These were left to dry for 4 h and then overlaid with MRS soft agar and incubated as before. This assay was also used to examine the antimicrobial activity of *Lactococcus* isolates from samples of *nyarmie* after 48 h of fermentation as test strains against *L.* isolates from unpasteurised and 0 h fermented milk and isolates of *Lactococcus* selected from PFGE type 1 as test strains against selected *L.* isolates from other PFGE types (2 to 7).

Testing for the nature of antimicrobial properties

Hydrogen peroxide activity was determined by the assay of Mante et al. (2003). Supernatants (100 µl) of test strains were treated for 2 h with filter sterilised (0.2 µm filter; Sartorius

Minisart).catalase enzyme (1 mg ml^{-1}). The effects of acid production were examined by neutralisation of the supernatants with 0.1 M NaOH to a pH of 6.8±0.1. Treated supernatants were tested against indicator strains using the agar well diffusion assay to look for residual antimicrobial activity. All experiments were duplicated.

Heat resistance of *Lactococcus* isolates

Broth cultures of *Lactococcus* strains were prepared by inoculating one colony of 24 h growth on an appropriate agar into 5 ml of its appropriate broth. Thermal inactivation was examined by replicating the conditions used in *nyarmie* production. Aliquots (1 ml) between 10^6 and 10^8 cfu ml^{-1} of the 24 h culture was inoculated into 5 ml of pre-heated reconstituted skim milk powder at 65°C. The samples were maintained at this temperature in a water bath for 40 min and 20 µl of each sample was taken at 5 min intervals for viable counts using the method of Miles & Misra (1938). Recovery of the cultures after heating was studied by storing the inoculated milk batches at 28±2°C for 24 and 48 h to simulate the conditions of the traditional preparation of *nyarmie*. After these times, viable counts were again performed.

RESULTS

Speciation and sub-species typing for the lactococci

Assimilation and fermentation profiles for lactococci

Seventy-two *L. lactis* identified during the different fermentation times of *nyarmie* preparation showed that 56 of these isolates were *L. lactis* subsp. *lactis* and 16 were *L. lactis* subsp. *cremoris* based on API identification. The percentage identification of *L. lactis* ranged between 73% and 98%. From the *L. lactis* subsp. *lactis* identified, all the strains produced acetoin, β-glucosidase, arginine dehydrolase and could assimilate lactose. Variations in production of leucine,

arylamidase, alkaline phosphatase, β-galactosidase and assimilation of ribose, trehalose, L-arabinose, mannitol and starch resulted in 11 different assimilation profiles (Table 1: A-K). The most dominant assimilation profile (profile F) constituted 30% of the isolates with production of acetoin, β-glucosidase, leucine, arylamidase, arginine dehydrolase and assimilation of ribose, mannitol, lactose, trehalose and starch. All the *L. lactis* subsp. *cremoris* isolates produced acetoin and fermented lactose and variation in the other carbon sources resulted in eight different assimilation profiles (Table 1; L-S). None of the strains produced carbon dioxide during glucose assimilation (Table 1).

Pulsed field gel electrophoresis of lactococci.

PFGE was carried out on *L. lactis* subsp. *lactis* NCIMB 8586 (reference strain) and the 72 API characterised *L. lactis* strains. To obtain optimal separation of the fragments produced by *Sma*I digestion, the switch time and the total run times were varied from 4 s to 45 s for 21 h, 1.5s to 18s for 21 h and 4s to 45s for 16 h. Best separation was obtained for the majority of the *Sma*I fragments (50 to 500 kb) when a pulse time ramped at 4s to 45s for 16 h was used and this was adopted as the standard setting (results not shown).

A visual comparison of PFGE profiles of the 72 isolates of *L. lactis* and the reference strain gave seven different characteristic restriction patterns, with the number of fragments ranging from 9 to 14 (Figure 1). The molecular sizes of the chromosomal DNA estimated by *Sma*I digestion for the different PFGE types ranged from 1.0 to 2.8 Mb (Table 2). PFGE types 1, 3, 4, 6 and 7 were *L. lactis* subsp. *lactis* by the API 20 Strep kit, whilst types 2 and 5 were *L. lactis* subsp. *cremoris*. .

Occurrences of PFGE types during *nyarmie* fermentation

The relationship of the seven PFGE types with their source and time of isolation during the fermentation (Table 3) showed that PFGE type 1 strains were found at the beginning, during, and at the end of fermentation in Processor A but not in Processor B. Some types like Type 4, which was infrequently isolated (1% isolates), appeared only in the unpasteurised milk and then disappeared after heating. Others like types 6 and 7 were found in the unpasteurised milk and then at the end of fermentation. This distribution of the PFGE types suggested possible differences in heat tolerance to the pasteurisation stage and/or that types like PFGE type 1 had a competitive advantage due to the production of antimicrobial agents. These features were examined further (see below). It was interesting to note that in Processor A no isolates were found post pasteurisation but these reappeared after 12 h of fermentation or later, which could reflect a post-process contamination event. In contrast isolates were evident at 0 h ie post pasteurisation in Processor B, although none survived to the final product, where the flora was dominated by lactobacilli (Obodai and Dodd 2006).

