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Antimicrobial activity of lactic acid bacteria isolated from locally fermented cow milk products sold in Keffi, Nigeria on clinical bacteria

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Abstract

Lactic acid bacteria (LAB) are renowned for the potential of producing antimicrobial compounds. This study focuses on isolation and antimicrobial effect of lactic acid bacteria isolated from locally fermented cow milk products (Nono, Kindirmo and Maishanu). The antimicrobial effect of the LAB isolates against clinical test isolates was also investigated. Fifty (50) samples each of Nono and Kindirmo, and twenty-five (25) of Maishanu were collected from cow milk products sellers in Keffi, Nasarawa State Nigeria. Standard microbiological methods were used for the isolation and identification of Lactic acid bacteria from fermented cow milk products. The percentage isolation rate of *Lactobacillus* species from Nono and Kindirmo showed that 52.0% were isolated from Nono and 58.0% from kindirmo. The highest percentage occurring *Lactobacillus* species from Nono was *Lactobacillus plantarum* (14.0%) and the highest percentage occurring *Lactobacillus* species from kindirmo was *Lactobacillus delbrueckii* (16.0%). The *Lactobacillus* species isolated were screened for antibacterial activity and it was observed that seven (7) were able to show inhibition zones. The antimicrobial effect of the LAB culture supernatant against clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Shigella dysenteriae* was investigated using agar diffusion technique. The highest 22.00mm was observed by *Lactobacillus delbrueckii* and *Lactobacillus delbrueckii* against *Shigella dysenteriae*, while the least inhibition zone of 15.00mm was observed by *Lactobacillus delbrueckii* and *Lactobacillus acidophilus* against *E. coli*. In conclusion, the zones of inhibition observed in this research strongly suggest that various antimicrobial compounds (lactic acid, acetic acid, propionic acid and bacteriocins) produced during the fermentation process are potent in the control of growth of the test bacteria.

Keywords: Fermented; Cow; Milk; Products; Antimicrobial; Activity; Clinical; Isolates

1. Introduction

Lactic Acid Bacteria which is gram-positive, non-sporing, catalase-negative, devoid of cytochromes, of non-aerobic habitat but aero-tolerant, fastidious, acid tolerant and strictly fermentative with lactic acid as the major end product during sugar fermentation Khalid [1].

Lactic acid bacteria have significant potential for use in food processing and especially dairy products because they are safe and naturally dominate the microflora of many foods during storage Bennani *et al.* [2]. Lactic acid bacteria (LAB) are candidate probiotic bacteria that are widely distributed in nature and can be used in the food industry Masalam *et al.* [3].

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Lactic acid bacteria are good flavoring and texturizing agents which also serve as preservatives in food. LAB such as *Lactobacillus lactis* and *Streptococcus thermophilus* inhibit food spoilage and the growth of pathogenic bacteria, thereby preserving the nutritive qualities of raw food material for an extended shelf life O'sullivan *et al.* [4].

Milk and milk products constitute important nutritional components for human diet and plays a prominent role in human nutrition Javaid *et al.* [5]. Fermented milks as examples of cow milk products have a semi-solid and curdled texture, due to the distribution of the milk casein in the liquid medium of the product; Both Physical and chemical changes occurs during the fermentation process which leads to the enhanced viscosity of the product.

Nono (sour milk), maishanu (local butter) and kindirmo (yoghurt) are some indigenous cow milk products locally sold in Keffi, Nigeria. Their production is as a result of fermentation which is a good source of Lactic acid bacteria. The indigenous fermentation processes are natural or spontaneous i.e attributable to chance inocula from the environment, the vessels used and/or the microbial flora on the substrates Osuntoki *et al.* [6]. They are highly consumed by Nigerians and numerous health benefits have been reported due to the consumption of these products.

It is evident that various strains of Lactic acid bacteria exert important antimicrobial roles in food, animal and human health which is beneficial. It is then most imperative to identify the specific strains and characterize to molecular level, those with potential antimicrobial effects. Molecular techniques, especially polymerase chain reaction (PCR)-based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE) are regarded important for the specific characterization and detection of LAB strains Mohania *et al.* [7].

