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CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM TWO FERMENTED FOOD PRODUCTS AND THE STOMACH OF RUMINANT ANIMAL

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ABSTRACT

Fifteen lactic acid bacteria (LABs), comprising two genera: *Lactobacillus* and *Lactococcus spp* were isolated from the intestine of ruminant animal, nono and kunun-zaki using MRS agar supplemented with 0.02% sodium azide; the fifteen isolates were tested on various salt concentration, temperature and pH. All the organisms grew at the pH of 4.5, salt concentration (%) of 1.5-5.0 and temperature of 15^{o}C, only one of the isolates grew at the pH of 9 and salt concentration (%) of 10, when all the isolates were tested on blood agar plate, it was observed that none was haemolytic. When the LAB isolates were used to produce yoghurt only the isolates from nono produced quality yoghurt; however, the other isolates did not. None of the 15 isolates shows heamolytic activity on blood agar plate which signifies that LAB are not pathogenic to human and therefore they could be good probiotic material.

Keywords: lactic acid bacteria, ruminant animals, nono, kunun-zaki.

INTRODUCTION

Lactic acid bacteria (LAB), which are primarily used by the dairy industry, are extensively utilized in the fermentation of a wide variety of food production and are known for their preservative and therapeutic effects. *Streptococus thermophilus and Lactobacillus Bulgaricus* are used for manufacturing of yoghurt. *Lactococcus lactis, Lactococcus cremoris, Lactococcus diactylactis, Leuconostoc cremoris, Leuconostoc lactis, Lactobacillus helveticus, lactobaccilllus acidophilus, lactobacillus casei, and lactobacillus spp* are used for the production of ripened cheese, cultured milk, cream and ripened butter while *L, acidophilus* and *L. casei* are widely used as probiotic bacteria in human and animal health.

There has been tremendous research on isolation and identification of microorganism responsible for the fermentation of commercial yoghurt by (Silvia *et al*., 2001) in Brazil. Several works have indicated the "nono" (Local yogurt) marketed by Fulani women and other in Bida has poor microbial quality and could constitute a health hazard. Agarry *et al*. (2010) reported the production of kunun-zaki (a Nigerian fermented non-alcoholic cereal beverage) using LAB isolated from previous kunun-zaki in order to enhance the quality of the product. Adnan and Tan (2007) isolated and characterized 20 industrially important LABs from 10 Malaysian food products. There seem to be no information on the isolation and characterization of microorganism from ruminant animal in Bida. This research needs to be extended to the importance of lactic bacteria associated with indigenous fermented food and using the isolates, isolated from the fermented food for the production of yoghurt. The purpose of this study is to characterize industrially important LABs from ruminant animals and locally fermented foods and evaluate its potential for used for the development of starter cultures.

Scientists have embarked on extensive studies to isolate and characterised microorganisms associated with production of indigenous fermented foods with the possibility of exploiting their industrial potentials (Leisner *et al*., 2001; Adnan and Tan, 2007). Banigo *et al*. (1974) developed a starter culture comprising of mixtures of *Lactobacillus plantarum, Streptococcus lactis* and *Saccharomyces rouxi* for the controlled production of ogi (a porridge from fermented cereal). Olasupo *et al*. (1997) produced ogi using a bacteriocin-producing Lactobacillus strain as starter culture.

MATERIAL AND METHOD

Source of materials

A sterilized container was used to collect sample from the stomach of ruminant animal (cow) at the abattoir in Bida, Nigeria; Kunun-zaki and nono was bought from commercial vendors at the small market in Bida

Isolation of LAB from the stomach of ruminant animal (cow) using Sodium azide enriched BHI broth.

Ten grams (10g) of the sample collected from the large intestine of cow was mixed with 100ml sterile Brain Heart Infusion broth (BHI; Biotech Laboratories Ltd, Ipswich, UK) supplemented with 0.02% (w/v) Sodium azide (Sigma, UK) and incubated at 30±2^{o}C for 24h (Lindquist, 1998). Sodium azide inhibits cytochrome activity therefore selectively enriched for lactic acid bacteria. Sub-cultures were made from the 24h enriched medium by streaking them on prepared MRS agar (LAB M, Lancashire, UK) plates; these were incubated for 24h at 30±2^{o}C. Following incubation discreet colonies (typical pin point) were randomly picked and purified on fresh MRS agar plates. Cultures of the isolates were considered to be pure after three successive subcultures on MRS agar plate; pure cultures of microbial isolates were

subsequently sub-cultured on MRS agar slants in Bijou bottles; these were covered with sterile mineral oil and kept in the refrigerator for further studies.