Comparison of PFGE types with the assimilation profiles.

Table 4 shows the comparison of the seven PFGE types with assimilation profiles of 45 *L. lactis* strains obtained with the API identifications (a subset of the 72 identified). Within each PFGE type different assimilation profiles of the strains occurred (Table 4). It was also observed that PFGE type 1 also showed the most dominant assimilation profile (F). PFGE type 1 represented a total of 58% of all the isolates.

Estimation of the degree of genomic relatedness for *Lactococcus lactis.*

A detailed genotypic analysis of the 72 strains is presented in Figure 2. Cluster analysis revealed the presence of 59 different genotypes separated at a similarity level ranging from 43% to 100%. At a similarity level of 75%, 16 clusters were observed (Figure 2). Fifty seven processor A strains were split amongst 14 clusters whilst 15 processor B strains were among four clusters (Figure 2 and Table 5). Some relationship of the cluster groups with processor and the time of fermentation (Figure 2: clusters b, e, g, j and n) was evident: cluster b isolates were from processor A at 48 h of fermentation; clusters c, d, e, f and g forming at a similarity level of 80% also from processor A were found to be mainly of PFGE type 1.

Clusters i and j were PFGE types 2 and 5 (*L. lactis* subsp. *cremoris*) from processor B. Interestingly, these did not cluster with PFGE type 2 strains from processor A (clusters a and b) showing a strong association with source. However most PFGE type 6 strains (clusters m and n, forming at a similarity of 95%) did cluster together although they were mainly from processor B and so also showed a relationship with source. Clusters o and p were from processor A and mainly of PFGE types 1 and 3 (Table 5).

Production of antimicrobial substances

The *Lactococcus* strains (72) were tested for their antimicrobial activity. Fifty percent of the isolates produced inhibition zones of up to 3 mm on both *Lb. casei* DSM 20011 and *Lb. sakei* DSM 20017, demonstrating antimicrobial activity.

In order to determine why some *Lactoccocus* isolates prevail throughout fermentation and others do not, *Lactoccocus* strains isolated at the end of fermentation (48h) were tested against *Lactoccocus* isolated at the start of fermentation (unpasteurised milk and 0h fermentation). When antimicrobial activity was examined between *Lactoccocus* isolates, widespread antimicrobial activity was observed between *Lactoccocus* isolated at the end of *nyarmie* production against

those found at the start; all the *Lactoccocus* isolated at the end of fermentation were able to inhibit all isolates from the unpasteurised and 0 h fermented milk, with the exception of isolates AU-2 and AU-15 (Table 6). Antimicrobial activity was also observed between PFGE types isolated at different stages of *nyarmie* production. All the PFGE type 1 isolates were able to inhibit all the isolates of PFGE types 3, 5 & 7. Only PFGE type 1 isolate A4-8, from 48 h of fermentation, inhibited all the other PFGE type isolates (Table 7).

Testing for the nature of the antimicrobial property

Several antimicrobial agents may be producing the inhibitory effects observed. The addition of catalase to the supernatant eliminates hydrogen peroxide secreted by some LAB; this showed that for some strains the inhibitory effect against the indicator strains still remained as in the case of isolate BO-12 (Table 8), however in some instances it was lost, indicating the action of H_2O_2 as the main antimicrobial activity. Neutralising the pH resulted in no inhibitory activity (Table 8), however, indicating acid production is a major factor causing inhibition in all cases. In no instance was there residual activity after these treatments, which demonstrates a lack of bacteriocin production.

Heat tolerance

Preliminary studies on heat tolerance were carried out on six *L. lactis* PFGE type 1 strains from different stages of fermentation which showed antimicrobial properties. Strain BO-4 isolated at 0 h fermentation was able to withstand heat stress at 65°C for 30 min and thus was selected for comparison with the other PFGE types. Subsequently two strains from each PFGE type showing antimicrobial properties were selected and tested for heat tolerance and the most tolerant was selected for comparison. PFGE type 1 strain BO-4 and PFGE type 5 strain BO-11 were both

found to be able to withstand heat stress at 65°C for 30 min (Figure 3). *L. cremoris* PFGE type 2 strain BO-3 survived the heat stress for 15 min. PFGE type 3 strain A2-22 isolated at 12 h of fermentation and PFGE type 6 strain BU-16 isolated from unpasteurised milk survived the heat stress for less than 5 min (Figure 3). With the exception of PFGE type 6 strain BU-16, all the PFGE types recovered after 24 h; however by 48 h after heating all the strains were at 10^9 cfu ml^{-1} (Table 9). This suggests that failure to isolate strains after pasteurisation is due to heat injury and their re-emergence later in the process is due to their recovery from injury and not through reintroduction in a post-process contamination event. It is also notable that the most dominant type in the final flora is amongst the most heat resistant.