This study focus on isolation of Lactic acid bacteria from locally fermented cow milk products (Nono, maishanu and kindirmo) sold in Keffi, Nigeria and evaluates their antimicrobial effect against indicator organisms.

2. Material and methods

2.1. Study Area

The study area was Keffi metropolis. Keffi is a cosmopolitan town in Nasarawa state in Northern part of Nigeria. It is about 58km from Abuja, the Federal Capital Territory and 128km from Lafia, the state capital of Nasarawa state. Keffi is located on latitude 80 32' North and longitude 80 18' East. The town is situated on an altitude of 850m above sea level Awka *et al.* [8].

2.1.1. Sample Size

One hundred and twenty-five (125) samples of the cow milk products were collected from the Fulani women at different locations of the main and old Markets in Keffi, Nasarawa state Nigeria.

2.1.2. Sample Collection

The cow milk product samples used for this research were collected from different locations at the main and old markets in Keffi, Nassarawa State, Nigeria using sterile bottles. The samples were stored at 4°C until use within 72 hours. The clinical isolates used were collected from the medical microbiology laboratory, National Biotechnology Development Agency Abuja, Nigeria.

2.1.3. Isolation of Lactic Acid Bacteria Species

Serial dilution of the samples was carried out by taking 1gram of sample into 10mL distilled water in a test tube. This was shaken properly and 1mL transferred to another test tube containing 10mL distilled water until the 6th dilution. One milliliter (1mL) of the 6th dilution was now introduced into sterile Petri dishes and 25 mL of MRS agar was added. The Petri dishes were gently swirled to enhance homogeneity and then incubated in a pre-set aerobic incubator at 37°C for 24 hours. At the end of the incubation, visible colonies formed in the petri dishes were observed. The bacterial cultures were subjected to macroscopic examination for colonial morphology as described by Cheesbrough [9]. Pure cultures were obtained by repeated sub-culturing on media used for primary isolation, and preserved on MRS agar slants for further use.

2.2. Identification and Characterization of Lactic Acid Bacteria

The isolated bacteria were identified by their colony morphology, gram staining characteristics, biochemical tests such as catalase, indole, methyl red, motility, citrate and using 16srRNA molecular Identification.

3. Screening of LABs Potential as an Antimicrobial Agent

The Screening to find isolates with antimicrobial effect was performed using the spot-on-lawn method described by Schillinger and Lucke [10] with some modifications. Briefly, overnight cultures of the isolates were spotted onto the surface of agar plates with MRS broth and 1.2% agar-agar and incubated for 24 h at 30°C to allow colonies to develop and produce their metabolites. Approximately 5×10^7 CFU/mL of the indicator strains was inoculated into 100 ml of peptone broth (containing 0.7% agar). Colonies from the MRS medium was then spotted on freshly prepared Muller Hilton agar after which indicator strains inoculated into the peptone broth was poured over the plate to form a lawn. After incubation at 37°C during 24 h, diameter of inhibition zones was measured from the edge of the zone with a caliper, and expressed in mm.

3.1. Antimicrobial Activity of Lactic Acid Bacteria Against Test Organisms

3.1.1. Source of test organisms

Three pathogens including *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae* were obtained from the department of medical microbiology, National Biotechnology Development Agency, Abuja. Biochemical test and molecular test were carried out to confirm the identity of the isolate.

Lactic Acid Bacteria Antimicrobial Activity

LABs isolated from cow milk products were grown in de Man Rogosa Sharpe (MRS) Agar medium (Oxoid, Milan, Italy) at 35°C overnight. 2% of LAB cells was inoculated into a fresh broth medium and propagated for 24 h. The cells were harvested by centrifugation (6000 g, 10 min, 4°C). The culture supernatants were filtered through a 0.2 mm sterile Millipore filter to remove all cells. The supernatants were used for the determination of antimicrobial activities of LAB supernatant (total metabolites)