Isolation of LABs from nono and kunun-zaki using BHI broth supplemented with Sodium azide

Ten millilitres (10ml) each of either nono or kunun-zaki was added separately to 100mls of sterilized BHI broth enriched with 0.02% Sodium azide (NaN_3) and incubated as reported previously.

Identification of LAB isolates

The purified isolates were identified on the basis of standard cultural, morphological and biochemical characteristics as described by (Sneath *et al*.1986).

Antimicrobial susceptibility test

Each of the hardened MRS agars plate was inoculated with 0.1ml of the test organism (LAB isolates) by spread plating technique to ensure a uniform growth. Commercial disk containing Amoxycilin (0-25µg), Ofloxacin (0-5µg), Streptomycin (0-10µg), Chloramphenicol (0-30µg), Ceftriaxone (0-30µg), Gentamycin (0-10µg), Pefloxacin (0-5µg), Co-trimoxazole (0-25µg), Ciprofloxacin (0-10µg), Erytromycin (0-5µg) was placed at the centre of the inoculated plate using a sterile forceps, following which it was incubated at 37^{o}C for 18-24h. The resulting zone of inhibition was measured; interpretative categories (susceptible >17mm, intermediate 16-17mm and resistant <16mm of zone of inhibition) were calculated for each zone of inhibition measurement in accordance with the NCCLS (1993) guideline table.

Haemolytic activity

To determine bacteria haemolytic activity, blood haemolysis was evaluated on MRS agar plate supplemented with 5% human blood; each bacterial suspension was streaked on the blood agar plate. After 24h incubation at 37^{o}C, the plates were examined for sign of β–heamolysis (clear zone around colonies), α–heamolysis (a green–hued zone around colonies) or γ-heamolysis (no halo around colonies).

Screening for tolerance of LAB isolate on varying temperatures, pH and Sodium chloride concentrations

A basal MRS medium was used in these series of studies but without beef extract and with 0.17g/l bromothymol blue added as pH indicator (pH 7). Universal bottle with screw caps were each filled with 20ml of the basal MRS medium and autoclaved. A 24h culture of each isolate was used as the inoculum whereby the cells were spun down, re-suspended in 0.85% normal saline and a loopful of the suspension was inoculated into each of the test tubes. The temperature tested were 15, 30, 34, 45, and 50^{o}C, the concentration of NaCl tested were 1.5%, 2.5%, 5%, 7.5% and 10% (w/v), while the pH tested was 4.5, 7 and 9. The basal MRS medium was adjusted with 1M phosphoric acid and 1M NaOH to prepare the initial pH. At the end of 24h the colour change of each test tube was noted as a simple indication of growth or no growth.

Selection of Lactic acid bacteria with potentials for use as starter culture for the production of yoghurt

Pure cultures of the fifteen identified LAB isolates were tested (singly and or in various combinations) for their potential use as starter cultures for yoghurt production. Fresh cultures of the isolates grown on MRS agar plates were harvested with sterile cotton swabs (Osawa *et al.*, 2000) and suspended (separately) in 1ml of sterile saline (0.85% NaCl) to prepare a dense suspension; this was used to inoculate four LAB isolates into 100ml of pasteurized milk (15% milk solid) and incubated at ambient temperature (30±2^{o}C) for 24h. After incubation, the samples were subjected to sensory evaluation by a 10 member trained expert profile panellist that can distinguish between intensity of aroma differences.

