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Screening For Probiotic Features Among Microorganisms Associated With Garri Fermentation In Agbani Town

Chinedu A. Okafor*, Chinemerem D. Nwobodo and Ebuka E. David+
Department of Microbiology
Renaissance University, Ugbawka, Enugu State, Nigeria.
E-mail : Okafor-edu@yahoo.com Tel. : +2348033386722

+ Present address : Department of Biochemistry, Renaissance University, Ugbawka, Enugu State, Nigeria.

* To whom all correspondence should be addressed

Abstract
Thirteen microorganisms were isolated from 5 samples of fermenting cassava mash obtained from cassava processing plants in Agbani town, Enugu State. They included the following genera: Streptococcus, Staphylococcus, Corynebacterium, Bacillus and Alcaligenes, which are non-lactic acid bacteria. Others were Propionibacterium, Lactobacillus and Leuconostoc, which are lactic acid bacteria. The identity of one isolate could not be determined. Some in vitro tests to check their ability to tolerate probiotic colonization factors were carried out which were: survival at pH of 4.0, tolerance to bovine bile, swine bile and antibiotic susceptibility profile. The results showed that only 4 of the isolates could not survive at pH of 4.0 (Propionibacterium A, B, C and Staphylococcus). All isolates survived bovine and swine biles except some isolates of the genera: Staphylococcus, Corynebacterium and Propionibacterium. Majority of the isolates were sensitive to the 10 antibiotics used in this project, while Corynebacterium B was sensitive to only 3 of the antibiotics. Generally, 4 lactic acid isolates and 4 non-lactic acid isolates were concluded to be likely probiotic, based on their ability to withstand these colonization factors.

Keywords: Probiotics, cassava mash, colonization factors, safety.

Introduction

The word "probiotic" is derived from a Greek word, which means "pro-life" (Ukeyima *et al.*, 2010). Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits on the host (FAO/WHO, 2002). For an organism to be classified as a probiotic, it must be resistant to gastric acidity; tolerance to bile, ability to adhere to epithelial cells of the mucosa, the organism must be non-pathogenic and non-toxic, resistant to antibiotics, ability to maintain viability in large numbers, it must be tolerant to food additives and also be stable in the food matrix (Soccol *et al.*, 2010; Oyetayo and Oyetayo, 2005). These criteria may serve as suitable index for screening a probiotic from the environment.

Microorganisms regarded as probiotics are the members of the genera *Lactobacillus, Bifidobacterium, Streptococcus, Escherichia, Lactococcus, Enterococcus,* and *Saccharomyces* (Javadi *et al.*, 2012; Mercenier *et al.*, 2003; Oyetayo and Oyetayo, 2005). Some of the benefits of probiotics include; maintaining balance of the intestinal flora, increasing lactose tolerance (Ukeyima *et al.*, 2010), anti-mutagenic/anticancer activity, cholesterol

lowering effect and enhancement of the immune system (Nagpal *et al.*, 2012). These probiotic microorganisms are generally recognized as safe (GRAS).

Over the years, there have been evaluations of the probiotic properties of microorganisms isolated from milk and milk products such as yoghurt, cheese, etc. These probiotic microorganisms contained in these foods are not destroyed before consumption and are mostly lactic acid bacteria. Since there are other indigenous naturally fermenting foods, there is need to screen for the probiotic features of microorganisms isolated from these sources, considering both the lactic acid bacteria and the non-lactic acid bacteria.

Cassava (*Manihot esculenta* Crantz) is a staple food for more than 500 million people in the developing world. It ranks fourth after rice, wheat and maize on the list of major food crops in developing countries (Edward *et al.*, 2012). Bacteria involved in cassava fermentation include; *Alcaligens faecalis*, *Corynebacterium*, *Lactobacillus plantarum*, and *Leuconostoc cremoris*, while *Geotrichum candidum* and *S. cerevisiae* as fungi (Ahaotu *et al.*, 2013; Okafor and Ejiofor, 1986; Oyewole and Odunfa, 1988). Other researchers have been able to isolate probiotics from fermenting foods that do not involve processes that destroy the organisms before consumption and in most cases have isolated lactic acid bacteria. Therefore, it is necessary to check for the probiotic features of both lactic and non-lactic acid bacteria isolated from some fermenting foods like cassava mash. It is also necessary to study the probiotic features of these microorganisms because the processing of cassava for garri involves steps that destroy the microorganisms present in the cassava mash. This makes them unavailable for human consumption in order to exert health benefits. These organisms, if found to possess probiotic features, could be incorporated into functional foods such as yoghurt and soy-yoghurt.