DISCUSSION

Most of the *L. lactis* isolates had the same biochemical assimilation of carbon compounds with some variations observed giving rise to the 11 different assimilation profiles obtained. This is an indication that different strains may have different effects on the product quality. Thus *L. lactis* could be considered for use as a starter culture, but the choice of strain might influence the outcome of the *nyarmie* product. The % ID ranged between 73-98% showing that in some cases the identifications as *L. lactis* were tentative. Although the biochemical profiling was used to differentiate between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, these routine identification methods largely based on phenotypic and biochemical properties were not enough to definitely assign a strain to a particular species since these phenotypic attributes can change as a result of storage conditions, incubation temperature and the particular API kit used among others. These results are in agreement with Salama et al. (1993), who found that simple biochemical tests do not provide sufficient evidence to distinguish between sub species of *Lactococcus*.

The use of this conventional method however provided some information about the technological properties of these isolates, in that all the strains could assimilate lactose, the main carbohydrate in milk, thus giving an indication that the isolates contain the lactose operon. Also the ability of *L. lactis* subsp. *lactis* to ferment ribose and trehalose, and hydrolyse arginine to ammonia differentiated it from *L. lactis* subsp. *cremoris*, confirming the findings of Carr et al. (2002). Other information obtained based on the production of carbon dioxide from the fermentation of glucose of the isolates indicated that the isolates are homolactic fermenters, with lactic acid as the sole end product. In recent years, the use of DNA specific probes directed at nucleic acid targets of the cell have been used in distinguishing between these two subspecies (Schleifer 1985; Le Bourgeois et al.1992) and between lactococci and enterococci (Betzl et al. 1990). Also the use of PFGE, where the banding patterns obtained from the different strains are subjected to multivariate data analysis, provides a high resolution capacity which can be used in classification at both species and genus level (Ståhl et al. 1990). These approaches reduce the reliance on phenotypic characteristics for strain characterisation.

With the use of PFGE, seven major PFGE patterns were observed for *L. lactis* and the dominance of type 1 at the end of fermentation and its presence throughout fermentation gives an indication that these strains are able to withstand the varying conditions of the product. These are, among others: low pH; competition with other microbiota such as yeast and *Klebsiella* species; the production of antimicrobials, probably bacteriocins, acids or hydrogen peroxide and their sensitivity to foodborne pathogens (Adams and Nicolaides 1997).

Based on PFGE analyses the genome size of the *L. lactis* subsp. *lactis* type 1 was 2.8 Mb whilst *L. lactis* subsp. *cremoris* type 2 was 2.3 Mb. These are comparable with the sizes already determined for other LAB: 2.6 Mb for *L. lactis* subsp. *lactis* DL11 (Tulloch et al. 1991), 2.6 Mb *L. lactis* subsp. *cremoris*, 1.75Mb for *Strep. thermophilus* ST1 (Leong-Morgenthaler et al. 1990)

and 2.3 Mb for *Lb. delbrueckii* subsp. *bulgaricus* (Lim et al. 1994.). This correlates well with the requirement of these bacteria for numerous growth factors and their lack of a higher developed secondary metabolism (Roussel et al. 1993). The low genome sizes observed for *L. lactis* - PFGE types 5 and 6 could have been due to partial digestion as a result of two or in some cases three fragments co-migrating together and thus giving an underestimation (Oana et al. 2002). It could also have been that the differences of the genome sizes of *L. lactis* which was 36% between the largest (2.8Mb) and the lowest (1.0 Mb) showed that there is a significant variation in the genome sizes of *L. lactis* strains. This has also been observed for the genome sizes of *E. faecium* (Oana et al. 2002) which showed a 17% variation. The presence of a large amount of strain or lineage specific DNA could account for the genome size variation (Hayashi et al. 2001).

The differences in fragment numbers and positions which lead to the different PFGE types could be attributed to changes in some cases with a single genetic event that is a point mutation or an insertion or deletion of DNA. This was the case with *L. lactis* subsp. *cremoris* PFGE types 2 and 5 which had two band differences thus indicating that these strains are closely related. It is therefore interesting to observe that *L. lactis* subsp. *cremoris* PFGE types 2 and 5 were dominant in the freshly pasteurised milk produced only from processor B suggesting a common source. Changes consistent with two independent genetic events leading to four to six band differences can also be explained by simple insertions or deletions of DNA or the gain or loss of restriction sites thus indicating that they are possibly related (Tenover 1995).

