Microbiological Screening of a Starter Culture of Probiotic Status from Formulated Non-Dairy Yoghurt Analogue from Natural Fermentation of Soymilk-Achamilk Blends

[1]Mbaeyi, I. E., [2]Ogbonna, J. C. and [3]Onwuka, N. D.
[1, 2]Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[3]Department of Food Science and Technology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Corresponding author: Mbaeyi, I. E. Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria. **Email:** miphie2003@yahoo.co.uk, ifeoma.mbaeyi-nwaoha@unn.edu.ng

Abstract

Non-dairy probiotic yoghurt analogue was formulated using cream variety of soybean (Glycine max L.) and white variety of acha (Digitaria exilis). One kilogram of cleaned soybean and 500grams of cleaned acha were used to produce soymilk and acha milk. Soymilk and acha milk were blended in different ratios (100:0; 90:10; 80:20: 70:30; 60:40; 50:50; 0:100), respectively. The various blends were pasteurized at 80^0C for 30 minutes, cooled to 44 ± 2^0C fermented naturally for 8 hours until desired degree of acidity was achieved, cooled rapidly to 8 ± 2^0C, packaged in sealed plastic bottles and stored in the refrigerator. Microbiological analyses were done on the isolates obtained from incubation in an anaerobic jar at 37^0C for 48hours. The isolates were stored in sterile agar slants after purification by successive streaking on MRS and ST agar before being subjected to characterization. Microbiological screening (morphological, physiological and biochemical tests) was done to identify characterized a working isolate of probiotic status. Further assessment of performance of probiotic strains {acidifying activity and exopolysaccharide (EPS) production} were performed. Results show that the isolates were Gram positive, catalase negative, oxidase negative and non-sporing. A total of 7 isolates produced lactic acid. Among the 5 rods,3 were able to grow at 45^0C ,produced acid and gas from glucose (characteristic of Lactobacillus spp.);2 isolates grew between 15 and 37^0C,at 4%NaCl and pH of 9.6 (characteristic of Streptococcus spp) while 2 isolates grew between 15 and 42^0C, produced acid from glucose and lactose, citrate positive (characteristic of Lactococcus spp). All the isolates showed tolerance to 5% bile and 3.0% NaCl (inhibitory substances) .There was no significant difference ($p>0.05$) in the acidifying activities of Lactobacillus, Lactococcus and Streptococcus species. After 6 hours of incubation, an increase in pH from 4.78 to 5.87(for Lactobacillus spp), 4.12 to 5.86(for Lactocccus spp) and 4.20 to 5.60(for Streptococcus spp) were observed. Lactococcus spp showed an acid value of $0.29g100m^{-1}$lactic acid after 6 hours and increased from 0.40 to 0.75g $100ml^{-1}$ after 12 and 24 hours respectively. These isolates were used for the formulation of probiotic yoghurt analogue.

Keyword: Microbiological screening, Starter culture, Probiotic status, Non-dairy yoghurt analogue, Soymilk-achamilk blends

Introduction

Fermented products are mainly used in human diet in developing and developed countries, which have been derived from plant or animal materials. It is an acceptable and essential part of diet in most parts of the world for several centuries. According to Hassan and Amjad (2010), yoghurt is one of the oldest fermented products known. Fermentation of milk involves the action of micro-organisms, principally the lactic acid bacteria. These microorganisms convert milk lactose into lactic acid and make milk sour (Kagan, 1985). The popularity of yoghurt is due to its characteristics; the pleasant aromatic flavor, thick creamy consistency and its reputation as food associated with good health (Domagla, 2005).

The acidity of yoghurt varies from 0.7 to 1.1%lactic acid with pH approximately 4.0 to 4.2 (Wanda and Salauen, 2005).Yoghurt is more nutritive than milk in vitamin contents for its digestibility. It is also used as source of calcium and phosphorus. It is believed that yoghurt has valuable therapeutic properties and helps in curing gastrointestinal disorders (Adolfsson, 2004; Perdigon, 2005). The specific health benefits depend on the strain and viability of the culture in yoghurt (Miller *et al*., 2008; Saudy, 2008). Probiotic bacteria are completely non-toxic and have been consumed as part of cultured food such as yoghurt.

In present days, most yoghurt is prepared by either using special lactic acid producing organism or by direct acidification of milk by acidulant (Nobuo, 2002). High quality yoghurt with a pleasant taste depends very much on the ratio of two bacterial species: *Streptococcus thermophilus* and *Lactobacillus bulgaricus (*Fuller, 1989*;* Abubakar *et al.,* 2005*).* However, the diary protein composition is known to influence the structure and texture character of yoghurt (Saint *et al.,* 2006). *Lactobacillus* species are employed in the production of a wide range of fermented milk, meat and plant products and are also routinely isolated from the vagina and gastro-intestinal tract (Callanan *et al.,* 2008).

Isolation of exopolysaccharide-producing Lactobacillus cultures from traditional fermented foods of Thailand (Smitinont *et al.,* 1999), in oat-based nondairy milk substitutes (Marensson *et al.,* 2000), Burkina Faso fermented milk (Savadogo *et al*., 2004), fermented green olives (Sanchez *et al*., 2006) and a traditional fermented dairy product, 'dahi' (Vijayendra *et al*., 2009) was reported.

Bacterial starters have also been produced for a variety of fermented products to improve their sensory and other quality characteristics (Saeed *et al*., 2009). Natural or spontaneous fermentation has been used for the production of fermented foods based on the microflora preset in the raw material(Vogel *et al.,* 2002).The quality of end products was dependent on the types and numbers of microorganism in the raw material. Natural fermentation was optimized through back slopping (that is the inoculation of the raw material with a small quantity of a previously performed successful fermentation) which means dominance of the best adapted strains (Harris,1998). The direct addition of selected starter cultures to raw materials is a milestone in the production of fermented foods which may help control the overall standardization of the fermentation process and quality of the end product. Strains with specific physiological and metabolic properties were isolated from natural habitats or from successfully fermented products for use in the industrial productions (Oberman and Libudzisz, 1998).