RESULTS AND DISCUSSION

The result of this study has shown that industrially important lactic acid bacteria (LAB) could be isolated from the stomach of ruminant animal (cow), kunun-zaki and nono. In all, 15 LABs were isolated 8 of these were *Lactococcous spp* and 7 *Lactobacillus spp*. When the LAB isolates were used to produce yoghurt only the isolates from nono produced quality yoghurt; however, the other isolates did not (data not shown but summarized as a footnote in Table 1). Adnan and Tan (2007) isolated 126 LABs from two traditional Malaysian foods: "tapai" (fermented tapioca) and "tempoyak" (fermented durian flesh made from chilli pure and fresh goat's milk). These workers reported that most of the isolates were of industrial importance. Agarry *et al.* (2010) isolated three lactic acid bacteria (*Lactobacillus plantarum, L. fermentum and Lactococcus lactics*) from kunun-zaki which they used to develop a starter culture for enhanced production of kunun-zaki.

Table 1: Cultural, morphological and biochemical characteristics[1] of Lactic Acid Bacteria isolated from, ruminant animal, nono and kunun-zaki

Isolates	Gram reaction /cell shape	Cultural characteristics	Hot loop test[1]	Catalase	Sugar fermentation[2] Inositol	D-xylose	D-Ribose	Mannitol	Arabinose	Mannose	Lactose	Possible isolate
RA001	+ (rod)	ppc*	+	-	+	+	+	+	+	+	+	*Lactobacillus spp*
RA002	+ (rod)	Ppc	+	-	+	-	+	+	+	+	+	*Lactobacillus spp*
RA003	+ (rod)	Ppc	+	-	+	-	+	+	+	+	+	*Lactobacillus spp*
RA004	+ (rod)	Ppc	-	-	+	+	+	+	+	+	+	*Lactobacillus spp*
RA005	+ (cocci)	Ppc	-	-	-	-	+	+	+	+	+	*Lactococcus spp*
RA006	+ (cocci)	Ppc	+	-	+	-	+	+	+	+	-	*Lactococcus spp*
KZ001	+ (cocci)	Ppc	-	-	+	+	+	+	+*	+	+	*Lactococcus spp*
KZ002	+ (rod)	Ppc	+	-	+*	+	-	+	+	+	+	*Lactobacillus spp*
KZ003	+ (rod)	Ppc	+	-	+	+	+	+	+	+	+	*Lactobacillus spp*
KZ004	+ (cocci)	Ppc	+	-	+	+	+	+	+	+	+*	*Lactococcus spp*
N001	+ (cocci)	Ppc	+	-	+	+*	+	+	+	+	+*	*Lactococcus spp*
N002	+ (cocci)	Ppc	+	-	+	-*	+	+	+*	+	+	*Lactococcus spp*
N003	+ (rod)	Ppc	+	-	+	+*	+	+	+	+	-	*Lactobacillus spp*
N004	+ (cocci)	Ppc	-	-	+*	+	-	+	-	+	+	*Lactococcus spp*
N005	+ (cocci)	Ppc	-	-	-*	+	+	+	-	+	+	*Lactococcus spp*

[1]Gas (CO_2) evolution , + Heterofermentative, - homofermentative; [2] +=Acid production (+* = weak positive, -* = weak negative); ppc: Pin Point Colony, [3]LAB isolates:-RA001-RA006- from stomach of ruminant animals- could not ferment milk during yoghurt production; Kz001-KZ004-from kunun-zaki; N001-N005- from nono-fermented milk which produce quality yoghurt

As observed in this study, five out of the fifteen isolates were found to be homolactics following hot loop test (Table 1). Homo-fermentatives generally ferments carbohydrates with the production of 100% lactic acid which lowers the pH close to 4.0-4.5 while hetero-fermentatives produces carbon-dioxide and other organic compounds (acetic acid, alcohol, acetaldehyde, diacetyl) which further lowers the pH to about 3.5 and impart characteristics flavour to the fermented food (Anderson, 1988; Steinkraus, 2002). The tolerance of the 15 LAB isolates as shown in Table 2 revealed that all the organisms grew at the temperature of 15^{o}C and salt concentrations of 1.5-5%; the isolate that grew at 50^{o}C, salt concentration of 7.5 and 10% could be useful in industrial fermentations especially, where the temperature of growth medium is elevated, thereby edging out other competing organism in the fermenting substrate.