Strain differentiation was established more rapidly using the PFGE analysis than by biochemical profiling. The discriminatory power of PFGE has been shown to be excellent with the ability to distinguish between strains and thus show their occurrences through the fermentation process. However, a comparison of carbohydrate fermentation abilities with PFGE type showed that there were five variations in the utilisation of carbohydrates by isolates of the

same PFGE types. It would be interesting to observe to what extent the strains showing identical restriction patterns differed in their other technological properties (pH tolerance, temperature tolerance and flavour production). It may mean that it may be impossible to predict any technological behaviour from these macro-restriction profiles. One can only follow the occurrence of strains in a complex ecosystem such as milk. A more structured approach would be to use PFGE as a preliminary screen to distinguish genomically similar isolates and then screen a reduced number of strains for their technologically important abilities thus providing a more rational approach (Lortal 1997) to strain choice. The overall genetic polymorphism detected allowed the identification of 59 different genotypes among 72 analysed strains, highlighting a high degree of variability within the species.

Bacteriocin production can be found among LAB present in milk and dairy products in general (Conventry 1997). Antimicrobial activity produced by the *nyarmie* isolates could be attributed mainly to the production of acids with just a little production of hydrogen peroxide activity by *L. lactis* strains, rather than through the production of bacteriocins, hydrogen peroxide or any other antimicrobial compound. Similar findings were d emonstrated by *L actobacillus plantarum* in *agbelima* (a Ghanaian smooth- textured sour cassava dough; Mante 2003). The bactericidal effect produced by acids is particularly relevant in developing countries where most of the fermented milk and foods are produced under semi-hygienic conditions. In other studies the frequency of bacteriocin production amongst *Lactococcus* strains has been shown to be 10%, thus it is less surprising that this was not found in the relatively low number of *Lactococcus* strains studied here. The antimicrobial activity may account for the presence of the different *L. lactis* strains isolated at the beginning and end of fermentations as some of the *L. lactis* strains isolated at the end of fermentation caused inhibition of those found at the start. This antimicrobial interaction has also been reported in other studies involving LAB in *agbelima*

fermentation (Mante 2003), maize dough fermentation (Olsen et al. 1995), yoghurt and sauerkraut fermentations (Schillinger and Lücke 1989).

Responses of the two subspecies of *L. lactis* to heat stress showed different thermal sensitivities. Although both subspecies survived for 30 min at 65°C, *L. lactis* subsp. *lactis* strain BO-4 (PFGE Type 1), had a reduction value of 4 log cfu ml^{-1} whilst *L. lactis* subsp. *cremoris* strain BO-11 had a reduction value of 3.5 log cfu ml^{-1} over the same period. The *D* value of *L. lactis* subsp. *lactis* was 5 min at 65°C whilst a *D* value of 25 min was recorded for *L. lactis* subsp. *cremoris* at this temperature. *Streptococcus thermophilus* strain B3-1, which had a reduction level of 3.5 log cfu ml^{-1} at 65°C for 30 min and a $D_{65°C}$ value of 20 min was comparable to the heat tolerance of *L. lactis* PFGE type 1. The differences observed amongst all the strains' heat tolerances are probably innate as this has been found to vary amongst strains of the same species (Stumbo 1973), as observed for *L. lactis* subsp. *cremoris* PFGE types 2 and 5. The tolerance levels of the different PFGE types showed some correspondence to the points of isolation of the isolates. PFGE type 1 was heat tolerant and hence was isolated immediately after pasteurisation. In contrast the more heat sensitive PFGE type 6 was found prior to pasteurisation and then not found again until 48 h, suggesting that the late reappearance of isolates was in fact due to recovery from heat injury followed by growth to a detectable level and not reintroduction by post-process contamination.

The preparation of *nyarmie* may play a major role in selecting dominant strains as was the case with *L. lactis* PFGE type 1 which was found throughout fermentation and had been naturally selected on the basis of heat tolerance and tolerance to antimicrobials. Such characteristics are important to establish in the choice of biotypes with different biotechnological characteristics from within a species which have the ability to improve the level of control in a fermentation process, thereby producing end products which have unique characteristics (Fortina

et al. 1998). The typing of LAB both genetically and phenotypically is developing to a point where this is of increasing value to manufacturers of starter cultures and food microbiologists (Giraffa and Neviani 1999), allowing the development of more tailored starter cultures for specific food types.

