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PHENOTYPIC CHARACTERIZATION AND *IN VITRO* SCREENING OF LACTIC ACID BACTERIA FROM GOAT MILK FOR PROBIOTIC USE

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ABSTRACT

A study was carried out to isolate and identify probiotic Lactic acid bacteria (LAB) from milk of West African Dwarf (WAD) goats. Fifty LAB strains were isolated from WAD goat milks and tested for *in-vitro* antibiotics susceptibility, tolerance to bile, resistance to low pH values and haemolytic activity. Sixteen isolates were found to possess probiotic characteristics and these isolates were identified as *Lactobacillus plantarum* (44%), *L. acidophilus* (38%) and *L. fermentum* (18%). These isolates were resistant to most of antibiotics tested, showed the survivability (8.00 ± 0.05 to 72.60 ± 0.1%) at high bile acid concentration and resistance to pH 1.5 (0.00 to 46.00 ± 0.2%), pH 2.0 (30.60 ± 0.15 to 63.00 ± 0.6%) and pH 2.5 (48.60 ± 0.03 to 85.20 ± 0.6 %). None of the LAB isolates produced hemolysin. Among the probiotic isolates, *Lactobacillus acidophilus* displayed strong bile acid and low pH tolerance, followed by *Lactobacillus plantarum*. From the results obtained, *L. acidophilus* and *L. plantarum* could be used as probiotic starter cultures for fermented dairy foods as well as feed additives in livestock production due to high tolerance to high bile and acidic medium.

***Keywords*:** Probiotic, Lactic acid bacteria, West African Dwarf goats, acid and bile tolerance

INTRODUCTION

Lactic acid bacteria (LAB) have been exploited for centuries in food fermentations, preservations and as probiotics to promote good human and animal health (Bhattacharyya, 2009). These organisms are gram positive, usually non- motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism (Nair and Surendram, 2005). They play important role in food fermentation, primarily by causing the characteristic flavour changes and contributing a preservative effect on the fermented product. These bacteria are widely used as starter cultures in most fermented foods such as dairy products, alcoholic beverages, fermented vegetables and fermented meat (Antara *et al*., 2009). However, some strains of lactic acid bacteria could be used as probiotic.

Probiotic bacteria (Direct fed microbials) are live, non-pathogenic bacteria that contribute to the health and balance of the intestinal tract. Fuller (1989) defined probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". When these

microorganisms are ingested, they exert beneficial effects beyond that of their nutritional value (Weese and Arroyo, 2003). The major groups of probiotics are *Lactobacilli*, *Bifidobacteria* and some minor groups are *Saccharomyces* and *Streptococcus. Escherichia coli* Nissle 1917 and *E. coli* H22 strains have also been reported to possess probiotic activities.

Probiotic bacteria possess the ability to survive in the host depending on their metabolic activity, resistant to gastric acidity, adhesion to the mucosal surface, friendly to the host and protecting the host against infection (Kumar *et al*., 2009). Probiotics have been used as feed additives to improve the performance of poultry (Donoghue, 2004) and also as feed supplements in livestock to replace antibiotics in animal rations. Some properly selected probiotic bacteria can also increase nutrient utilization by providing enzymes in the gut capable of converting certain components of the diet into more digestible nutrients for the host animal. It has also been reported that probiotics control intestinal pathogens by production of antibacterial compounds, including short chain fatty acids such as acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, lactic acid; hydrogen peroxide and bacteriocin (Elizete *et al*., 2000). Other mechanisms of controlling the intestinal pathogens include the production of antibiotic- like substances, competition for nutrients and adhesion sites, increased and decreased enzyme activity, increased antibody levels and increased macrophage activity (Elizete *et al*., 2000). Other therapeutic functions of probiotics include anticholesterol activity, improved lactose utilization and anticarcinogenic activity (Zhu *et al*., 2009).

Today, lactic acid bacteria play an essential role in fermented food especially for their ability to produce various antimicrobial compounds promoting probiotic properties. Thus, this study aims to isolate, characterize and screen strains of LAB from goat milk for probiotic use for goats.