Lactic acid bacteria (LAB) are a group of Gram positive bacteria, catalase–negative, non-motile, non-spore forming rods or cocci and produce lactic acid as the major end product during fermentation. They are strictly fermentative, microaerophilic, acidophilic, salt-tolerant with complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives and vitamins. The natural habitat of these organisms includes humans, animals and plants. Their long history of safe use (Holzapfel *et al.,* 2001), commonly referred to as the GRAS (Generally Recognized as Safe) status ,has led to a wide range of industrial application ,that is flavour, texture and preservative qualities of many fermented foods such as yoghurt, cheese, sausages and sour bread. The overall flavour of yoghurt is affected by the content of lactic and acetic acids. Homofermentative lactic acid bacteria are able to convert hexoses into lactic and acetic (ethanoic) acid and CO_2. The temperature also affects the ratio of lactic to acetic acid in addition to type of starter culture. Lactic and acetic acids are also produced by heterofermentative lactic acid bacteria from pentoses (Hammes and Vogel, 1995).

Lactic acid fermented foods have made up a significant portion of food taken by humans for a long time and still do in many developing countries in Africa(Molin,2001). Lactic acid fermentation is the simplest and often the safest way of preserving food and before the industrial revolution. Lactic acid fermentation was use just as much in Europe as it still is in Africa. In this way, humans consumed large numbers of live lactic acid bacteria and presumably those associated with milk. Thus, it may well be that the human gastrointestinal tract evolved to adapt to a more or less daily supply of live lactic acid bacteria . This supply ceased in industrialized countries during the 20th century, which may have led to intestinal problems, perhaps even immunologically dependent ones.

The starter cultures for the production of fermented products are not presently produced in Nigeria and are imported for industrial use. The use of lactic acid bacteria as starter helps to improve the quality and shelf life of the products. The lactic acid bacteria of the naturally fermented dairy yoghurt may be used in the production of novel non-dairy yoghurt of probiotic status, which is likely to have superior quality and longer shelf life. Therefore, the present study was designed to isolate and characterize the suitable starter culture for the production of non-dairy probiotic yoghurt.

Materials and Methods

Procurement of strains: Bacterial strains were isolated from the natural fermentation of the formulated yoghurt analogue from soybean-acha blends in different ratios.

Growth media of bacterial strains: deMan Rogosa Sharpe (MRS) agar CM 361 and broth (M17) both manufactured by Oxoid usually designated for anaerobes was measured out, weighed and autoclaved before use.

Isolation and characterization of microorganisms: Lactic acid producing bacteria were isolated from the formulated yoghurt analogue. Serial ten-fold dilutions of the yoghurt analogue samples were prepared. A 0.1ml aliquot of 10^{-5} dilution was spread-inoculated on sterile plates of deMan Rogosa Sharpe agar CM361 (Oxoid). The plates were incubated anaerobically in an anaerobic jar (containing a gaspak used to evacuate all traces of oxygen thereby creating an environment having only carbon IV oxide). The incubation was done for 24-72hours. Several developed colonies were repeatedly sub-cultured to obtain pure isolates anaerobically.

Identification of working isolate: The taxonomic identification of the working isolate was based on its colony and morphological characteristics and standard biochemical tests as described in the identification schemes of Holt *et al.*(1994). The colony morphological characteristics of the isolates were examined by observing the colonies on MRS agar plates while chromogenesis for the morphological characteristics, an 18-hour, MRS broth culture of the isolate was used in making a hanging drop preparation to determine the shape and motility of the isolate. The morphology was further determined by the reaction of the isolate to Gram Stain and all were viewed microscopically.

Gram's staining methods: Gram's stain was done using the method described by Harrigan and McCance (1976). A heat-fixed smear was prepared from an 18-24 hours culture in the usual way from MRS broth and stained with crystal violet solution for 1-2 minutes. Then, rinsed rapidly with water and Gram's iodine solution was added and left for 1 minute. The iodine was poured off and blotted dry. The slide was washed with 95% ethanol (or industrial methylated spirits) until no more violet stain runs from the slide (only 5-15 seconds in the case of well-prepared thin smears).The slide was rinsed under the tap and stained with dilute carbol fuschin solution for 20 seconds. The slide was washed well and blotted dry. The slide was viewed under a microscope using oil immersion.

Motility test: The motility of isolate was determined using the method described by Harrigan and McCance (1976).A small drop of suspension was placed on a slide and covered with a cover slip (glass). The preparation was not made too thick. It was advisable to seal the preparation with nail varnish or molten petroleum jelly to prevent it from drying out. The preparation was examined microscopically for motile organisms using the 10 x and 40x objectives. The movement of small motile bacteria must be distinguished from the on-the-spot vibratory movement (Brownian movement) which is shown by all micro-organisms and particles when suspended in a fluid.

Slime production: For slime production, strains were inoculated on the suitable media (MRS broth) and incubated at 42^0C for 24 hours. Ropiness of colonies was tested on agar surfaces were tested with a loop to observe the formation of slime (Zambou *et al.*, 2007).

Biochemical tests on the working isolates: The ability to utilize certain substrates based on the presence (or absence) of certain enzymes are characteristics important in the systematic studying for the identification of bacteria (Jideani and Jideani, 2006). The biochemical tests carried out to identify the isolates included the following: voges-proskaeur, indole, citrate, growth in NaCl (of different percentages), growth on sugars (namely glucose, sucrose, lactose, mannitol, maltose) and production of enzymes (namely oxidase, catalase).

Catalase test: Catalase test was done using the method described by Harrigan and McCance (1976). About 2-3ml of the hydrogen peroxide (H_2O_2) solution was poured into a test tube. Using a sterile wooden stick or a glass rod, a good growth (a loopful) of the test organism was removed and immersed it in the H_2O_2 solution. The presence or absence of immediate bubbling (effervescence caused by the liberation of free oxygen as gas bubbles indicate the presence of catalase in the culture under test) was looked for. Active bubbling (positive test) showed catalase was produced as shown in the equation below while no release of bubbles indicate negative test and no catalase was produced. $2H_2O_2 \rightarrow 2H_2O + O_2$

Indole test: Freshly prepared Kovac's indole reagent was used. Two grammes of 4-dimethylaminobenz-aldehyde was dissolved in 30ml of isoamyl alcohol (3-methyl-1-butanol) solution. Then 10ml of concentrated

hydrochloric acid was added and mixed well according to Harrigan and McCance (1976). A loopful of the pure isolates was grown in sterile peptone water for 48 hours at 37^{0}C. The 2-day old cultures were used by the addition of 0.2ml freshly prepared Kovac's reagent shaken and allowed to stand for 5 minutes. The appearances of a red coloration on the amyl alcohol layers indicate positive indole test (that is a decomposition of tryptophan to indole). A negative test remains as a yellow layer on the interface.