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TABLE 1. Assimilation profiles of *Lactococcus* species from *nyarmie* samples

Phenotypic characteristics	Assimilation profiles of *Lactococcus* species*																		
	Lc. lactis subsp. *lactis*										*Lc. lactis* subsp. *cremoris*								
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Acetoin Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hippurate hydrolysis	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
ß-glucosidase	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	+
α-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ß-glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ß-galactosidase	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+
Alkaline phosphatase	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+
Leucine arylamidase	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Starch	-	-	-	+	-	+	+	+	-	-	-	-	-	+	-	-	+	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Lancefield grouping	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
CO_2 from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth at 10°C	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on KF agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+) positive reaction; (-) negative reaction

TABLE 2. Genome size analysis of *Lactococcus* strains by pulse field gel electrophoresis

Species /PFGE types		GS (Mb)	Trf	Size of *Sma*1 fragments (Kb)													
L. lactis strains	1	2.8	14	599	425	257	249	228	204	221	170	143	101	98	93	78	58
	2	2.4	12	457	395	310	231	221	181	161	140	101	93	78	66		
	3	2.5	14	498	257	248	227	219	170	165	146	143	121	100	80	78	30
	4	2.5	11	425	310	257	251	248	231	198	165	148	139	101	-	-	-
	5	1.7	10	396	227	221	181	161	140	121	78	65	58	-	-	-	-
	6	1.0	9	181	170	161	139	100	89	62	56	48			-	-	-
	7	2.6	13	588	320	298	254	229	221	180	161	148	121	111	100	78	
Ref. strain		2.3	14	598	301	257	161	153	148	121	111	100	89	73	62	56	48

GS Genome size
Trf Total number of restriction fragments
Ref. strain *Lactococcus lactis* subsp. *lactis* NCIMB 8586

TABLE 3. Distribution of PFGE types of 72 *L. lactis* strains at the different fermentation times of *nyarmie* production

Species		Fermentation time (h)									
		Processor A					Processor B				
			PM					PM			
		UPM	0 h	12 h	24 h	48 h	UPM	0 h	12 h	24 h	48 h
L. lactis PFGE types	1	9	0	6	9	13		1	0	0	0
	2	0	0	0	1	7		2	0	1	0
	3	1	0	1	3	1		0	0	1	0
	4	0	0	0	0	0	1	0	0	0	0
	5	0	0	0	0	0		5	0	0	0
	6	2	0	0	0	2	2	0	0	0	2
	7	1	0	0	0	1		0	0	0	0

UPM unpasteurised milk
PM pasteurised milk

TABLE 4. Comparison of 45 *L. lactis* strains in relation to PFGE types and their assimilation profiles.

Strains used	PFGE types	Assimilation profiles
Au-9	1	A
Au-8	1	A
Au-4	1	B
Au-14	1	F
Au-2	1	A
Au-24	1	F
Au-7	1	A
A2-6	1	F
A2-7	1	F
A2-10	1	F
A2-16	1	F
A2-18	1	F
A2-19	1	F
A3-6	1	G
A3-7	1	A
A3-12	1	C
A4-6	1	H
A4-24	1	J
A4-8	1	I
A4-10	1	I
Bu-14	1	E
Bo-4	1	G
A4-5	2	C
A4-3	2	P
Bo-3	2	L
Bo-7	2	M
B3-2	2	Q
B3-4	2	R
B3-7	2	K
B3-5	3	R
A2-22	3	F
A3-20	3	H
Bu-9	4	D
Bo-12	5	M
B0-14	5	L
Bo-9	5	L

TABLE 5. Comparison of 72 *L. lactis* strains based on PFGE types and cluster groupings

Species	Strain number	PFGE type	Cluster grouping
L. lactis subsp. *cremoris*	A4-15	2	
	A4-17	2	a
	A3-10	2	
	A4-16	2	b
	A4-25	2	
L. lactis subsp. *lactis*	A3-15	3	outlier
	A3-14	1	
	A3-17	1	
	A2-6	1	
	A3-11	1	c
	A3-24	1	
	A4-20	1	
	A4-6	1	
	AU-9	1	
	A4-8	1	
	AU-8	1	d
	A4-10	1	
	A4-29	1	
	A4-9	1	
	A4-21	7	e
	A4-17	1	
	A4-30	1	
L. lactis subsp. *cremoris*	A4-5	2	
L. lactis subsp. *lactis*	A2-18	1	
	AU-21	1	f
	AU-4	1	
	A3-29	1	
	A4-13	1	g
	A4-12	1	
	A4-26	1	
L. lactis subsp. *cremoris*	A4-28	2	outlier
L. lactis subsp. *lactis*	AU-26	6	outlier
	A3-30	3	h
	AU-10	3	
	B3-5	3	outlier
L. lactis subsp. *cremoris*	A4-3	2	
	BO-3	2	i
	BO-7	2	
	BO-12	5	
	BO-14	5	
	BO-9	5	j
	BO-11	5	
	BO-1	5	
L. lactis subsp. *lactis*	A2-4	6	outlier
L. lactis subsp. *lactis*	A3-7	1	