This study was aimed at evaluating the survival of lactic and non-lactic acid bacteria, isolated from fermenting cassava mash, towards probiotic colonization factors.

Materials and Methods

Source of isolates: Cassava mash was collected from different cassava processing plants in Agbani town, Enugu State. Each sample (10g) was aseptically obtained, after thorough mixing, and placed in test tubes containing 9ml of sterile peptone water and allowed to soak for 2-3min with occasional stirring using a sterile glass rod. Ten fold serial dilutions were subsequently prepared by transferring 1ml aliquot of the mash into 9ml of sterile peptone water. Further serial dilution was carried out and thereafter, 0.1ml of appropriate dilution factors were aseptically plated out, using spread plate technique on nutrient agar (Titan, China) for isolation of non-lactic acid bacteria (non-LAB) and incubated for 24hr at room temperature, while 0.1ml was plated out on de Mann Rogosa Sharpe (MRS) broth (Titan, China) and incubated micro-aerophilically for 48hr at room temperature. The isolates were identified based on their morphological and biochemical properties (Holt et al., 1994).

Acid tolerance test: One ml of overnight culture of each of the isolates was aseptically transferred into 9ml of freshly prepared de Mann Rogosa Sharpe (MRS) broth (for LAB) and nutrient broth (for non-LAB), with pH adjusted to 4.0. Using the spectrophotometer (Midfield, China) at wavelength of 600nm, the absorbance of the culture media were taken, and repeated 2hr later (Khali, 2009).

Bile tolerance test: Overnight culture of the isolates were aseptically plated out on nutrient agar and MRS agar media. Wells of 6mm in

diameter were aseptically made in the agar plates. The wells were filled with Bovine bile and Swine bile separately. The plates were incubated at 35°C for 24 - 48hr (Okafor and Umeh, 2013).

Antibiotic susceptibility test: The antibiotic susceptibility profile of all isolates was tested by the Kirby-Bauer method on nutrient agar and MRS agar for non-LAB and the LAB respectively. The isolates were assayed for possible resistance to 10 commonly used antibiotics. Since the isolates were mainly Gram positive microorganisms, the following antibiotics were used : ciprofloxacin, norfloxacin, gentamycin, amoxicillin, streptomycin, rifampicin, erythromycin, chloramphenicol, ampicloxacin and levofloxacin. The test was carried out using multiple discs on the same plate to eliminate differential effects from growth time and temperature. Overnight cultures of the isolates were aseptically plated on the appropriate medium. The antibiotic discs were aseptically placed on the plates with the aid of forceps. It was then allowed to stand for 20 min for diffusion of the discs. The plates were inverted and incubated at 35°C for 24 - 48hr.

Results and Discussion

Thirteen microorganisms were isolated from the samples. Seven were non-LAB, and included *Streptococcus*, *Staphylococcus*, *Corynebacterium* A, *Corynebacterium* B, *Bacillus*, *Alcaligenes* and an undetermined isolate. The LAB isolates were *Propionibacterim* A, *Propionibacterim* B, *Propionibacterim* C, *Lactobacillus* A, *Lactobacillus* B and *Leuconostoc*. Most of the bacterial isolates such as *Lactobacillus*, *Leuconostoc*, *Alcaligenes*, *Bacillus*, and *Corynebacterium* had also been isolated by several other workers from fermenting cassava (Ahaotu *et al.*, 2013; Amao-Awun *et al.*, 1996; Essers *et al.*, 1995; Okafor, 1977; Oyewole and Odunfa, 1988).

Before reaching the intestinal tract and exerting beneficial effects on host, probiotic bacteria must first survive in the stomach (Nawaz *et al.*, 2011). Most of the microorganisms used in this project were shown to be resistant to acidity at pH 4.0 except *Staphylococcus*, *Propionibacterium* A, *Propionibacterium* B and *Propionibacterium* C (figs. 1 and 2). This implies that the genera *Staphylococcus*, and *Propionibacterium* would not be able to survive in the stomach when consumed as probiotics. *Streptococcus* and *Lactobacillus* A exhibited the highest viability which supports similar findings (Maragkoudakis *et al.*, 2006, 2009; Mathara *et al.*, 2008; Okafor and Umeh, 2013). Survival of *Alcaligenes*, *Bacillus*, *Corynebacterium* A, *Corynebacterium* B, *Lactobacillus* A, *Lactobacillus* B, *Leuconostoc* and *Streptococcus* at pH of 4.0 is a good attribute.