Test for sugar fermentation (triple sugar iron agar): About 65g of the dehydrated TSI medium was dissolved in 1 litre of distilled water and homogenized by boiling. The medium was mixed well and dispersed into cotton-plugged test tubes. These tubes were sterilized by autoclaving at 151b pressure and 121^{0}C for 15 minutes. The medium was allowed to set in sloped form with a butt of about 1inch long. The slants were inoculated with test isolate by streaking the slope and stabbing the butt. The test tubes were incubated at 37^{0}C for 18-24 hours. The results were taken immediately after incubation as follows:

- Yellow slant with yellow butt → Fermentation of lactose and glucose by bacteria
- Red slant and yellow butt → Fermentation of glucose and not lactose and sucrose

Voges-Proskaeur test: The Voges-Proskaeur test was done. Glucose phosphate peptone water was used as the base. The isolates were inoculated into this medium and incubated for 72 hours at 37^{0}C. Five millilitres of 40% potassium hydroxide was added. A knife edge or trace of creatinine was then added and shaken well. A deep pink colour within 5 minutes indicated a positive test of butanediol fermentation. A negative test was detected by a faint pink colour after 5 minutes.

Citrate utilization: The Simon citrate medium was used. About 23g of the dehydrated medium was suspended in one litre of distilled water and homogenized. Eight millilitres of the medium was dispensed into test tubes plugged with cotton wool and kept in slants. Pure isolates were inoculated into the sterile medium and incubated at 37^{0}C for 24-48 hours. Growth and blue coloration show positive test because of the change in indicator colour from green to blue indicating the use of the citrate as sole carbon source. No change in the colour of the medium (green) and no growth indicate a negative test.

Oxidase test: One percent of oxidase reagent was prepared by the dissolution of 0.1g of tetramethyl phenylenediamine hydrochloride in 10ml of distilled water. A drop of the freshly prepared oxidase reagent was added to a 24-hour old culture agar plates. A positive test was observed by the production of a purple colouration within 5-10 seconds while a delayed purple colour (10-16 seconds) indicated negative test.

Preliminary investigation/identification of fermenting strains for probiotic yoghurt analogue from soymilk-'acha' milk blends: For the isolation and identification of the strains, the milk samples were incubated at these respective temperatures. Coagulated samples were then streaked on MRS agar (Oxoid) according to DeMan *et al*. (1960) and were used for the isolation of the lactic acid bacteria (LAB) such as *Lactobacillus* while M17 was used for *Lactococcus* (Terzaghi and Sandine, 1975). The streaked samples on MRS were incubated under anaerobic conditions using the Gas Pak Plus Anaerobic System (Beckon Dickinson Microbiology System, Cockeyville, MD, USA) at 30, 37 and 42^{0}C, respectively, for 48 hours. The isolates were purified by successive streaking on the appropriate agar media before being subjected to characterization. The purified isolates were stored at –20^{0}C in sterile formulated vegetable milk containing analytical lactose (12.5% w/v) for preservation and further analysis. (Notably, the storage of animal milk is usually in 12.5% w/v re-constituted Skim milk containing 15% glycerol for further analysis Working cultures were also kept on MRS agar slant at 4^{0}C and restreaked every 4 weeks (Samelis *et al*., 1994; Herrero *et al*., 1996).

Selection of isolates after preliminary identification: Isolates were selected on the basis of Gram reaction, morphology, catalase activity and spore formation (Harrigan and McCance, 1976). Catalase-negative and gram positive rods and cocci were selected and screened for 2-5 days; arginine hydrolysis was performed on selected strains according to Schillinger and Lucke (1987). Growth at different temperature 15, 37 and 45^{0}C was observed in MRS agar and broth, respectively, after 5 days of incubation and 12 days at 4 and 10^{0}C (Schillinger and Lucke, 1987; Samelis *et al*., 1994). For coccoid strains, growth on *Streptococcus thermophilus* (ST) broth medium and in the presence of 6.5%

NaCl was also considered. The ability to grow at pH 3.9 and 9.6 was tested on MRS agar and broth. Citrate Utilization in the presence of carbohydrates was performed on the media of Kempler and McKay (1980). Acetoine production from glucose was determined by Voges-Proskauer test (Harrigan and McCance, 1976).

Phenotypic characterization of the isolates: The carbohydrate fermentation was analyzed in a MRS broth with bromocresol purple mixed at 0.02% as a pH indicator according to the procedure described by Cheriguene *et al.* (2006). Sugars were sterilized by filtration and added to make final concentration of 1% (w/v). Samples were incubated at 37^0C for 48 hours on MRS broth without glucose but supplemented at a final concentration of 1% (w/v) with fructose, galactose, glucose, mannitol, lactose and sorbitol. To ensure anaerobic conditions, each tube was supplemented with two drops of sterile liquid paraffin after inoculation.

Further characterization of 25% of the isolates was carried out using the API 50 CH System (API System bioMerieux, Marcy L'Etoile, France). Cells used as inoculum were prepared as recommended by the manufacturer. The results were recorded after 24 and 48 hours and interpreted using the APILAB Plus computer-aided identification program (bioMerieux). A percentage correct-identification (%Id) value was obtained for each strain and standard taxonomic descriptions from Wood and Holzapfel (1995).

Performance tests for the isolate acidifying activity: Acidifying activity of the strains was measured according to the International Dairy Federation (IDF) standard 306(IDF, 1995; Allonso-Calleja *et al.*, 2002). The strains were subcultured in MRS broth at 30^0C for 24 hours. The microbial culture was inoculated at a level of 1millilitre per 100millilitre in sterile formulated soymilk-acha milk blends containing analytical lactose 10% (w/v). Titrable acidity and pH were determined after 6, 12 and 24 hours of incubation at 30^0C.

Microbiological screening for the selection of a working isolate: A working isolate was screened for from the formulated probiotic yoghurt analogue obtained from the fermentation of blended samples of soymilk and 'acha' milk in different ratios [SOAC (0:100; 100:0; 90:10; 80:20; 70:30; 60:40; 50:50)] and a commercial yoghurt(Farm Fresh Yoghurt) which served as the positive control.

