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Management of spoilage and pathogenic organisms during fermentation of *nono* - an indigenous fermented milk product in Nigeria

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ABSTRACT
Objectives: The ability of lactic acid bacteria to suppress the growth of spoilage and pathogenic organisms during *nono* fermentation was investigated.
Methodology and results: Pasteurized milk samples were inoculated with selected spoilage and pathogenic organisms. Lactic acid bacteria (1%) were then inoculated singly or in-combination into the milk samples for evaluation as protective cultures. The treated milk was fermented at 30°C for 24h. Changes in pH, titratable acidity (TA) and viable counts of the inoculated indicator organisms were determined at specific intervals during *nono* fermentation. The pH of fermenting *nono* ranges between pH 4.08 and 5.9. A significant increase ($P \leqq 0.05$) in TA values was observed ranging between 0.4 and 1%. There was a significant increase ($P \leq 0.05$) in the viable counts of *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterobacter aerogenes* and *Staphylococuss aureus*. However, a significant decrease ($P \leq 0.05$) in the viable counts of *E. coli, Bacillus cereus, Salmonella typhi* and *Klebsiella sp*. were recorded after fermentation for 24 hours.
Conclusion and application of findings: This study shows that spoilage and pathogenic organisms could survive in milk during *nono* production. A mixture of *L. plantarum* and *L. bulgaricus* could be useful as protective culture against some of the organisms. However, more appropriate quality control measures should be put in place to prevent contamination during *nono* production.

Key words: *Lactobacillus* spp., milk, fermentation, protective cultures

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INTRODUCTION

Milk is highly valued among natural foods since it provides essential nutrients in higher amounts than other staple foods (Oyawoye *et al*., 1997). Milk is utilized in the production of at least 400 different fermented products all over the world (Willey *et al*., 2008). *Nono* is a traditional fermented milk product that is commonly produced by the Hausa-Fulani people of Northern Nigeria (Atanda & Ikenebomeh, 1989). *Nono* is produced by spontaneous fermentation of milk, and numerous attempts have been made to study microorganisms that are associated with the process (Akinyanju, 1989; Bankole, 1990; Okagbue & Bankole, 1992).

Milk and its fermented products have been identified as important means of spreading human pathogenic organisms. Recently, interest has increased on the potential of incorporating lactic acid bacteria (LAB) strains as protective cultures into food products (Ogunbanwo *et al*., 2004). Protective cultures are selected based on their ability to grow in a product and inhibit food poisoning or spoilage organisms (Modzelewska-Kapitola *et al*., 2005). Many researchers have investigated the antimicrobial activity of lactic acid bacteria against undesirable microorganisms, e.g. *Escherichia coli*, Salmonella, Staphylococci, Yersiniae, Bacilli, and Pseudomonads (Northolt, 1984; Garriga *et al*., 1993; Larsen *et al*., 1993; Farias *et al*., 1993; Pazakova *et al.,* 1997; Pepe *et al*., 2003). Alternative methods of preventing the growth of spoilage organisms include vacuum packing and refrigeration of food. Treatments like ionizing radiation can destroy pathogens non-chemically, but may affect taste and do not protect food against post-treatment contamination (Ogunbanwo *et al*., 2004). Nowadays consumers are concerned about the synthetic chemicals used as preservatives in food, leading to a trend towards less processed food (Soomro *et al*., 2002). A potential solution to this problem is in the use of protective cultures and/or their metabolites in food preservation. Cells of lactic acid bacteria (LAB) and their metabolites have been consumed by humans for long and have known adverse effect. The present work investigated the use of wild lactic acid bacteria as protective cultures in nono.

MATERIALS AND METHODS

Nono samples: *Nono* samples were collected in sterile containers from Bodija market, Ibadan, South West Nigeria and transported to the laboratory for immediately analysis.