	A4-11	1	k
	A2-19	1	
	A3-12	1	outlier
	A3-9	1	l
	A4-24	1	
	AU-29	1	
	BO-4	1	
	AU-14	1	
	AU-2	1	
	AU-24	1	
	AU-7	1	
	A2-1	6	m
	AU-13	6	
	B4-3	6	n
	B4-6	6	
	BU-16	6	
	BU-6	6	outlier
	A2-16	1	outlier
L. lactis subsp. *cremoris*	B3-9	2	outlier
L. lactis subsp. *lactis*	A2-10	1	o
	A3-19	1	
	A2-17	1	outlier
	A2-22	3	p
	A3-20	3	
	BU-9	4	outlier
	A4-2	3	outlier
	AU-15	7	outlier

Note: Strain notation was based on production sites (processors A & B) followed by fermentation time (U=unpasteurised milk, 0=zero h, 2=12 h, 3=24 h, 4=48 h) and then individual isolate number eg. A3-9 is processor A, at 24 h fermentation and isolate 9.

TABLE 6. Antimicrobial interactions between *Lactoccocus* isolates from different stages of fermentation

Test strains	Indicator strains					
	Unpasteurised milk			0 h fermentation		
	AU-4	AU-2	AU-15	BO-6	BO-11	BO-12
A4-2	+	-	-	++	++	++
A4-6	+	-	-	+	+	++
A4-8	+	-	-	++	++	++
A4-9	+	-	-	+	++	++
A4-15	+	-	+	++	++	++
A4-16	+	-	-	++	+	++
A4-21	+	-	-	++	+	++

Key -: no inhibition, +: 3 mm inhibition zone, ++: 5 mm inhibition zone,
PFGE type 1 isolates: A4-6; A4-8; A4-9; AU-2; AU-4; BO-6; PFGE type 2 isolates : A4-15; A4-16; PFGE type 3 isolates : A4-2; PFGE type 5 isolates : BO-11; BO-12;
PFGE type 7 isolates: A4-21; AU-15.

TABLE 7. Antimicrobial interactions within PFGE types of *Lactoccocus* isolates from different stages of fermentation

Test strains (PFGE type 1)	Indicator strains (PFGE types)				
	Type 2	Type 3	Type5	Type 6	Type 7
AU-2	-	++	+	-	+
BO-1	-	+	+	-	+
A2-18	-	+	+	-	+
A3-17	-	+	+	-	+
A4-8	++	++	++	++	++

Key -: no inhibition, +: 3 mm inhibition zone, ++: 5 mm inhibition zone,
Indicator strains used: PFGE type 2, BO-7; PFGE type 3, A2-22; PFGE type 5, BO-11; PFGE type 6, A2-4; PFGE type 7, AU-15.

TABLE 8. Test for hydrogen peroxide and acid production as antimicrobial properties

Test strains	Hydrogen peroxide as an antimicrobial property			Acid production as an antimicrobial property		
	Indicator strains at 0 h fermentation			Indicator strains at 0 h fermentation		
	BO-6	BO-11	BO-12	BO-6	BO-11	BO-12
A4-2	-	-	+	-	-	-
A4-6	-	+	+	-	-	-
A4-9	+	-	+	-	-	-
A4-16	-	-	+	-	-	-
A4-21	-	+	+	-	-	-

Key -: no inhibition; +: 3 mm inhibition zone
Controls are shown in Table 6

TABLE 9. Recovery of *Lactococcus* PFGE types (log_{10} cfu ml^{-1}) stored at 28±2°C for 48 h after heat stress treatments.

PFGE types	Fermentation time (h)	
	24	48
1	9.73 (±0.03)	9.62 (±0.05)
2	9.72 (±0.07)	9.61 (±0.24)
3	6.51 (±0.23)	9.59 (±0.35)
5	8.52 (±0.31)	9.61(±0.56)
6	7.91 (±0.03)	nd
7	6.51 (±0.42)	9.98(±0.51)

(n = 3), nd-not detected

Isolates used were: PFGE type 1: *L. lactis* subsp. *lactis* isolate BO-4; PFGE type 2: *L. lactis* subsp. *cremoris* isolate BO-3; PFGE type 3: *L. lactis* subsp. *lactis* isolate A2-22; PFGE type 5: *L. lactis* subsp. *cremoris* isolate BO-11; PFGE type 6: *L. lactis* subsp. *lactis* isolate BU-16; PFGE type 7: *L. lactis* subsp. *lactis* isolate AU-15.