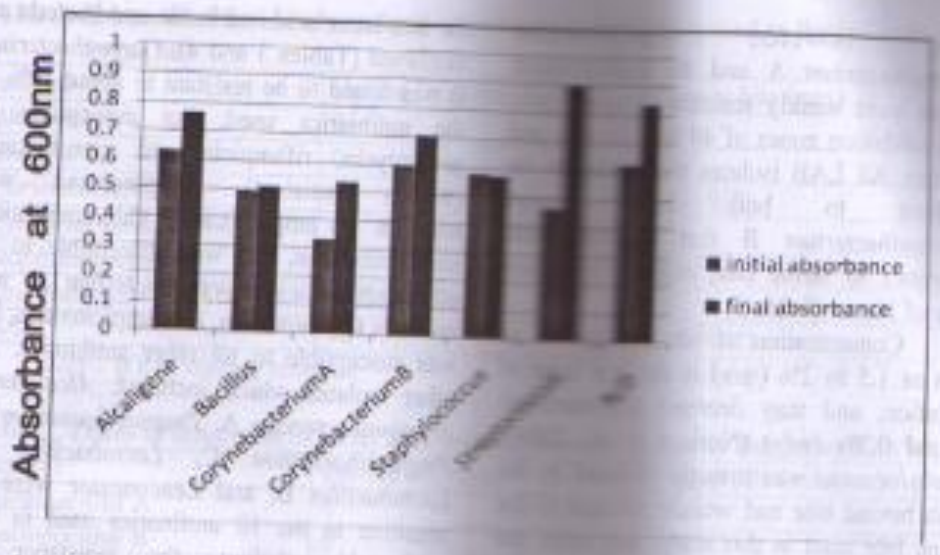

Fig. 1. Initial and final absorbance of non-LAB exposed to pH 4.0 in nutrient broth for 2hr.

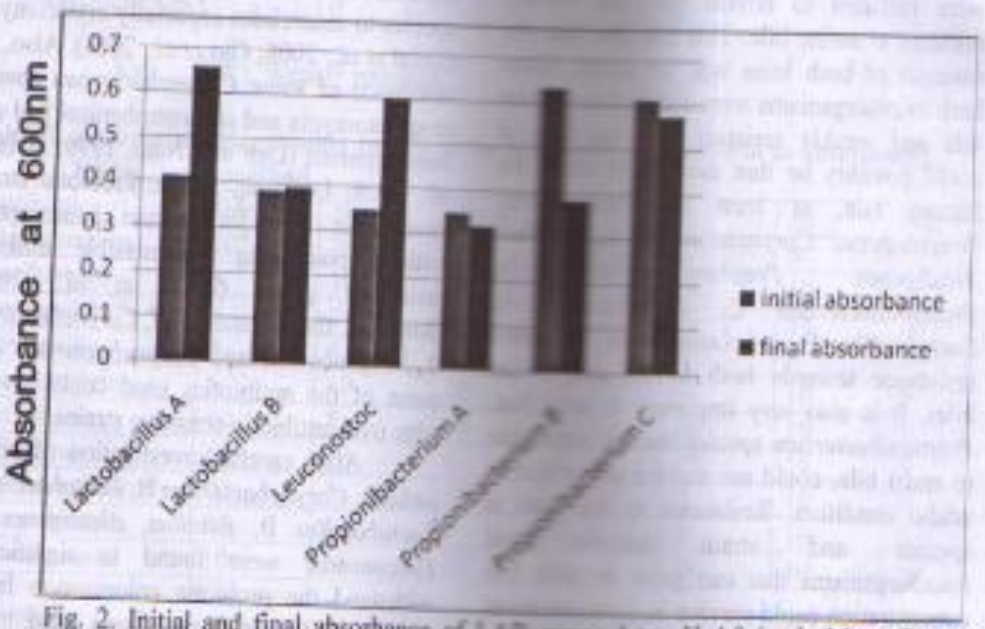

Fig. 2. Initial and final absorbance of LAB exposed to pH 4.0 in de Mann Rogosa Sharpe broth for 2hr

Tables 1 and 2 show results obtained from resistance to fresh bovine and swine bile among the isolates. Five non-LAB isolates *Streptococcus*, *Corynebacterium* A, *Corynebacterium* B, *Bacillus* and *Alcaligenes* were resistant to bovine bile (Table 1). *Staphylococcus* and the undetermined isolate were sensitive with inhibition zone of 10 mm

each. However, *Staphylococcus*, *Corynebacterium* A and the undetermined isolate were weakly resistant to swine bile, with inhibition zones of 40 mm, 14 mm and 28 mm. All LAB isolates were found to be resistant to both biles, except *Propionibacterium* B that showed weak resistance to swine bile, with an inhibition zone of 16mm (Table 2).