MATERIALS AND METHODS

Collection of samples:

Raw milk samples were collected from lactating West African Dwarf (WAD) goats from Livestock farm, University of Agriculture, Abeokuta. The milk samples were collected aseptically by swabbing the udders of the goats with absolute ethanol to eliminate possible contaminants from the udders. The milk samples were then collected with sterile MacCartney bottles by aseptically squeezing the udders.

Isolation of Lactic acid strains

Lactic acid bacteria were isolated from milk samples by using De Mann Rogosa Sharpe (MRS) agar supplemented with 0.05% (w/v) cysteine. One millilitre (1.0ml) of each sample was mixed with 9.0ml of sterile peptone water to obtain 10^{-1} dilution. The dilution was then made to 10^{-2}, 10^{-3} until 10^{-7}. 1.0ml of 10^{-7} dilutions were inoculated on MRS agar (BIOLAB) supplemented with cysteine and incubated at 37^{o}C for 48 hours in an anaerobic condition. Pure cultures of the isolates were maintained in MRS agar at 4^{o}C.

Identification of the isolates

The isolated LAB were identified by observing their morphological characteristics and by means of Gram staining, catalase test, endospore test, capsule staining, oxidase test, coagulase test, citrate test, methyl red test, indole test, voges proskauer test, arginine hydrolysis test, nitrate reduction test, growth at 2%, 4% and 6.5% sodium chloride con-

centrations, growth at 15°C and 45°C, acid and gas production from carbohydrates (glucose, rhamnose, lactose, mannitol, sorbitol, xylose, sucrose, D-ribose and D-arabinose). The isolates were identified using Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Further characterization was carried out using Analytical Profile Index (API) identification kit (API 50 CH kit, bio Merieux, France) as per manufacturer's instructions.

In vitro Evaluation of Probiotic Activity

Assay for Gastric juice Resistance

Gastric juice resistance of isolated LAB from goat milk were assayed using the methods of Kacem and Karam, 2006, Raz *et al*., 2007 and Antara *et al*., 2009; with little modification. Strains were grown in MRS broth at 37°C overnight. 100µl of each overnight culture was inoculated into MRS broth adjusted to pH 1.5, 2.0 and 2.5 with 5M HCl; and pH 6.0 (which served as control) and incubated anaerobically at 37°C for 2, 3 and 4 hours. 1.0ml of each broth culture was then diluted with sterile peptone water to 10^{-7} and 1.0ml of these dilutions were inoculated on MRS agar plates. Pour plate method was adopted. The plates were then incubated at 37°C for 48 hours under anaerobic conditions. The experiment was repeated and each reading represents the means of two observations. The number of surviving colonies were counted, recorded and selected. The survival percentage (%) of each strain to different pH values was then calculated as:

$$\text{Survival percentage (\%) of strains} = \frac{\text{Viable counts after acid exposure}}{\text{Viable counts of control}} \times 100$$

Significant differences between the treatment means were established by Duncan's Multiple Range test at 5% level of significance.

Bile tolerance test

Selected strains were propagated in a MRS broth and incubated at 37°C for 24 hours in an anaerobic condition. Serial dilutions of the cultures were prepared with sterile peptone water to 10^{-7}. 1.0ml of 10^{-7} dilutions were inoculated on MRS agar containing 0% and 10.0% sterilized bovine bile acids. The plates were incubated at 37°C for 48 hours under anaerobic conditions. The number of surviving colonies were counted and selected for further studies. The experiment was repeated and each reading represents the means for duplicate samples. Bacterial growth was expressed in colony forming units per millilitre (cfu/ml) and the survival percentage (%) of each strain to bile was then calculated.

Note: MRS agar plates containing 0% sterilized bovine bile acids served as control plates.

$$\text{Survival percentage (\%) of strains} = \frac{\text{Viable counts (cfu/ml) after bile exposure}}{\text{Viable counts (cfu/ml) of control}} \times 100$$

Significant differences between the treatment means were established by Duncan's Multiple Range test at 5% level of significance.