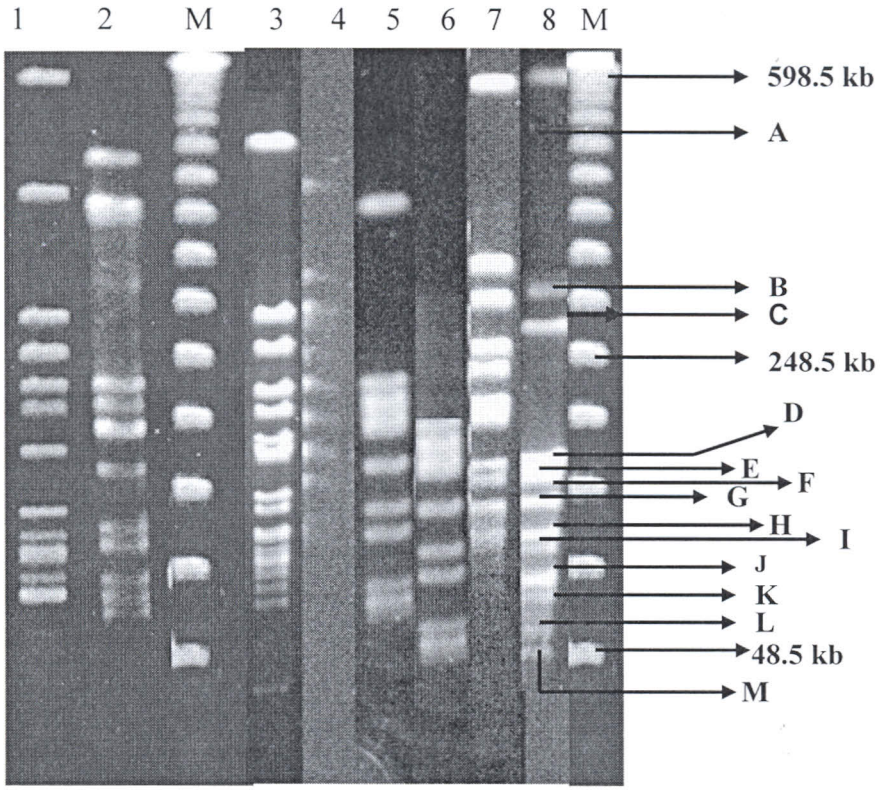

Figure 1: Comparison of *L. lactis* isolates with *L. lactis* reference strain using pulsed–field gel electrophoresis and *Sma*I restriction digestion. Genomic DNA obtained with *Sma*I and separated on 1% PFGE gel using electrophoretic parameters of initial switch time 4s, final switch time 45s, at 6V for 16h. The sizes of the fragments are indicated in kilobases. Fragments A to M of the reference strain were used as a standard for comparison with the fragments of the different PFGE types. Lane 1 - PFGE type 1 *L. lactis* subsp. *lactis;*

Lane 2 - PFGE type 2 *L. lactis* subsp. *cremoris;* Lane 3 - PFGE type 3 *L. lactis* subsp. *lactis;* Lane 4 - PFGE type 4 *L. lactis* subsp. *lactis ;* Lane 5 - PFGE type 5 *L. lactis* subsp. *cremoris ;* Lane 6 - PFGE type 6 *L. lactis* subsp. *lactis ;* Lane 7 - PFGE type 7 *L. lactis* subsp. *lactis;* Lane 8 - *L. lactis* subsp. *lactis* NCIMB 8586 (reference strain) ; Lane M-50-1000kb molecular marker