Isolation of the micro-organisms: One millilitre of each sample was pipetted aseptically into nine millilitre of sterile, peptone physiological saline solution [1% peptone (oxoid), 0.9% NaCl, pH 7.0]. Serial dilutions (10^{-1} to 10^{-8}) were made for each sample and 0.1millilitre of the appropriate dilutions (10^{-3} and 10^{-4}) were spread plated on deMan, Rogosa, Sharpe [MRS agar CM361 (Oxoid)] which was used for the enumeration and isolation of the lactic acid bacteria (*Lactobacillus* or *Lactococci species*). The plates were incubated anaerobically for 24-72 hours at 42^0C in a carbon IV oxide jar (Anaerobic jar). A gaspak systems (Gener box anae indicator Biomerieux) was used evacuate every trace of oxygen. Isolations were obtained randomly from the countable plates of MRS and purity was checked by successive streaking on the same media and stored on slants (of the same media) at 4^0C for further investigation.

Identification of lactic acid bacteria (lab) to the genus level: The bacteria were characterized by microscopic examination and by conventional biochemical and physiological tests (Zeinab *et al.*, 2008). The cultures were examined for colony and cell morphology, motility, Gram stain and production of acid from glucose (Harrigan and McCance, 1976), in addition to the oxidation and fermentation tests according to Hugh and Leifson (1953).

Identification of *lactobacilli:* After their microscopic examination, Gram positive and catalase negative, *Lactobacilli* were tested for their sugar fermentation pattern, production of ammonia from arginine in addition to their ability of growth at 15^0C and 45^0C according to Harrigan and McCance (1976).

Identification of *streptococci* group: The cocci strains were grown on ST (*Streptococcus thermophilus*) broth at 42^0C (Ronald *et al.*, 1996). Streptococci were further identified by their production of carbon IV oxide (CO_2) from glucose using Gibson Litmus milk, production of acid from sugars and production of ammonia from arginine (Harrigan and McCance, 1976). The salt tolerance test was done using MRS broth (Oxoid) containing 4% (w/v) NaCl with anaerobic incubation. Growth at pH 9.2 and at 40^0C was determined in MRS broth.

Selection of probiotic strains: The selection of the probiotic strains for the formulated probiotic yoghurt analogue was carried out in according with biosafety aspect, bacterioscopy

(morphology), Gram staining, viability of strains kept in deMan, Rogosa Sharpe (MRS) agar while stored at 4-7^{0}C (Reque *et al.*, 2000), bile tolerance (5%) and best survival rate at room temperature

Biochemical Characterization of Screened Working Probiotic Isolate/Strain

Gas production from glucose: Overnight isolated cultures were inoculated at 10% (v/v) in MRS broth containing inverted Durham tubes and incubated at 37^{0}C. The gas production from glucose was observed after 24 hours (Davis, 1955).

Growth at different temperatures: Overnight isolated cultures were inoculated at 10% (v/v) in MRS and incubated at 15^{0}C, 37^{0}C, 40^{0}C, 42^{0}C and 45^{0}C for 24 hours. Total populations were determined by the pour plate method, incubating the plates at 37^{0}C for 48 hours (Buchanan and Gibbons, 1974).

Tolerance to inhibitory substances: Inhibitory substances such as bile (Sigma), phenol (Merck) and sodium chloride (Biotec) were tested. MRS broth containing 5% bile, 0.4% phenol and 5% and 6.5% sodium chloride (Davis, 1955) were determined by inoculation at 10% of the overnight isolated cultures and incubated at 37^{0}C for 24 hours. Total populations were determined by the pour plate method, incubating the plates at 37^{0}c for 48 hours.

Fermentation of Different Carbon Sources: A suspension was made in the medium with the microorganism to be tested and each tube of the strain was inoculated. During incubation, carbohydrates were fermented to acids, which cause a decrease in the pH, detected by the colour change of the indicator (Biomerieux, 2005). The carbohydrate fermentation profiles of the purified isolates were determined using API 50 CH system (Biomerieux, 2005). Interpretations of these fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studies with information from the computer-aided database API LAB Plus v3. 2.2. (Zambou *et al.*, 2007).

Assessment of technological performance of strains acidifying activity: The acidifying activity was determined by Zambou *et al.* (2007) procedure. Before use, the *Streptococcus* and *Lactobacilli* strains/cultures were propagated twice in ST broth and MRS broth, respectively and incubated at 42^{0}C for 16 hours. Seventy millilitres of broth media (MRS) for rods and ST broth (for cocci) were inoculated with 10% overnight, activated subcultures of LAB strains. The growth of cultures in broth media was monitored at 650nm using Spectrophotometer (Pharmacia LKB NOVASPEC II) for 6 hours. At the early stationary growth phase, bacterial cells were harvested from the media by centrifugation of the culture (4000g, 10 minutes, 4^{0}C). The cell pellet was washed twice with 10ml ringer solution and suspended in sterile reconstituted skim milk (12.5w/v) and formulated probiotic yoghurt analogue containing analytical lactose (12.5w/v). This mixture was stored in ice at 4^{0}C overnight. Sterile reconstituted skim milk and the formulate probiotic analogue containing analytical lactose was inoculated with 2% of the cell pellet suspended in milk/ milk analogue. The change in pH was determined using pH meter (Microcomputer pH-vision, model 05669.20) during six hours incubation at 42^{0}C. The variation of pH (ΔpH) was calculated as the difference between the value immediately after inoculation and values at different time. The cultures were considered fast, when change in pH (ΔPH) of 0.4 unit was achieved after 3 hours.

Exopolysaccharide (EPS) production: The screening of the exopolysaccharide production was performed on the strains showing weak pellet formation after centrifugation according to the procedure described by Zambou *et al.* (2007). The screening of exopolysaccharide consisted to reveal the presence of diffuse capsules surrounding bacterial cells as described by Prescott *et al.* (1996). For slime production, strains were streaked on the suitable media (MRS or ST) and incubated at 42^{0}C for 24 hours. Ropiness of colonies on agar surfaces was tested with a loop to observe the formation of slime.