Testing for resistance to antibiotics

Antibiotic susceptibility of the selected strains were analysed by using the disc diffusion method as described by Sreekumar and Krishnan (2010) with little modification. An overnight culture was standardized to a turbidity equivalent to 1.0 McFarland standard (3.0×10^8 cfu/ml) with sterile distilled water. 100µl was spread on MRS agar plates using swabs. Antibiotic- impregnated discs (Abtek,

U.K) were placed on seeded plates and the plates were incubated at 37°C under anaerobic condition. All the assays were carried out in triplicates. Zones of inhibition were measured after 24h of incubation. The results (average of three readings) were expressed as Sensitive (S) or Resistant (R).

Significant differences between the treatment means were established by Duncan's Multiple Range test at 5% level of significance.

Note: Zones lower than 5.0mm were regarded as resistance while zones higher than 5.0mm were regarded as sensitive.

Haemolytic activity

Blood haemolysis of the isolates was evaluated by streaking each bacterial strain on nutrient agar supplemented with 5% human blood (Blood agar). The plates were incubated at 37°C for 48 hours. The appearance of clear zones around the bacterial colonies indicated the presence of haemolysis.

RESULTS

Fifty lactic acid bacteria isolates were isolated on MRS medium from goat milks. Sixteen possessed probiotic characteristics. These sixteen isolates were identified as *Lactobacillus plantarum* (44%), *Lactobacillus acidophilus* (38%) and *Lactobacillus fermentum* (18%) based on their colony morphology, physiological, gram staining, catalase test, gas production from glucose, growth on medium containing 2%, 4% and 6.5% Sodium chloride as well as biochemical tests. Microscopically, they were Gram positive rods, non- motile, catalase negative and absence of endospore. The isolates have the abilities to grow in MRS containing 2, 4 and 6.5% Sodium chloride and they produced acid from glucose fermentation. Their characteristics are shown in Tables 1 and 2.

Tolerance to bile acids and low pH are considered to be pre-requisite for colonization and metabolic activity of bacteria in the small intestine of the host. Therefore, when evaluating the potential of using LAB as effective probiotics, it is generally considered necessary to evaluate their ability to resist the effects of bile acids. In this study, bile tolerance of the LAB was investigated and the results are shown in Table 3. The LAB strains demonstrated variable tolerance to 10% fresh bovine acids. *Lactobacillus fermentum* was the most sensitive isolates to 10% bovine bile with survival percentage of 8.00± 0.05%. *Lactobacillus acidophilus* showed the highest tolerance (72.60 ± 0.1%).

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2.0. Table 3 also shows the results of acid tolerance (survival percentage of LAB isolates at various pH values). All the isolates possessing probiotic activities survived an incubation periods of 2h to 4h at pH 1.5 to 2.5 with decrease in survival percentage when the exposure time progresses. *Lactobacillus acidophilus* survived the acidic conditions better than other isolates. At pH 1.5, *Lactobacillus acidophilus* showed the highest survival percentage (46.0%, 28.6% and 26.5%), followed by *Lactobacillus plantarum* (34.7%, 22.8% and 19.5%) at 2, 3 and 4h incubation periods respectively. No growth occurred when *Lactobacillus fermentum* were exposed to pH 1.5 for 4h.

Table 4 shows the results obtained for antibiotic susceptibility of the LAB isolates. All the isolates were totally susceptible to augmentin antibiotic. *Lactobacillus acidophilus* showed total resistance to minimum of 7 of the 8 antibiotics tested (cotrimoxazole, cloxacillin, erythromycin, gentamicin, streptomycin, tetracycline and chloramphenicol). The

resistance of this isolate to most of the common antibiotics make it very safe for use as probiotic. Finally, none of the isolates produced haemolysis on animal blood.