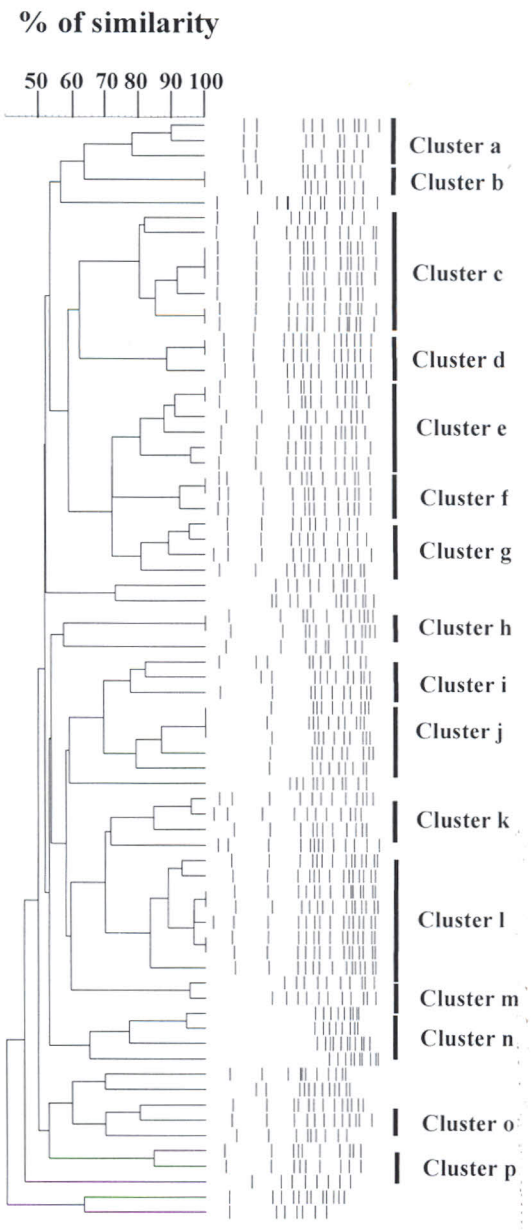

Figure 2: Cluster analysis of 72 isolates of *L. lactis* from processors A and B based on their PFGE profiles evaluated using the Dice association coefficient and the unweighted pair group algorithm with arithmetic averages (UPGMA).

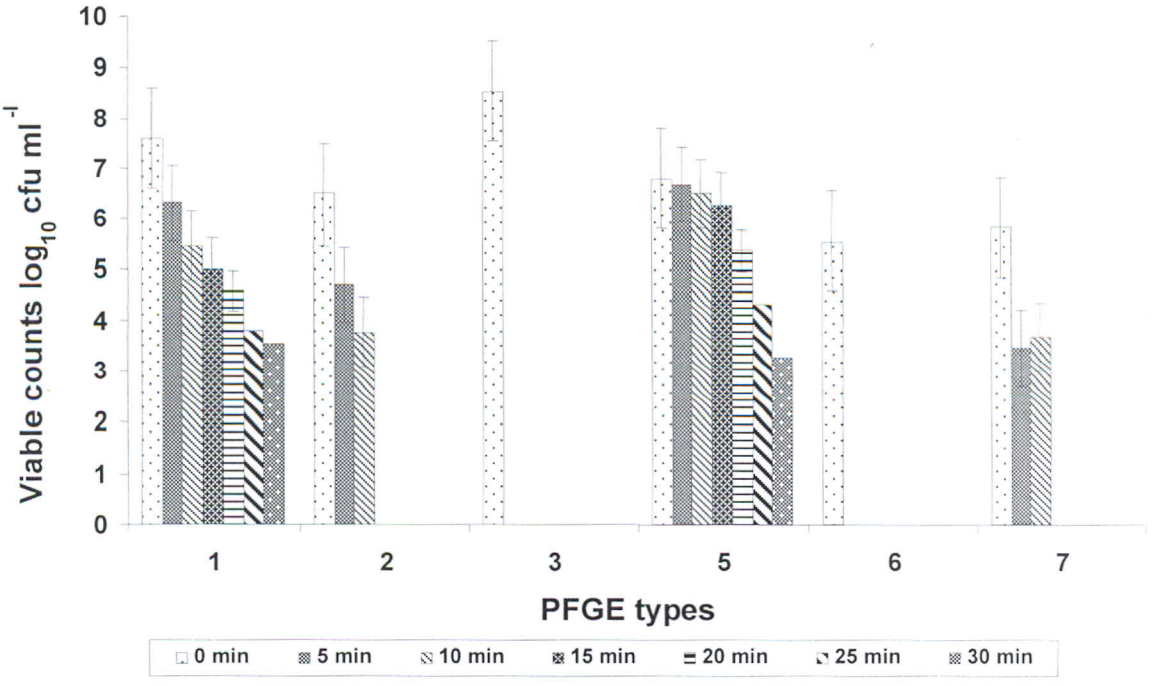

Figure 3: Effect of heat stress on different *Lactococcus* PFGE types heated at 65°C in reconstituted skim milk for 30 min and recovered on M17 agar at 28±2°C (n = 3). Isolates used are PFGE type 1: *L. lactis* subsp. *lactis* isolate BO-4; PFGE type 2: *L. lactis* subsp. *cremoris* isolate BO-3; PFGE type 3: *L. lactis* subsp. *lactis* isolate A2-22, PFGE type 5: *L. lactis* subsp. *cremoris* isolate BO-11, PFGE type 6: *L. lactis* subsp. *lactis* isolate BU-16, PFGE type 7: *L. lactis* subsp. *lactis* isolate A