Technological properties of fermented milk and yoghurt manufactured with single or mixed selected strains of probiotic status: The isolates from the fermentation of soymilk-acha milk blends for yoghurt analogue were selected on their acidifying and exopolysaccharide (EPS) production abilities according to the procedure described by Zambou *et al.* (2007). The strains, stored at –20^{0}C, were reactivated in ST broth and MRS broth and incubated overnight at 42^{0}C. Inocula were prepared by incubating cultures in 12.5% reconstituted skim milk and formulated probiotic yoghurt analogue containing analytical lactose for 16 hours at 42^{0}C and the freshly curdled cultures were used for the

preparation of fermented soymilk-acha milk blends into probiotic yoghurt analogues.

Results

Isolation, characterization and identification of starter cultures: Table 1 shows the isolation, characterization and identification of starter culture from the natural Fermentation of soymilk- 'acha' milk blends in the formulation of probiotic yoghurt analogue

Microbiological screening for the selection of a working isolate: The results for the isolation and characterization of the starter culture from the natural fermentation of soymilk-'acha' milk blends used in the formulation of probiotic yoghurt analogue are presented in Table 1. The data obtained constitute the profile of the strains and are also used for the identification or determination of their genus. All strains reacted positively to Gram straining (based on the bacillary morphology. The morphological, physiological and biochemical tests revealed a diversity of lactic acid bacteria (LAB) which were classified based on the genera-*Lactococcus*, *Lactobacillus* and *Streptococcus*. All the strains/ isolates were Gram positive, catalase negative, oxidase negative and non-spore forming.

Five isolates of lactic acid bacteria were isolated from the formulated probiotic yoghurt analogue from soymilk-'acha' milk blended in difference ratios (Table 1). The seven isolates obtained from MRS plates were grouped as lactic acid bacteria according to the fact that they were Gram positive, catalase negative, oxidase negative and non-spore forming rods and cocci. Among the suspected lactic acid bacteria isolated, 2 isolates produced gas from glucose. Of the 5 rods, 3 were able to grow at 45^0C and were tentatively identified as *Lactobacillus* spp while from the tentative identification of *Streptococcus,* 2 isolates were able to grow at 4% NaCl and at pH 9.6. In relation to the inhibitory substances, the isolates showed better tolerance to 5% bile, 3.0% NaCl but not 6.5% NaCl when compared with the control (farm fresh yoghurt) grown on MRS and M17 and these characteristics compared with other researchers.

Among the cocci, 2 isolates were able to grow at between 10^0C and 42^0C but not at 45^0C and at a pH 9.6 broth. These strains did not survive beyond 45^0C for 30 minutes. About 2 isolates could grow in 3 and 5% but not in 6.5% NaCl broth, produced acid from glucose lactose but acid production from sorbitol was negative.

Two isolates of rods were classified as belonging to the genus *Lactobacillus* as suggested by their sugar fermentation patterns. All strains fermented lactose, sucrose and glucose. There was an evidence of acid and gas production from glucose. These isolates were also classified by their incapacity to grow below 10 and characterized by their ability to form acid from most of all the sugars. Further characterization was done by their sensibility to NaCl and their inability to ferment sorbitol. Two isolates were considered as species to their ability to grow between 15 and 30^0C because they are heat-resistant. These organisms (species) were included in starter cultures during the production of yoghurt and cheese manufacture according to literature. In this present study, results of acid-producing activity of the isolates are presented in Table 2. No significant (p>0.05) differences in the acid-producing ability was found between the strains even within the same species. After 6 hours of incubation, a decrease in pH among the isolates ranged from 4.12 to 5.87. All these strains were described as highly acidifying since the variation of pH by 0.4 units were achieved within 4 hours.

The genus *Lactococcus* had those that showed the highest acidifying capacity, developing an acidity of 0.29g $100ml^{-1}$ lactic acid after 6 hours and 0.40-0.75g $100ml^{-1}$ after 12 hours (Table 2). After 24 hours, the pH of the culture medium decreased to values lower than 4.1 and then the acidity reached values around 0.7-0.8 $g100ml^{-1}$. Basically, most of the differences with respect to acidifying capacity were observed after 6 hours. All the *Lactobacillus* isolates had similar behaviour after 24 hours, although 2 isolates showed the highest acidifying capacity throughout the incubation time. *Lactobacillus* isolates showed a relatively low acidifying capacity, with average values of acidity of 0.31-0.55g $100ml^{-1}$ after 6 hours. The strains of this species showed differences after 12 and 24 hours. After 24 hours, one isolate with highest acidifying capacity.

Discussion

From these results, Lactobacilli metabolize lactose more slowly than *Lactococci* but the final acid production can be similar to or even higher than, that of the Lactococci. The isolates of Lactococcus from the formulated probiotic yoghurt analogue showed an acidifying activity after 6 hours of incubation

Table 1: Isolation, characterization and identification of starter cultures

Colony morphology	Presence of pigment	Gram stain reaction	Motility	Presence of capsule	Spore formation	Colony diameter (mm)	Slime production	Growth at 15^{0}C	Growth at 37^{0}C	Growth at 40^{0}C	Growth at 42^{0}C	Growth at 45^{0}C	Growth at NaCl(0.5%)	Growth at NaCl(1.0%)	Growth at NaCl(3.0%)	Growth at NaCl(5.0%)	Growth at NaCl(6.5%)	Bile tolerance (5%)	Acid/Gas production with glucose	Acid production with lactose	Acid production with mannitol	Acid production with sorbitol	NH_3 production with arginine	Catalase production	Oxidase production	Citrate utilization	Indole production	Voges-proskaeur reaction	Growth at pH of 3.9	Growth at pH 9.6	Probable organisms
Rod-shaped cells (straight) and convex	-	+	-	-	-	1.5	-	+	+	+	+	+	-	-	-	-	-	-	A,G	A	-	-	-	-	-	-	-	-	-	-	*Lactobacilli spp(3)*
Ovoid or spherical cells in pairs	-	+	-	-	-	0.7	-	+	+	+	-	-	-	-	+	+	+	+	A	A	-	-	-	-	-	+	-	-	-	-	*Lactococcus* spp((1)
Ovoid or spherical cells in short chains	-	+	-	-	-	1.0	-	+	+	+	+	-	-	-	-	+	+	+	A	A	A	-	-	-	-	+	-	-	-	-	*Lactococcus spp.(1)*
Spherical or ovoid cells in pairs or chains	+	+	-	+	-	1.3	-	-	+	+	+	-	-	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-	-	+	*Streptococcus spp.(2)*