Bacterial strains and cultures: Lactic acid bacteria strains were isolated from *nono* samples and characterized using the API 50 CH strips and API 50 CHL medium (API systems, Biomeriex Sa, France). The pathogenic and spoilage bacteria used as indicator organisms were obtained from the culture collection of the Department of Biology, The Polytechnic, Ibadan, Nigeria. These included *E. coli, Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa* and *Enterobacter aerogenes.*

Antimicrobial activity of lactic acid bacteria isolates: Agar well diffusion assay was carried out as described by Schillinger and Lucke (1989). A 0.1ml aliquot of the over-night broth culture of the indicator organisms was seeded into sterile molten nutrient agar (45°C) and poured into sterile Petri-dishes (10cm in diameter) and allowed to solidify. Holes (4 holes per plate, placed 4cm apart) were bored on the agar plates using a sterile cork borer (6mm diameter). A 0.1ml volume of the supernatant from a 24 hour-old broth culture of the lactic acid bacteria was dispensed into the holes, allowed to diffuse and was incubated at 37°C for 24 h. After incubation, the zones of inhibition around the agar wells were measured and the diameter of the well was subtracted from the total zone diameter (Modzelewska-Kapitola *et al*., 2005). The LAB were selected according to their antagonistic ability and those with strong antimicrobial activity were studied further as protective cultures in this study.

Milk fermentation and inoculation of indicator organisms: Fresh milk was pasteurized at 72°C for 20 seconds (Willey *et al*., 2008). The LAB were propagated in MRS broth over night and centrifuged at 3,500rpm for 20 minutes. The cells were washed in 10ml sterile 0.9 (w/v) NaCl solution. The cells were re-suspended in the same NaCl solution and 1ml of each suspension was inoculated singly in 99ml of pasteurized milk held in sterile conical flasks. When mixed cultures of the LAB were used, 0.5ml suspension of each organism was inoculated into the milk.

Uninoculated pasteurized milk (without LAB) was used as the control (Okagbue & Bankole, 1992). Pure cultures of spoilage and pathogenic organisms with initial microbial load of 10 cfu/ml were singly used to inoculate the milk samples. Fermentation was carried out at 30°C for 24 h.

Microbiological and physico-chemical analyses: Samples of the fermenting milk taken at 0, 12 and 24 h were serially diluted in distilled water for enumeration of the inoculated enteropathogens by the pour plate method using appropriate culture media for each organism (Ogunbanwo *et al*., 2004). The pH of the fermenting milk was measured at the time of sampling using a pH meter (Crison MicropH). The titratable

acidity expressed as percentage lactic acid was determined according to the method described by Achi and Akubor (2000).

RESULTS

Fifty strains of lactic acid bacteria were isolated from *nono* samples collected from different local producers. The LAB isolated were identified as belonging to *Lactobacillus brevis*, *L. bulgaricus*, *L. plantarum*, *L. casei* and *L. fermentum*. The diameter of zones of inhibition ranged between 2 and 14mm (table 1). A significant decrease in pH (P≤ 0.05) was observed for all the fermented milk samples (table 2). After fermenting for 24 h, the lowest pH (4.08) was attained in milk inoculated with a mixture of *L. bulgaricus* and *L. plantarum* as protective culture while the highest pH (4.8) was in spontaneously fermented *nono.* A significant increase (p≤ 0.05) in titratable acidity was observed during fermentation of all the milk samples. The TA ranged between 0.5 and 1.0% after fermenting for 24 h.

Table 1: Antagonistic activity of *Lactobacillus* spp. metabolites against selected indicator organisms.

Starters	*Staphylococcus aureus*	*E. coli*	*Pseudomonas aeruginosa*
L. bulgaricus	12mm	7mm	4mm
L. plantarum	14mm	12mm	6mm
L. brevis	10mm	8mm	4mm
L. fermentum	10mm	4mm	2mm
L. casei	10mm	6mm	2mm

Table 2: Changes in pH and titratable acidity (TA) during fermentation of *nono* inoculated with different *Lactobacillus species*.