Table 1: Morphological, Physiological and Biochemical properties of Probiotic Lactic acid bacteria isolated from milk of West African Dwarf goats

Properties	Lactobacillus plantarum	Lactobacillus acidophilus	Lactobacillus fermentum
Gram reaction	+ve	+ve	+ve
Morphology	Rods	Rods	Rods
Motility	-ve	-ve	-ve
Growth at 15oC	+ve	-ve	-ve
Growth at 45oC	-ve	+ve	-ve
Growth in 2% NaCl	+ve	+ve	+ve
Growth in 4% NaCl	+ve	+ve	+ve
Growth in 6.5% NaCl	+ve	+ve	+ve
Capsule	-ve	-ve	-ve
Catalase	-ve	-ve	-ve
Spore	-ve	-ve	-ve
Coagulase	-ve	-ve	-ve
Indole	-ve	-ve	+ve
Oxidase	-ve	-ve	-ve
Citrate	-ve	+ve	-ve
Methyl red	-ve	-ve	-ve
Voges proskauer	+ve	+ve	+ve
Nitrate reduction	-ve	-ve	-ve
Arginine hydrolysis	-ve	-ve	-ve
Acid & gas production			
Glucose	A	A	A
Mannitol	AG	A	A
Lactose	-ve	A	A
Rhamnose	-ve	-ve	-ve
Sorbitol	-ve	-ve	A
Xylose	-ve	A	A
Sucrose	A	A	A
D- ribose	A	-ve	A
D- arabinose	-ve	-ve	-ve

Note:
-ve: negative reaction +ve: positive reaction
A: acid production G: gas production

Table 2: Carbohydrate fermentation patterns (using API 50 CH kit) of probiotic lactic acid bacteria isolated from milk of West African Dwarf goats

Sugars	L. plantarum	L. acidophilus	L. fermentum
Glycerol	-ve	-ve	-ve
Erythritol	-ve	-ve	-ve
Salicin	+ve	+ve	+ve
L- arabinose	+ve	+ve	+ve
D- maltose	+ve	+ve	+ve
D- xylose	+ve	-ve	+ve
L- xylose	-ve	-ve	+
D- adonitol	-ve	-ve	-ve
Methyl-βD- xylopyranoside	-ve	-ve	-ve
D- galactose	+ve	+ve	+ve
D- glucose	+ve	+ve	+ve
D- fructose	+ve	+ve	+ve
Arbutin	+ve	+ve	-ve
Esculin	+ve	+ve	+ve
D- mannose	+ve	+ve	+ve
D- trehalose	+ve	+ve	+ve
Inositol	-ve	-ve	-ve
L- sorbose	+ve	-ve	-ve
Dulcitol	-ve	-ve	-ve
Inulin	-ve	+ve	-ve
D- raffinose	+ve	+ve	+ve

Note:
-ve: no fermentation
+ve: fermentation

Table 3: Bile and Low pH tolerance of Lactic acid bacteria isolated from goat milks

LAB isolates	Survival percentage (% ± S.D) to low pH after incubation									Survival percentage (% ± S.D) to 10% bile
	pH 1.5			pH 2.0			pH 2.5			
	2h	3h	4h	2h	3h	4h	2h	3h	4h	
Lactobacillus acidophilus	46.00 ± 0.2a	28.60 ± 0.5a	26.50 ±0.11a	63.00 ± 0.6a	55.80 ±0.05a	51.00 ±0.14a	84.20 ± 1.0a	78.50 ±0.2a	76.00 ±1.1a	72.60 ± 0.1a
Lactobacillus plantarum	34.70 ±1.6b	22.80 ±0.1[a]	19.50 ±0.07a	56.50 ±0.3ab	43.00 ±1.1b	38.00 ±0.03b	85.20 ±0.6a	74.00 ±0.3a	73.20 ±1.2a	68.00 ±0.06a
Lactobacillus fermentum	12.90 ±0.3c	4.80 ±0.16[b]	0.00± 0.00[b]	49.00 ±0.1[b]	41.30 ±0.01[b]	30.60 ±0.15b	66.00 ±2.1[b]	58.40 ±0.2b	48.60 ±0.03[b]	8.00 ±0.05b

Note: Means with the different letters along the columns are significantly different ($p<0.05$)

Table 4: Antibiotic susceptibility of Lactic acid bacteria isolated from goat milk