Concentration of bile salt can be as high as 1.5 to 2% (w/v) in the first hour of digestion, and may decrease afterwards to around 0.3% (w/v) (Noriega *et al.*, 2004). *Staphylococcus* was strongly inhibited by the fresh bovine bile and weakly resistant to the swine bile used in this study, and could not survive low pH of 4.0 which indicates a very poor colonization potential if consumed. *Corynebacterium* A and *Propionibacterium* B were resistant to bovine bile, but weakly resistant to swine bile. This indicates that the strength of both biles was not equal. Since both microorganisms were resistant to bovine bile and weakly resistant to swine bile, it could possibly be that they might resist the human bile, at least to some extent. *Streptococcus*, *Corynebacterium* B, *Bacillus*, *Alcaligenes*, *Propionibacterium* A, *Propionibacterium* C, *Lactobacillus* A, *Lactobacillus* B and *Leuconostoc* exhibited resistance towards both bovine and swine biles. It is also very important to note that, *Propionibacterium* species though were able to resist bile, could not survive subjection to acidic condition. Resistance to bile salt is species and strain specific and microorganisms that can grow in such bile concentration could survive in gastrointestinal tract (Bao *et al.*, 2010 and Morelli, 2000).

Antibiotics susceptibility profiles of the non-lactic acid and lactic acid bacteria are displayed (Tables 3 and 4). *Corynebacterium* B was found to be resistant to about 70% of the antibiotics used, but susceptible to gentamycin, rifampicin and ciprofloxacin (weakly resistant). *Streptococcus* was resistant to ampicloxacin, chloramphenicol and amoxicilin, but was susceptible to all other antibiotics. *Corynebacterium* A was resistant to amoxicilin, and ampicloxacin, but was susceptible to all other antibiotics. All other isolates which included; *Alcaligenes*, *Propionibacterium* A, *Propionibacterium* B, *Propionibacterium* C, *Lactobacillus* A, *Lactobacillus* B, and *Leuconostoc* were all sensitive to the 10 antibiotics used in this work. Nevertheless, the resistance of *Corynebacterium* B to most antibiotics was not surprising, since several researchers have reported the resistance of *Corynebacterium* species to macrolides especially erythromycin (Delal *et al.*, 2008; Ojo *et al.*, 2006). Also, the resistance of some *Corynebacterium* species to streptomycin and chloramphenicol had also been reported (Deb and Nath, 1999; Delal *et al.*, 2008; Leclereq, 2002). Probiotic strains should be safe for human consumption, without possessing transmissible antibiotic resistance genes (Zhou *et al.*, 2005). However, the resistance of *Corynebacterium* B, *Streptococcus* and *Corynebacterium* A to some of the antibiotics used could give an edge over antibiotic-sensitive strains.

After careful investigation of all the isolates, *Corynebacterium* B, *Lactobacillus* A, *Lactobacillus* B, *Bacillus*, *Alcaligenes* and *Leuconostoc* were found to significantly withstand the probiotic colonization factors among all the microorganisms used in this study.

Table 1. Zones of inhibition of non-lactic acid bacteria isolates exposed to Bovine and Swine bile

Isolates	Bovine bile (mm)	Swine bile (mm)
Alcaligenes	0	0
Bacillus	0	0
Corynebacterium A	0	14 (WR)
Corynebacterium B	0	0
Staphylococcus	10	40 (WR)
Streptococcus	0	0
N/D	10	28 (WR)

Key : WR = Weakly resistant N/D : Not determined

Table 2. Zones of inhibition of lactic acid bacteria isolates exposed to Bovine and Swine bile

Isolates	Bovine bile (mm)	Swine bile (mm)
Lactobacillus A	0	0
Lactobacillus B	0	0
Leuconostoc	0	0
Propionibacterium A	0	0
Propionibacterium B	0	16 (WR)
Propionibacterium C	0	0

Key : WR : Weakly resistant.