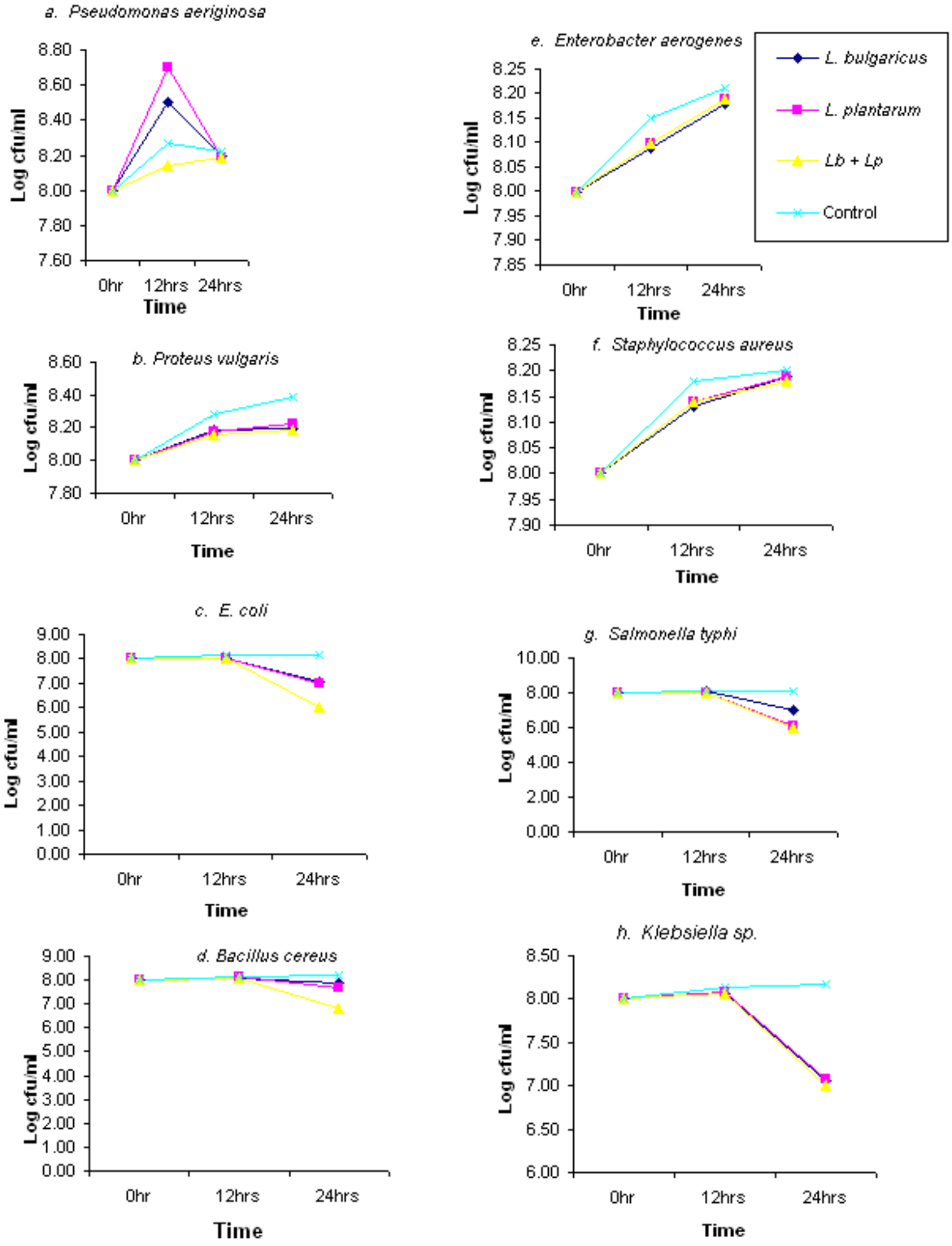
Starter	pH			TA		
	0h	12h	24h	0h	12h	24h
L.bulgaricus(Lb)	$5.95^{*}\pm0.005^{aa}$	4.34 ± 0.005^{ab}	4.27 ± 0.01^{ac}	0.30 ± 0.05^{ad}	0.60 ± 0.08^{ae}	0.90 ± 0.07^{af}
L.plantarum(Lp)	5.95 ± 0.005^{aa}	4.67 ± 0.008^{bb}	4.52 ± 0.008^{bc}	0.20 ± 0.05^{ad}	0.50 ± 0.05^{be}	0.80 ± 0.07^{bf}
Lb + Lp	5.96 ± 0.005^{aa}	4.25 ± 0.006^{cb}	4.08 ± 0.02^{cc}	0.20 ± 0.05^{ad}	0.60 ± 0.07^{ce}	1.00 ± 0.08^{cf}
Control**	5.95 ± 0.005^{aa}	4.89 ± 0.01^{db}	4.80 ± 0.005^{dc}	0.30 ± 0.05^{ad}	0.40 ± 0.06^{de}	0.50 ± 0.05^{df}

**Spontaneous fermentation;*Values are means (n=3) ± standard deviation. Means with different superscripts are significantly different along the row and columns.