Antibiotics	Zones of inhibition (mm ± S.D.)		
	Lactobacillus acidophilus	Lactobacillus plantarum	Lactobacillus fermentum
Cotrimoxazole (25.0µg)	2.70±0.1a (R)	2.90±0.05a (R)	3.50±0.02a (R)
Cloxacillin (5.0µg)	3.30±0.3a (R)	1.50±0.01b (R)	2.60±0.01a (R)
Erythromycin (5.0µg)	1.30±0.05a (R)	11.00±0.3b (S)	18.00±1.5c (S)
Gentamycin (10.0µg)	3.80±0.2a (R)	3.20±0.01a (R)	1.30±0.5b (R)
Augmentin (30.0µg)	11.80±0.03a (S)	16.50±0.5b (S)	12.50±1.00a (S)
Streptomycin (10.0µg)	4.00±0.1a (R)	9.80±0.2b (S)	2.40±0.05c (R)
Tetracycline (10.0µg)	3.70±0.04a (R)	3.30±0.08a (R)	10.00±1.00b (S)
Chloramphenicol (10.0µg)	3.20±0.01a (R)	14.00±1.5b (S)	13.50±0.6b (S)

Note: Means with the different superscripts along the rows are significantly different ($p<0.05$)

(R): Resistance (S): Sensitive

DISCUSSION

The criteria for the *in vitro* selection of organisms to be used for probiotic include antibiotic tolerance, bile tolerance, gastric juice resistance as well as the production of lactic acid that inhibits the growth of other microorganisms (Hoque *et al*., 2010). These characteristics allow the probiotic organisms to be established in the intestinal tract as well as enable them to survive, grow and perform their beneficial action in the gastrointestinal tract (GIT) of the hosts. Thus, probiotic cultures must survive in the environment with gastric and bile acids, with pH as low as 1.5 to 2.0 and 0.3% bile acids. Results from this study revealed that *Lactobacillus acidophilus* and *L. plantarum* could tolerate more acidic medium and gastric bile than *L. fermentum* which exhibited low acid and bile tolerance and thus, *L. acidophilus* and *L. plantarum* would be able to survive high acidic environment in the stomach and high concentration of bile components in the intestine when animals consumed feeds containing these organisms. These results support the previous findings of Rashid *et al*. (2007) who reported that *Lactobacillus delbrueckii subsp. bulgaricus* M340-3 strain has good acid tolerance but, less bile acid tolerance.

Bile resistance of some organisms is related to specific enzyme activity – bile salt hydrolase (BSH) which helps to hydrolyse conjugated bile, thus reducing its toxic effect. This shows that *Lactobacillus acidophilus* and *L.*

plantarum which were able to survive high concentration of bile acid had high bile salt hydrolase (BSH) activity and very low in *L. fermentum*. BSH activity has mostly been found in organisms isolated from the intestines or faeces of animals (Parvez *et al*., 2006), which is in accordance with the results of this study since the LAB strains were isolated from goat milk.

In addition, results indicated that *Lactobacillus acidophilus* is resistant to most of antibiotics tested and low multiple susceptible were observed. This is in accordance with various reports that LAB strains are normally resistant to the principal antibiotics such as chloramphenicol, penicillin G, ampicillin and vancomycin (Lonkar *et al*., 2005; Hoque *et al*., 2010 and Marroki *et al*., 2011). The broad range of resistances of most of the antibiotics tested could be due to wide use of these antibiotics in the treatment of many diseases of human and animals. However, the results of antibiotic sensitivity pattern of this study revealed that *Lactobacillus plantarum* and *L. fermentum* isolated from goat milks were susceptible to some antibiotics and this result corroborates with the previous report of Kacem and Karam (2006) who reported that *L. plantarum* strains were susceptible to most antibiotics.

CONCLUSION

The results of the present study revealed the ability of LAB isolated from goat milks to survive the conditions of high bile salt concentration and low pH values. This will help the organisms to reach the gastro intestinal tract (GIT) of the host and contribute to the balance of the intestinal microflora. Among the LAB isolates, *Lactobacillus acidophilus* and *L. plantarum* have high probiotic activities and these bacteria are favourable for use as probiotics. Additional research needs to be carried out on the *in vivo* selection of these organisms.

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