3.1.2. Effect of Lactic Acid Bacteria Antimicrobial Metabolite on Test Organisms

The ability of LAB strains to produce antimicrobial metabolites was tested by an agar well diffusion assay. Individual colonies were suspended in normal saline to 0.5 McFarland standards using sterile swabs. The suspension was inoculated on Muller Hinton agar (MHA) using a sterile swab stick. A 10 mm cork borer was used to puncture holes in the solidified medium; two well were punctured on each petri plate. Thereafter, 160 µL LAB supernatant (total metabolites) was introduced into each well using microtiter-pipette. This was allowed to diffuse at room temperature for one hour. The plates was incubated at 37°C for 24 hours aerobically. The activities of the extracts were compared with the positive control antibiotic discs (Amoxicillin (30 µg)). Zones of inhibition observed after 24 hours' incubation was measured and recorded. Sterile 6 mm Whatman filter paper No. 3 (Germany), impregnated with sterile distilled water was used as negative control CLSI, [11].

3.2. Statistical Analysis of Data

Data collected was analyzed using Statistical Package for the Social Sciences (SPSS) version 21. Data was expressed as mean ± standard deviation while mean comparison was carried out using Duncan multiple range test (DRMT) Ling and Roberts [12].

4. Results and discussion

4.1. Occurrence of *Lactobacillus* Isolates

The isolation rate of *Lactobacillus* species isolated from cow milk products sold in Keffi is as given in Table 1. Out of 125 collected samples of cow milk products namely (Nono, Kindirmo and Maishanu) a total of 55 (44.0%) *Lactobacillus* species were isolated. The percentage isolation rate showed that 52.0% were isolated from Nono, 58.0% were isolated from kindirmo and none was isolated from maishanu.

The cultural, morphological and biochemical characterization of the *Lactobacillus* isolated from cow milk products (Nono and Kindrimo) sold in Keffi is as shown in Table 2. Creamy-white on MRS agar was gram-positive, rod-shaped

bacilli/coccobacilli, occurring singly. There was variation in the sizes with some of the isolates being either long thick rods, short thick rods or short thin rods, non-endospore formers, glucose positive and had biochemical reactions.

Table 1 Isolation Rate of *Lactobacillus* species From Cow Milk Products

Cow Milk Products	Number of Samples	Number (%) of Isolates
Nono	50	26(52.0)
Kindirmo	50	29(58.0)
Maishanu	25	0(0.00)
Total	125	55(44.0)

Table 2 Cultural, Morphological and Biochemical Characteristics of *Lactobacillus* Isolated From Cow Milk Products

Isolate Code	Cultural morphology	ET	Gram stain	Biochemical characteristic				Sugar fermentation			Inference
				Cat	Ox	In	Nit	Fru	Mal	Glu	
KD1-3	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	+	+	-	+	-	+	<i>Lactobacillus delbrueckii</i>
KD5	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	-	+	+	+	+	+	<i>Lactobacillus plantarum</i>
KE1-2	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	+	-	+	-		-	+	<i>Lactobacillus rhamnosus</i>
KE4	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	-	-	+	+	+	+	<i>Lactobacillus delbrueckii</i>
ND1-2	Creamy-white, smooth elevated colonies and thick long bacilli	+	+	-	+	-	+	+	-	+	<i>Lactobacillus plantarum</i>
ND 3	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	+	+	-	+	-	+	<i>Lactobacillus acidophilus</i>
ND 4-5	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	+	+	-	+	-	+	<i>Lactobacillus crispatus</i>
NE1-3	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	-	-	+	+	+	+	<i>Lactobacillus delbrueckii</i>
NE4-5	Creamy-white, smooth elevated colonies and thick long bacilli	-	+	-	-	-	+	+	+	+	<i>Lactobacillus plantarum</i>

Key: ET= endospore test; cat= catalase; Ox= oxidase; In= indole; Nit= nitrate; fru= fructose; mal= maltose; Glu= glucose; += positive; -= negative

4.2. Percentage Occurrence of *Lactobacillus* Isolates

The percentage occurrence of different *Lactobacillus* species isolated from cow milk products (Nono and Kindirmo) sold in Keffi is as shown in Table 3. The highest percentage occurring *Lactobacillus* species from Nono was *Lactobacillus*

plantarum (14.0%) followed by *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* (12.0%), *Lactobacillus crispatus* (8.0%) and *Lactobacillus rhamnosus* (6.0%), similarly from kindirmo the highest was *Lactobacillus delbrueckii* (16.0%) followed by *Lactobacillus crispatus* (14.0%), *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* (10.0%) and *Lactobacillus plantarum* (10.0%).