The results indicated a significant increase (p≤ 0.05) in the viable counts of *Pseudomonas aeruginosa*, *Proteus vulgaris* , *Enterobacter aerogenes* and *Staphylococcus aureus* (fig 1 a,b,e,f, respectively) throughout the fermentation period regardless of protective bacteria. The highest viable counts of *P. vulgaris* (8.39 log cfu/ml) was obtained for the control (fig 1b) while the lowest viable counts of *S. typhi* (6.00 log cfu/ml) was detected when a mixture of *L. bulgaricus* and *L. plantarum* was used as protective cultures. A substantial decrease (p≤ 0.05) in viable counts of *E. coli*, *B. cereus*, *Salmonella typhi* and *Klebsiella sp* were recorded after 24 hours fermentation in the presence of protective bacteria (fig. 1c, d, g, h).

DISCUSSION

In this study the lactic acid bacteria isolated from *nono* samples were identified as belonging to the genus *Lactobacillus*. The species isolated were *Lactobacillus brevis*, *L. bulgaricus*, *L. plantarum*, *L. casei* and *L. fermentum*. Isolation of lactic acid bacteria has been reported from various foods including meat, milk, cheese (Randazzo *et al*., 2002; Velijovic *et al*., 2007), vegetables, *gari* and *fufu* (fermented cassava products) and yoghurt. Okagbue and Bankole (1992) reported isolation of LAB from *nono*, which they used singly and in combination with yeast to develop starter cultures for *nono* production. Furthermore, Gran *et al*. (2003) isolated LAB from Amasi, a Zimbabwean naturally fermented milk product.



Figures 1(a-h): Changes in viable counts of spoilage and pathogenic organisms during *nono* fermentation.

All the LAB isolates demonstrated antagonistic activity to varying degrees against the indicator organisms evaluated in the agar diffusion assay. Several studies have shown that LAB exhibit antimicrobial activity against many pathogenic and spoilage organisms (Daba *et al.*, 1991; Sanni *et al.,* 1999; Jin *et al.,* 2000; Martini *et al.,* 2001; Bromberg *et al.,* 2004; Ogunbanwo *et al*., 2004; Veljovic *et al*., 2007). The result further indicated that inoculation of LAB cultures led to significant increase in titratable acidity and corresponding decrease in pH. This observation is in agreement with reports of previous studies (Daeschel *et al*., 1989; Nout 1991; Achi & Akubor, 2000).

Some of the spoilage and pathogenic organisms inoculated into the milk samples survived and multiplied in the presence of the inoculated lactic protective cultures. This suggests that these organisms are insensitive to the antimicrobial compounds produced by the LAB isolates. The viable count of *Staphylococcus aureus* increased steadily throughout the period of fermentation regardless of the LAB present. Our finding is contrary to that of Pazakova *et al.* (1997) who reported a slight decrease in *S. aureus* population inoculated into fermenting milk for yoghurt production, though it could be due to differences in potential of the isolates evaluated. Many spoilage and pathogenic organisms have been reported to survive during fermentation by lactic cultures (Martinis *et al.,* 2001; Govaris *et al*., 2002; Ananou *et al.,* 2005).

The protective LAB cultures were able to suppress the growth of *E. coli*, *B. cereus*, *S. typhi* and *Klebsiella sp*. The suppressive effect could be due to decrease in pH of the fermenting milk samples coupled with ability of the lactic acid bacteria strains to produce hydrogen peroxide, diacetyl and/or bacteriocins (Sanni *et al*., 1999; Ogunbanwo & Okanlawon, 2006). Ogunbanwo *et al.* (2004) reported a decrease in the growth of *B. cereus* and *Salmonella typhi* after 24 h fermentation of *fufu*, a traditional fermented cassava product.

The results of this study indicated that both spoilage and pathogenic organisms could survive in *nono* during fermentation. However, the inoculated protective cultures indicated potential to suppress the growth of *E .coli, B .cereus, S. typhi* and *Klebsiella sp.* Considering that the evaluated protective cultures were not completely effective, we recommend that appropriate quality control measures should be put in place to prevent contamination of milk destined for *nono* production.

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