Table 3. Antibiotics susceptibility profile of the non-lactic acid bacteria as obtained by Kirby-Bauer method

Antibiotics	*Strep*	*Staph*	Cy A	Cy B	B.	Al	N/D
Ciprofloxacin	10*	20	20	4 (WR)	20	6	14
Norfloxacin	6	10	10	0	10	10	10
Gentamycin	6	16	6	12	14	14	20
Amoxicillin	0	6 (WR)	0	0	6	6	10
Streptomycin	6	10	10	0	14	10	6
Rifampicin	10	8	20	4	10	10	4
Erythromycin	6	12	6	0	12	10	4
Chloramphenicol	0	6	6	0	0	10	0
Ampicloxacin	0	6	0	0	0	10	0
Levofloxacin	10	16	20	0	10	10	10

* Inhibition zone measured in millimeters (mm)

Key : WR : Weakly resistant, *Strep: Streptococcus, Staph: Staphylococcus, Cy A: Corynebacterium, Cy B: Corynebacterium B, B: Bacillus, Al: Alcaligenes*

Table 4. **Antibiotics susceptibility profile of the lactic acid bacteria as obtained b Kirby-Bauer method**

Antibiotics	Prop. A	Lc. A	Leu	Prop. B	Lc. B	Prop. C
Ciprofloxacin	20	20	20	20	20	16
Norfloxacin	14	20	12	20	10	16
Gentamycin	14	20	14	14	16	16
Amoxicilin	16	16	12	14	20	12
Streptomycin	20	20	20	12	14	16
Rifampicin	20	14	4	14	16	16
Erythromycin	12	20	20	12	20	20
Chloramphenicol	20	16	16	20	20	20
Ampicloxacin	16	20	10	10	14	14
Levofloxacin	16	20	20	20	20	20

* Inhibition zone measured in millimeters (mm)

Key : *Lac A: Lactobacillus* A, *Lac B: Lactobacillus* B, *Leu: Leuconostoc*, *Prop A: Propionibacterium* A, *Prop B:Propionibacterium* B, *Prop C: Propionibacterium* C

References

Ahaotu, I., Ogueke, C. C., Owuamanam, C. I., Ahaotu, N. N., and Nwosu, J. N. (2013). Fermentation of undewatered cassava pulp by linamarase producing microorganisms: Effect on nutritional composition and residual cyanide. American Journal of Food and Nutrition. 3 (1): 1 - 8.

Amoa-Awua, W. K. A., Appoh, F., and Jacobsen, M. (1996). Lactic acid fermentation of cassava into 'Agbelima'. International Journal of Food Microbiology. 31: 87 - 98.

Bao, Y., Zhang, Y. C., Zhang, Y., Liu, Y., Wang, S. Q., Dong, X. M., Wang, Y. Y., Zhang, H. P. (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Journal of Food Control. 21(5) : 695 – 701.

Delal, A., Urban, C., and Segal-Maurer, S. (2008). Endocarditis due *Corynebacterium amycolatum*. Journal of Medical Microbiology. 57 (10): 1299-1302.

Deb, J. K., and Nath, N. (1999). Plasmids of Corynebacteria. FEMS Microbiology. 175 (1) : 11 - 20.

Edward, V. A., Egounlety, M., Huch, M., Vanzyl, P. J., Sigh, S., Nesengani, N. D., Haakuri, V. M., and Franz, M. A. P. (2012). Isolation and screening of microorganisms from a gari fermentation process for starter culture development. African Journal of Biotechnology. 11(65) : 12865 - 12877.

Ejiofor, M. A. N., and Okafor, N. (1981). Comparison of pressed and unpressed cassava pulp for garri making. In: Tropical Root Crop Research Strategies For The 1980, Terry, E. R., Ottawa, K. A. and Caveness, F. (eds). IDRC, Canada. PP. 154 - 158.

Essers, A. J. A., Bennik, M. H. J., and Nout, M. J. R. (1995). Mechanisms of increased linamarin degradation during solid substrate fermentation of cassava. World Journal of Microbiology and Biotechnology. 11: 118 - 128.

FAO/ WHO. (2002). Guidelines for the evaluation of probiotics in food, Report of a Joint FAO/ WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada.

Holt, J.G., editor-in-chief. (1994). Bergey's Manual of Determinative Bacteriology. 9th edn. The Williams and Wilkins Company, Baltimore.

Javadi, A., Mirzaei, H., Safarmashaei, S., and Vahdat-pour, S. (2012). Effects of probiotic (live and inactive *Saccharomyces cerevisiae*) on meat and intestinal microbial properties of Japanese quails. *African Journal of Biotechnology*. 11(54): 12083-12087.