Key: - = negative, + = positive, A = acid production, G = gas production

-

Table 2: Acidifying activity of some strains of lactic acid bacteria isolated from formulated probiotic yoghurt using soymilk +'acha' milk blends

	Strain	Incubation Time (Hours)					
		6		12		24	
		pH	TA*	pH	TA*	pH	TA*
I	*Lactobacillus spp*	5.87±0.01	0.31±0.03	5.28±0.01	0.48±0.00	4.78±0.03	0.55±0.01
II	*Lactococcus spp*	5.86±0.03	0.29±0.02	4.96±0.05	0.40±0.01	4.12±0.00	0.75±0.02
III	*Streptococcus spp*	5.60±0.01	0.31±0.02	4.45±0.02	0.67±0.00	4.20±0.01	0.75±0.01

*Key TA = Titrable acidity * = Titrable acidity expressed as g100ml^{-1} lactic acid*

similar to that detected for Lactococci isolated from milk cheeses by Requena *et al*. (1991) which suggested that strains of *Lactobacillus* whose acidifying capacity are higher than 0.25mg/100ml of lactic acid after 6 hours of incubation could be used as starter culture in cheese manufacture. The differences observed from one lactic acid bacteria species to another were explained by De Roissart (1986). In fact, the acidifying acidity of each strain is related to its specific capacity to break down the substances in the medium and render them capable of assimilation. In some occasions, differences are also due to the presence or absence of nutrient transport systems (Albenzio *et al*., 2001).

The results obtained were in accordance with the physiological and biochemical characteristics described by Kandler and Weiss (1986). According to Halm *et al*. (1997), Lactobacillus species could be isolated from cereal-based fermented product. Wardlow (1999) identified that the *Lactobacillus sp. (Lactobacillus acidophilus)* is used in the production of different types of fermented dairy products and could be utilized to correct lactose maldigestion and intolerance. Therefore, it could be used probiotic which upon ingestion exert health benefits beyond inherent basic nutrition (Wood, 1992). *Lactobacillus* sp. was similarly reported by Wood (1992) as obtained in this study.

The above identification results were confirmed by carbohydrate fermentation and assimilation profile obtained by API chart which correlated with Bergey's Manual (Teuber *et al*., 1986). Lactose and sucrose were fermented by all the isolates while the fermentation of glucose was strain/genus dependent. The fermentation of selected carbohydrates of the API 50 CH gallery (Biomerieux, 2005) was carried out, that is the catabolism of these sugars that results in the production of organic acid and detected by the pH turning point and titrable acidity.

Strain selection for industrial purposes must consider some specific technological activities such as acidification from the isolates, the lactic acid bacteria with probiotic potential were considered particularly *Streptococcus*, *Lactococcus* and *Lactobacillus*. Among the cocci, 7 isolates were able to grow at between 15^{0}C and 40^{0}C but not at 45^{0}C. According to other reports, *Lactococcus* spp. was more frequently isolated from raw milk samples (Moreno and Busani, 1990) and Dahi and buttermilk samples from India (Padmanabha-Reddy *et al*., 1994). According to Crow *et al.* (1993), Lactococcus spp. were isolated only from natural sources. According to WeerKamp *et al*. (1996), *Lactococci* isolated from natural sources were usually identified as *Lactococcus lactis subsp lactis* where as the phenotype *Lactococcus lactis subsp cremoris,* which is common in industrial mixed strain starter cultures, was isolated rarely (Beukes *et al*., 2001).

Streptococcus spp. play a vital role in commercial milk fermentation. Selected isolates were deliberately added as starters and their primary role is to ferment lactose to lactic acid (Gisele *et al*., 2006). *Streptococcus* isolates / strains are used in commercial milk fermentation in which the temperatures reached during manufacture are relatively high (up to 55^{0}C). This organism is normally used as part of mixed starter culture, the other component being *Lactobacillus spp.* for yoghurt. Data obtained from pure cultures do not consider phenomena related to interactions between microorganisms typical of mixed culture. Yoghurt is a simple ecosystem whose successful manufacture relies on interaction between two thermophilic lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbruecki bulgaricus*). This bacterial association had significant effect on acid production by strain of *Lactobacillus delbruecki bulgaricus,* possibly because of a production of formic acid by *Streptococcus thermophilus* isolate (Zourari *et al.,* 1992; Courting and Rule, 2004). In previous study, Veringa *et al*. (1968) showed that all strains of *Streptococcus thermophilus* produced some formate, which is considered important in stimulating the growth of *Lactobacillus delbruecki subsp bulgaricus* in yoghurt cultures. Similar reports were obtained in the

formulated probiotic yoghurt analogue with slight modifications.

Conclusion: The increasing public awareness about the health promoting properties of probiotic foods is a commendable development that should be promoted. In America, Europe and oriental countries particularly Japan where there exist the trained manpower and available technologies for commercial production of various types of probiotic (both dairy and non-dairy) foods, consumers normally access a variety of such foods. In Africa, there is also a growing awareness about the health promoting properties of probiotic foods and peoples of different regions in Africa produce foods containing probiotic organisms, though at small scale level. Therefore, there is the need to train manpower and develop technologies that would optimize production processes beginning from reliable characterization of the probiotic organisms to strain level. An exploitation of nondairy materials as potential carriers of the probiotic organisms would assuredly be a viable alternative to consumers who are either lactose intolerant or vegetarians.

References

Abubakar, M.M., Adegbola, T.A. and Oyawole, E.(2005). Determination of physiochemical, microbial and organoleptic properties of yoghurt .*Journal of Texture Studies* 6:333.

Adolfsson, O.(2004). Yoghurt and gut function. *American Journal of Clinical Nutrition* 80(2): 245-56.

Albenzino, M., Corbo, M.R., Rehman, S.U., Fox, M., De Angelis, A., Corsetli, A., Sevi, A and Gobetii, M. (2001). Microbiological and Biochemical Characteristics of cane strato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food Microbiology* 67: 35-48.

Allonso-Calleja, C.J., Caballo, R., Capita, A., Bernardo, A. and Garcia-Lopez, M.L. (2002). Comparison of the acidifying activity of *Lactococcus lactis subsp. lactis* strain isolated from goat's milk and Valdeteja cheese. *Letters in Applied Microbiology* 34: 134-138.

Beukes, E.M., Bester, B.H. and Mostert, J.F. (2001). The Microbiology of South African Traditional fermented milks. *International Journal of Food Microbiology* 63: 189-197.