4.2.1. Antibacterial Activity of *Lactobacillus* species Against Clinical Test Isolates

Table 4 shows the antibacterial activity of *Lactobacillus* species isolates against clinical isolates were KDI *Lactobacillus delbrueckii* have inhibition zone of 16.00mm on *E. coli*, 17.00mm on *S. aureus* and 17.00mm on *S. dysenteriae*. KD2 *Lactobacillus acidophilus* against *E. coli* showed no inhibition zone, against *S. aureus* showed 16.07mm, and against *S. dysenteriae* showed 20.00mm inhibition zone. KE1 *Lactobacillus plantarum* showed inhibition zone of 17.00mm against *E. coli*, 16.00mm against *S. aureus* and 20.00mm against *S. dysenteriae*. KE4 *Lactobacillus plantarum* showed inhibition zone of 15.00mm against *E. coli*, 18.31mm against *S. aureus* and 20.00mm against *S. dysenteriae*. KE5 *Lactobacillus delbrueckii* showed inhibition zone of 15.70mm against *E. coli*, 19.00mm against *S. aureus* and 17.00mm against *S. dysenteriae*. NE1 *Lactobacillus acidophilus* showed inhibition zone of 15.00mm against *E. coli*, 18.00mm against *S. aureus* and 22.00mm against *S. dysenteriae*. NE3 *Lactobacillus delbrueckii* had no inhibition zone against *E. coli*, 18.00mm against *S. aureus* and 22.00mm against *S. dysenteriae*

Table 3 Percentage Occurrence of *Lactobacillus* species Isolated from The Cow Milk Products

Isolates	No sample	Nono (%)	Kindirmo	No sample	Maishanu
<i>Lactobacillus delbrueckii</i>	50	6(12.0)	4(8.0)	25	0(00.0)
<i>Lactobacillus plantarum</i>	50	7(14.0)	8(16.0)	25	0(00.0)
<i>Lactobacillus acidophilus</i>	50	6(12.0)	5(10.0)	25	0(00.0)
<i>Lactobacillus rhamnosus</i>	50	3(6.0)	5(10.0)	25	0(00.0)
<i>Lactobacillus crispatus</i>	50	4(8.0)	7(14.0)	25	0(00.0)

Table 4 Antibacterial Activity of Lactic Acid Bacteria against Clinical Test Organisms

Lactic Acid Bacteria	Mean diameter of inhibition zone (mm) produced by lactic acid bacteria		
	<i>E. coli</i>	<i>S. aureus</i>	<i>S. dysenteriae</i>
KD1	16.00 ^b ±0.57	17.00 ^d ±0.77	17.00 ^d ±0.77
KE1	17.00 ^a ±1.00	16.00 ^e ±0.15	20.00 ^c ±0.50
KE4	15.00 ^c ±1.73	18.31 ^{bc} ±0.57	20.00 ^c ±0.50
NE1	15.00 ^c ±0.00	18.00 ^c ±0.28	22.00 ^b ±0.23
NE3	00.00 ^d ±0.00	18.00 ^c ±0.00	22.00 ^b ±0.23
KE5	15.70 ^{bc} ±0.82	19.00 ^b ±0.06	17.00 ^d ±4.583
KD2	0.00 ^d ±0.00	16.07 ^e ±0.07	20.00 ^c ±0.50
Control	0.00 ^d ±0.00	31.00 ^a ±0.59	29.00 ^a ±2.80

Key: KD1 - *Lactobacillus delbrueckii*, KD2 - *Lactobacillus acidophilus*, KE1 - *Lactobacillus plantarum*, KE4 - *Lactobacillus plantarum*, KE5 - *Lactobacillus delbrueckii*, NE1 - *Lactobacillus acidophilus* and NE3 - *Lactobacillus delbrueckii*. Values are means of duplicate determinations. Means with dissimilar letter (s) differ significantly according to the Least Significant Difference. *Significant at $p \leq 0.05$; **Significant at $p \leq 0.01$