Khali, R. K. (2009). Evidence for probiotic potential of a capsule-producing *Streptococcus thermophilus* CHCC3534 strain. African Journal of Microbiology Research. 3 : 27 - 34.

Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. Journal of Clinical Infectious Diseases. 34 (4): 482 – 92.

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., and Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Journal of Dairy. 16: 189 - 199.

Maragkoudakis, P. A., Mountzouris, K. C., Psyrras, D., Cremonese, J., Fischer, J., Cantor, M. D., and Tsakalidou, E. (2009). Functional properties of novel probiotic lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Salmonella enteritidis*. *international journal of food microbiology*. 130 : 219 - 226.

Mathara, J. M., Schillinger, U., Guigas, C., Franz, C., Kutima, P. M., Mbugua, S. K., Shin, H. K., Holzapfel, W. H. (2008). Functional Characteristics of *Lactobacillus* spp. from Traditional Maasai Fermented Milk products in Kenya. International Journal of Food Microbiology. 126 (1) : 57 - 64.

Mattila-Sandholm, T., Matto, J., and Saavela, M. (1999). Lactic acid bacteria with health claims-interactions and interference with gastrointestinal flora. International Journal of Dairy. 91: 25-35.

Marteau, P., Pochart, S., and Pot, B. (2003). Probiotics as biotherapeutic agents: Present knowledge and future prospects. Journal of Current Pharmaceutical Distribution. 9 (2) : 175 - 91.

Morelli, L. (2000). *In vitro* Selection of probiotic lactobacilli: A critical appraisal. *journal of current issues and intestinal microbiology*. 1: 59-67.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., and Xu, J. (2011). Screening and characterization of non pathogenic probiotic lactobacilli from breastfed healthy babies in Pakistan. African Journal of Microbiology Research. 5(12) : 1428 - 1436.

Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., and Yadav, H. (2012). Probiotics. Their health benefits and application for developing healthier foods: A review. FEMS Microbiology. 334 : 1 - 15.

Noriega, L., Gueimonde, M., Sanchez, B., Margolles, A., and de los Reyes-Gavilan, C. G. (2004). Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross resistance to bile salts in *Bifidobacterium*. International Journal of Food Microbiology. 94 (1) : 79 - 86.

Olejnik, A., Lewandowska, M., Obarska, M., and Grajek, K. W. (2005). Tolerance of *Lactobacillus* and *Bifidobacterium* strains to Low pH, Bile Salt and Digestive Enzymes. *Electron Journal of Polish Agriculture University*. Volume 8.

Okafor, A. C., and Umeh, C. N. (2013). Studies on the probiotic properties of *Lactobacillus* species isolated from local raw cow milk. Pakistan Journal of Biological Sciences. (Accepted for publication). Accessed 22/06/2013.

Okafor, N. (1977). Microorganisms Associated with Cassava Fermentation for Garri Production. Journal of Applied Bacteriology. 41: 279 - 284.

Okafor, N. and Ejiofor, M.A.N. (1986). The microbial breakdown of linamarin in fermenting pulp of cassava. Journal of Applied Microbiology and Biotechnology. 2 (2) : 327 – 338.

Oyetayo, V. O., and Oyetayo, F. L. (2005). Potentials of Probiotic as Biotherapeutic agents Targeting the Innate Immune System: a review. *African Journal of Biotechnology*. 4(2): 123-127.

Oyewole, O. B., and Odunfa, S. A. (1988). Microbiological studies on cassava fermentation for 'lafun' Production. Journal of Food Microbiology. 5(3): 125-133.

Soccol, C. R., Vanderberghe, L. P., Spier, M. R., Medeiros, A. B., Yamaguishi, C. T., Lindner, J. D., Pandey, A., and Soccol, V. T. (2010). The Potential of Probiotics: A Review. Journal of Food Technology and Biotechnology. 48 : 413 - 434.

Ukeyima, M. T., Enujiugha, V. N., and Sanni, T A. (2010). Current applications of probiotic food in Africa. African Journal of Biotechnology. 9(4) 394-401.

Vinderola, G., Capellini, B., Vilarreal, F., Suarez V., Quiberoni, A., and Reinheimer, J. (2008) Usefulness of a set of simple *in vitro* tests for the screening and identification of probiotic candidate strains for dairy use. LWT-Food Science Technology. 41: 1678 - 1688.

Zhou, J. S., Pillidge, C. J., Gopal, P. K., and Gill H. S. (2005). Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. International Journal of Food Microbiology. 98 : 211 - 217.

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