Biomerieux (2005). API 50 CHL medium. http://courses.ag.uidaho.edu/fst/fstmmbb417/api50_CHLmedium.pdf

Buchanan, R.E. and Gibbons, N.E. (1974). Bergey's Manual of Determinative Bacteriology, 8th ed., Williams and Wilkins, Baltimore, 1268p.

Callanan, M.,K aleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAulliffe, O., Sangrador,-Vegas, A., Slattery, L., Fitzgerald, G.F., Beresford, T. and Ross, R.P.(2008). Genome sequence of *Lactobacillus helveticus,* an organism distinguished by selective Gene loss and Insertion sequence Element Expansion. *Journal of Bacteriology* 190(2): 727-735.

Cheriguene, A., Chougrani, F. and Bensoltane,A.(2006). Identification and Characterization of Lactic Acid Bacteria Isolated from Algerian Goat's milk. *Pakistan Journal of Biological Sciences* 9(7): 1242-1249.

Courting, P.and Rule, F. (2004). Interactions between microorganisms in a simple ecosystem: yoghurt bacteria as a study model. *Lait* 84: 125-134.

Crow, V.C., Coolbear, T., Holland, G.G., Pritchard, G.G. and Martley, F.G. (1993). Starters as finishers: Starter properties relevant to cheese ripening. *International Dairy Journal* 3:423-460.

Domagla, J.(2005).Texture of yoghurt, bio-yoghurt from goat's milk depending on starter culture type*. Milchwissenchraft* 60(3): 289-292.

Davis, G.H.G. (1955). The classification of Lactobacilli from the human mouth. *Journal of General Microbiology* 13(3): 481-493.

DeMan, H.B., Rogosa, M. and Sharpe, M.E. (1960). A medium for the cultivation of *Lactobacilli*. *Journal of Applied Bacteriology* 23: 130-135.

DeRoissart, H.B. (1986). Lactic Acid Bacteria. In: Milks and Dairy Products: Cow, Sheep and Goat. Ed. *Tec and Doc., Lavoisier, Paris*, 3: 343-408.

Fuller, R. (1989). Probiotic in man and animals. *Journal of Applied Bacteriology* 66: 365-378.

Gisele, I., Chammas, B., Rachad-Saliba, B., Corrieu, G. and Beal, C. (2006); Characterization of lactic acid bacteria isolated from fermented milk laban. *International Journal of Food Microbiology* 110: 52-61.

Halm, M., Lille, A., Sorensen, A.K. and Jakobsen, M. (1997). Microbiological and aromatic characterization of

fermented maize dough for Kinkey production in Ghana. *International Journal of Food Microbiology* 19: 134-143.

Harmes , W.P. and Vogel, R.F.(1995). The genus of Lactobacillus: The genera of lactic acid bacteria, Vol.1 ,Blackie Academic Professional, London, pp 19-54.

Harrigan, W.E. and McCance, M.E. (1976). Basic methods In: Laboratory Methods in Food and Dairy Microbiology Academic Press, London; pp 3-16, 78.

Harris,L.J.(1998). The Microbiology of vegetable fermentations: Microbiology of fermented foods,Vol.1. Blackie Academic Professional ,London, pp 45-72.

Hassan,A. And Amjad, I.(2010). Nutritional evaluation of yoghurt prepared by different starter cultures and their physiochemical analysis during storage. *African Journal of Microbiology Research* 4(1): 022-026.

Herrero, M.B., Mayo, B., Gonzalez, B. and Suavez, J.E. (1996). Evaluation of Technologically Important strains in lactic acid bacteria isolated from spontaneous fermentation. *Journal of Applied Bacteriology* 82: 565-570.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology, 9th Edn., William and Wilkins, Baltimore, pp 565-596.

Holzapfel, W.H. Haberer, P., Bjokroth, J. and Schillenger, U.(2001). Taxonomy and important features of probiotic microorganisms in food and nutrition . *American Journal of Clinical Nutrition*73: 365-373.

Hugh, R. and Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. *Journal of Bacteriology* 66: 24.

International Dairy Federation (IDF), (1995). IDF Guideline-Determination of acidifying activity of dairy cultures. Bulletin IDF 306, Brussels, Belgium.

Jideani, V.A. and Jideani, T.A (2006). Biochemical tests for identification of unknown bacteria. In: Laboratory Manual of Food Bacteriology, Amana Printing and Advertising Ltd, Kaduna, Nigeria pp 132-163.

Kagan, J.(1985). Yoghurt –Arising star in the dairy industry. *Culture Dairy Production Journal* 20(1):24-29.

Kandler, O. and Weiss, N. (1986). Genus Lactobacillus. In: Bergey's Manual of Systematic Bacteriology. 3rd edn., Sneath, P.A. and Holt, J.G. (Eds), Williams and Wilkins, Baltimore, pp 1209-1234.

Kempler, G.M. and Mckay, L.L.(1980). Improved medium for detection of citrate-fermenting *Streptococcus lactis subsp diacetylactis Applied Journal of Environmental Microbiology* 39:926 - 927.

Marensson,O. Oste,R., and Holst,O.(2000). Lactic acid bacteria in an oat-based nondairy milk substitute: Fermentation characteristics and exopolysaccharide formation. *LWT Food Science and Technology* 33: 525-530.

Miller, G.D.(2008). Benefits of dairy product consumption on blood pressure in humans. *Journal of American College of Nutrition* 19:25.

Molin,G.(2001). Probiotics in foods not containing milk or milk constituents with special reference to *Lactobacillus planturum 299v. American Journal of Clinical Nutrition 73(supplement):* 380s-385s.

Moreno, I. and Busani, S.F.B. (1990). Characterization of *Lactococci* isolated from raw milk and commercial lactic starters. *Coletanea do Instituto de Technologia de Alimentos* 20: 44-50.

Nobuo, J.(2002). Manufacturing of frozen yoghurt with smooth mouthfeel. *Journal of American College of Nutrition* 19(5): 277-281.

Oberman, H and Lidudzisz,Z.(1998). Fermented milks: Microbiology of fermented foods,Vol.1. Blackie Academic Professional, London, 308-350.