Table 2: Tolerance[1] of lactic acid bacteria isolates[2] to ranges of temperature, salt concentration and pH

	Temp ^{o}C				Salt concentration (%)					pH		
Organisms	15	27	37	50	1.5	2.5	5	7.5	10	4.5	7	9
RA001	+	+	+	+	+	+	+	+	-	+	-	+
RA002	+	-	-	-	+	+	+	+	-	+	-	-
RA003	+	+	-	+	+	+	+	+	-	+	-	-
RA004	+	+	+	+	+	+	+	+	-	+	-	-
RA005	+	-	+	+	+	+	+	-	+	+	-	-
RA006	+	+	+	-	+	+	+	-	+	+	-	-
KZ001	+	-	-	-	+	+	+	+	-	+	-	-
KZ002	+	+	-	-	+	+	+	-	-	+	-	-
KZ003	+	-	-	+	+	+	+	-	-	+	-	-
KZ004	+	+	-	+	+	+	+	+	-	+	-	-
N001	+	+	+	-	+	+	+	+	-	+	-	-
N002	+	+	-	+	+	+	+	-	-	+	-	-
N003	+	+	-	+	+	+	+	-	-	+	-	-
N004	+	-	-	+	+	+	+	-	-	+	-	-
N005	+	+	+	+	+	+	+	-	+	-	+	-

[1] +: indicate growth i.e. colour change; -: means no growth ie no colour change

[2] LAB isolates:-RA001-RA006- from stomach of ruminant animals- could not ferment milk during yoghurt production; Kz001-KZ004-from kunun-zaki; N001-N005- from nono-fermented milk

Furthermore, the isolates that grew at high salt concentration (7.5-10%) might be useful in the production of lactic acid on a large scale where it is precipitated as lactate [salt]. Bacteria cells cultivated in a high salt concentration would experience a lot of turgor pressure, which would then affect the physiology, enzyme activity, water activity and metabolism of the cells (Liu *et al.*, 1988). However, some of the cells could overcome this effect by regulating the osmotic pressure between the inside and outside of their cells (Kashket, 1998). It is of interest to note that none of the 15 isolates shows heamolytic activity on the blood agar plate (data not shown but summarized as a footnote in Table 3)

which signifies that LABs are not pathogenic to human and therefore they could be good probiotic material. It is true because some of the isolates were isolated from intestine of the ruminant animal.

Table 3: Antibiotics susceptibility of lactic acid bacteria isolated from ruminant animals, nono and kunun-zaki

	Antibiotics[2]/Zone of inhibition[3,4,5] (mm)									
Organisms[1]	AMX	OFL	STR	CHL	CEF	GEN	PEF	COT	CPX	ERY
RA001	-	-	-	-	-	-	9.5	-	-	-
RA002	-	-	-	-	-	-	-	-	-	-
RA003	16	-	-	17	-	-	17	-	20	18
RA004	-	14	-	16	-	-	13	-	17	9
RA005	-	-	-	21	-	-	-	-	-	12
RA006	-	-	-	-	-	-	-	-	-	-
KZ001	-	-	-	-	-	-	-	-	-	-
KZ002	-	11	-	-	-	-	-	-	-	-
KZ003	-	-	-	-	-	-	-	-	-	-
KZ004	-	11	-	-	-	-	-	11	12	-
N001	-	13	-	19	-	-	-	-	15	15
N002	-	-	-	-	-	-	-	-	-	-
N003	-	-	-	-	-	-	-	-	-	-
N004	-	-	-	-	-	-	-	-	-	-
N005	-	-	-	-	-	-	-	-	-	-

[1]On blood agar plate, the isolates did not show any clear zone around each colony after 24h incubation at 37^{o}C

[2]AMX-amoxycilin (25µg); OFL-ofloxacilin (5 µg); STR-streptomycin (10 µg); CHL- chloramphenicol (30 µg); CEF-ceftriazone (30 µg); GEN-gentamycin (10 µg); PEF-pefloxacin (5 µg); COT-cotrimoxazole (25 µg); CPX-ciprofloxacin (10 µg); ERY- erythromycin (5 µg)

[3]- no zone of inhibition

[4]NCCLS (1993) standard: >17mm-susceptible; 16-17mm-intermediate; <16mm-resistant

[5] LAB isolates:-RA001-RA006- from stomach of ruminant animals- could not ferment milk during yoghurt production; Kz001-KZ004-from kunun-zaki; N001-N005- from nono-fermented milk

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