5. Discussion

Cow milk products (*Nono* and *Kindirmo*) served as the source for the isolation of *Lactobacillus* in this study. *Lactobacillus* species are said to be present in dairy products such as cheeses, yoghurts and fermented milk Ranagath *et al.*, [13]. *Nono* and *Kindirmo* are local yoghurt (set and stirred yoghurt) in Nigeria and therefore termed a good source for the isolation

of *Lactobacillus* species. Earlier reports detected the presence of lactic acid bacteria in cow milk products Yelnetty *et al.* [14] and Arimah *et al.* [15]. Therefore, our finding is consistent with the previous reports.

Similarly, *Lactobacillus* species and their final products of metabolism act as bio-preservatives, increasing the shelf-life of foods and reducing the risks of foodborne diseases Arimah *et al.* [15]. Thus, the presence of *Lactobacillus* species may confer desirable qualities and increase the safety of fermented products. The lack of *Lactobacillus* species in Maishanu may not be unconnected to the fact that it is highly processed when compared with *Nono* and *Kindirmo*. Moreso, this product, unlike *Nono* and *Kindirmo* is composed of mainly fat and oil. Therefore, the absence of *Lactobacillus* species in Maishanu is not unexpected.

The three categories of *Lactobacillus* from the present study were identified as *Lactobacillus delbrueckii sp. bulgaricus*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*. Our finding is in line with previous reports from Nigerian indigenous dairy product by Adebayo-Tayo and Fashogbon [16], who opined that *Lactobacillus plantarum*, and *Lactobacillus delbrueckii* are the dominant LAB in Nigeria. The frequency of isolation of *Lactobacillus delbrueckii* in all the cow milk product samples from different location reflects the ubiquitous nature of this bacteria and its ability to survive in the products.

The fact that *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*, *delbrueckii sub sp. bulgaricus*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* predominated all investigated cow milk products from the study area point at the probable importance of incorporating these species in a starter culture for *Nono* and *Kindirmo*. Optimal combination of a *Nono* and *Kindirmo* starter culture will have to be determined by trying different combinations under laboratory and subsequently field conditions.

The continuous increase in multiple resistance pathogenic bacteria particularly in the clinical setting has led to the investigation of natural effective alternatives to known antibiotics. Lactic acid bacteria are well known producers of antimicrobial compounds especially bacteriocins which have high antimicrobial activity. As observed in this study, Table 4 showed zones of inhibition to the various bacteriocins and this indicates the efficacy of these *Lactobacillus* species against the test bacteria used. All LABs isolated from cow milk products possess very good antibacterial activity against target multidrug resistant bacteria with inhibition zone ranging from 15.70mm to 22.00 mm diameter as observed in this study. *Staphylococcus aureus* and *Shigella dysenteriae* were only sensitive to bacteriocin of *Lactobacillus* species isolated from *Kindirmo* and *Nono*. However, only *Escherichia coli* resists *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*. In all the bacteria used, *Shigella dysenteriae* was found to be more susceptible to the *Lactobacillus* species activity were the inhibition zones against the test bacteria range from 15.0 - 22.0mm. Ciprofloxacin used as positive control at the concentration of 30µg had antimicrobial activities against all the bacteria used with varied inhibition zones except for *Escherichia coli*.

The observed antibacterial effect of the isolated LAB could be due to the LAB potential to produce substances with inhibitory activity against members of the families of *Enterobacteriaceae* Maciel *et al.*, [17]. Konings *et al.*, [18] asserts that the action of various antimicrobial compounds produced during the fermentation process, such as lactic acid, acetic acid and propionic acid creates an unfavourable environment for pathogenic microorganisms. Besides, bacteriocins a microbial compound of a proteic nature with bactericidal or bacteriostatic effect have been previously isolated from Lactic acid bacteria.

6. Conclusion

The *Lactobacillus* species were isolated from locally fermented cow milks. The isolated *Lactobacillus* species namely *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* were observed to have inhibitory effect on selected clinical isolates tested.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no competing interests exist.

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