Padmanabha-Reddy, V., Habibulla-Khad, M.M. and Purushothaman,V.(1994). Plasmid linked starter characteristics in Lactococci isolated from dahi and buttermilk. *Culture Dairy Production Journal* 29: 25-30.

Perdigon,G.(2005).Anti-t.umor activity of yoghurt. Horizons in cancer research Nova science publications, pp97-122.

Prescott, L.M., Harley, J.P and Klein, D.E. (1996). The study of Microbial, Structure, Microscopy and Specimen Preparation. In: Microbiology, 7th ed. McGraw-Hill, New York, pp 17-36.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, D.K. (1984). Legume-based fermented foods: their preparation and

nutritional quality: *Critical Reviews in Food Science and Nutrition* 17: 335.

Reque, E.F., Pandey, A., Franco, S.G. and Soccol, C.R.(2000). Isolation, Identification and Physiological Study of *Lactobacillus fermentum* LPB for use as probiotic in chickens. *Brazilian Journal of Microbiology* 31: 303-307.

Requena, T., Pellaez, C and Desmazeaud, M.J. (1991). Characterization of *Lactococci* and *Lactobacilli* isolated from semi-hard goats' cheese. *Journal of Dairy Res.* 58: 137-145.

Ronalds, M., Atlas, C. and Lawrence, P.(1996). Handbook of Microbiological Media, 2nd edn., Atlas Publishing Co. Ltd, London.

Saauen, F. And Bernard, M.(2005). Buffering capacity of dairy products .*International Dairy Journal* 15(2): 95-109.

Saeed, M., Anjum, F.M., Zahoor, T. Nawaz, H. And Ur-Rehman, S.(2009). Isolation and characterization of starter culture form spontaneous fermentation of sourdough. *International Journal of Agriculture and Biology* 11:329-332.

Saint A., Juteau, A., Allan, S., Martin, N. And Souchon, I.(2006).Influence of proteins on the proteins on the perception of flavoured stirred yoghurts. *Journal of Social Dairy Technology* 30:31-32.

Saundy ,E.(2008). Probiotics in yoghurt may aid gut health. Digestive diseases and Sciences. Maryvale Hospital Medical Center, Phoenix, 48(10): 2085-2091.

Samelis, J., Maurogenakis, F. and Metaxopoulos, J. (1994). Characterization of lactic acid bacteria isolated from naturally fermented Greeck dry salami. *International Journal of Food Microbiology* 23: 179-196.

Sanchez, I., Martinez, B. Guillen, R., Jimmenaz-Diaz, R. and Rodriguez, A.(2006).Culture conditions determine the balance between two different exopolysaccharides produced by Lactobacillus pentosus .*African Journal of Biotechnology* 3: 189-194.

Savadago, A. Quattara, C.A.T., Savadago,P.W. Barro,,N.,Quattara,A.S. and Traore,A.S. (2004). Identification of exopolysaccharide –producing lactic acid bacteria from Burkina Faso fermente milk samples. *African Journal of Biotechnology* 3: 189-194

Schillinger, U. and Lucke, F.K. (1987). Identification of *Lactobacilli* from meat and meat product. *Food Microbiology* 4: 199-208.

Smitinont, T., Tansakul, C., Tanasupawat, D., Keeratipibul, S., Navarini, L. Bosco, M. and Cescatti, P.(1999). Exopolysaccharide-producing lactic acid bacteria strains from traditional Thai fermented foods: Isolation, identification and exopolysaccharide characterization. *International Journal of Food Microbiology* 51:105-111.

Terzaghi, B.E. and Sandine, W.E. (1975). Terzaghi, B.E. and Sandine, W.E. (1975). Improved medium for *lactic Streptococci* and their *bacteriophages. Applied Journal of Environmental Microbiology* 29: 807-813.

Teuber, M.A., Geis, A. and Neve, H. (1986). Genus *Lactobacillus*. In: Bergey's Manual of Systematic Bacteriology, 8th Edn., Waverly Press Inc., Baltimore US.A.

Veringa, H.A., Galesloot, T.E. and Davelaar, H. (1968). Symbiosis of yoghurt II: Isolation and Identification of a growth factor for *Lactobacillus bulgaricus* produced by *Streptococcus thermophilus. Netherlands Milk Dairy Journal* 22: 114-120.

Vijayendra,S.V.W., Palanivel,G., M ahadevamina, S.and Tharanathan,R.N.(2009). Physio-chemical characterization of the of a new heteropolysaccharide produced by a native isolate of heterofermentative Lactobacillus Sp.CFR-2182.*Archival Microbiology*191:303-310.

Vogel, R.F., Ehrmann, M.A. and Ganzle, M.G.(2002). Development and potential of starter lactobacillus resulting from exploration of the sourdough system. *International Journal of General and Molecular Microbiology* 81: 631-638.

Wanda, M. and Salauen, A.(2005). Changes in acidity of fermented milk products during their storage as exemplified by natural bio-yoghurt. *Milchwissenschraft* 60(3): 294-296.

Wardlow, G.M. (1999). Pespectives in Nutrition, 4th ed., McGraw Hill, U.S.A, pp 475-494.

Weerkamp, A.H., Klijn N., Neeter, R. and Smit, G. (1996). Properties of mesophilic lactic acid bacteria from raw milk and naturally fermented raw products.

Netherlands Milk Dairy Journal 50: 319-322.

Wood, B.J. (1992). The Lactic Acid Bacteria in Health and Disease. Elsevier Applied Science, London.

Wood, B.J.B. and Holzapfel, W.H. (1995). The Genera of Lactic Acid Bacteria. Blackie Academic and Professional, Glasglow, volume 2.

Zambou, N. F., El-Hoda, N., Foneh, A. F., Moundipa, F. P., Tchouanguep, M. F. and El-Soda, M. (2007). Biochemical Properties of some Thermophilic lactic Acid Bacteria Strains from Traditional Fermented milk Relevant to their Technological Performances as starter culture. *Biotechnology* 6(1): 14-21.

Zeinab, A. M. E., Warda, S. A. G. and Hamid, A. D. (2008). Isolation and Identification of Lactic Acid Bacteria and Yeast from raw milk in Khartoum State (Sudan). *Research Journal of Microbiology* 3(3): 163-168.

Zourari, A., Accolas, J. P., and Desmazeaud, M. J. (1992). Metabolism and Biochemical characteristics of yoghurt bacteria. *A. Rev. Lait* 72: 1-34.