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In vitro evaluation of the broad-spectrum antimicrobial potential of lactic acid bacteria isolated from milk whey and Nunu for veterinary applications

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Abstract
Zoonoses incidences continue to play significant roles in both human and veterinary medicine and their eradication especially in the developing country like Nigeria, is difficult. Zoonotic food-borne infections caused by Gram-positive and Gram-negative bacteria are re-emerging. Several findings indicated that the cell-free supernatant derived from some Lactic Acid Bacteria have some antibacterial effects when applied directly or added to food during packaging. The aim of this study was to investigate the *in vitro* antagonistic effect of cell-free supernatant derived from some LAB species isolated from whey and Nunu (obtained from a locally fermented milk product) against some zoonotic bacteria pathogens (*Escherichia coli*, *Salmonella sp. and Staphylococcus aureus*. *Streptococcus* sp. and *Salmonella* sp. Four (4) LAB isolates were isolated, identified using basic morphological and biochemical characterization and screened on the basis of functional and technological characterization. The Cell-free supernatant from these strains, *Lactobacillus casei* LB01, *Lactobacillus* sp. LB02, *Lactobacillus acidophilus* LB03 and *Lactobacillus plantarum* LB04 were prepared by centrifugation at 10,000 rpm for 5 minutes and used against the indicator organisms at 10^7 CFU/ml using agar well diffusion method. The cell-free supernatant inhibited the growth of both Gram- positive and Gram-negative bacteria strains of veterinary origins. Our findings suggest that the LAB strains have the potential for antibiotic alternatives for the control of several zoonotic diseases. This holds a great advantage in the current efforts to tackling and reducing the prevalence and spread of zoonotic diseases which are re-emerging in Nigeria.

Keywords: Zoonosis, Bio-active, Cell-free supernatant, Antimicrobial, Pathogens and Bacteria

Introduction

In spite of the global scientific and technological applications of principles and safety concepts to solving disease problems such as hazard analysis and critical control point (HACCP) system and other epidemiological control mechanisms, food-borne illnesses and intoxications originating from domesticated animals are on the rise (Karen and Wayne, 2002). For instance, the Council for Agricultural Science and Technology reported that microbial pathogens in food cause an estimated 6.5–33 million cases of human illness and up to 9000 are reported deaths annually and the main foods implicated being meat, poultry, eggs, seafood and dairy products. Among the implicated bacteria pathogens include *Salmonella sp., E. coli, staphylococcus aureus* among others (Nicoline *et al.*, 2015) [2] The world cannot afford not to take the issues of zoonotic diseases seriously owing to the devastating effects Covid-19 had on the global economy and its negative consequences which are yet to be recovered from.

Livestock are important reservoirs of zoonotic infections like brucellosis and Q fever, for which ruminants provide a major source of human infections. A study in Oyo State indicated that cows may be reservoirs of Q fever in Nigeria (Jacques Godfroid, 2017) [3].

In many countries, livestock are also sources of food-borne zoonotic pathogens like *Y. Pseudotuberculosis* and *Y. enterocolitica*, for which pigs, sheep, goats and cattle were identified as principal reservoirs of the pathogenic serotypes of human infection in Nigeria (Norma, 2018) [4]. Also, genomic analysis of *Salmonella enterica* Eko isolates, agents of salmonellosis, collected from different sources in Nigeria showed an association of camels with NTS outbreaks. This implicates camels, along with cattle, as a primary source of local human infections. *Lactobacilli* generally refers to as the Lactic Acid Bacteria (LAB) represent a significant part of intestinal microflora and their role in the general state of human health is being seriously investigated both as probiotics, bioconversion of many resources (fermentations) as well as being candidates of bio-active antimicrobial agents against some pathogenic bacteria (Mduduzi, 2017) [20]. Therefore, this genus (*Lactobacillus)* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance due to many metabolites they produce. Some important strains in this regards include L. *Acidophilus*, closely related species like *Lactobacillus casei*, *L. paracasei* subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus.* One striking characteristic of the *Lactobacilli* is that they are not reported to be associated with disease in both human and animal and as such, have been regarded as non-pathogenic members of intestinal, urogenital and milk fermenting flora (Raquel *et al*., 2014). As a results of the antagonistic interactions with some pathogenic bacteria they have been able to maintain the gastrointestinal ecosystem in a healthy state and this principle is being exploited in the preservation of some fermented food products. Cell-free extracts produced by species of *Lactobacillus* contains some antibacterial compounds such as lactic and other organic acids, hydrogen peroxide (H_2O_2) and bacteriocins are now being harnessed on a larger scale for the control of many zoonotic diseases. Investigation shows that the biologically active, low-molecular weight proteins or peptides produced by the LAB are able to inhibit the growth of a variety of pathogenic bacteria and may be of economic and safety importance if properly harnessed (Marina *et al*., 2021) [7]. However, the potentials of these LAB are strain dependence where different strains from different environment might be able to offer different and possibly better potentials bioactivities. Therefore, the aim of the present investigation is to isolate and identify and screen some indigenous Lactic acid determine the *in-vitro* antimicrobial potentials against some zoonotic disease- causing bacteria.

Materials and Methods

Samples Collection

In order to isolate the Lactic Acid Bacteria strains, samples of locally fermented dairy products; specifically, whey and Nunu were collected at Giri (Federal Capital Territory) and its surroundings. The samples were immediately preserved in an icebox and transported to the laboratory for further analysis. Having sterilized all the ware to be used, 10ml of each sample were aseptically suspended in 90 ml sterile normal saline (0.85% salt solution), homogenized and spread on sterile MRS agar plates and then incubated for 18–24 h at 37 °C (De Gregorio *et al*., 2014) [8].

Isolation of *Lactobacillus* spp.

After the incubation period, discrete colonies were were identified as *Lactobacillus* spp. with the help of various morphological, cultural and biochemical testing techniques as contained in Bergey's manual. The identification methods included simple Gram staining and cell morphological tests. The biochemical testing included catalase, oxidase test, carbohydrate fermentation and milk coagulation. Based on the various tests carried out, the presumptive *Lactobacillus* isolates were further compared with known standard LAB isolates and then preserved aseptically in MRS agar slants, kept at 4^0C until further needed (Juan *et al*., 2015) [10].

Screening of the isolated *Lactobacillus* strains

Determination of optimum pH

In order to examine the optimum pH of the respective isolate, a pH range of 2 to 8 was prepared in MRS broth adjusted with either NaOH (1.0M) or HCl (1.0M) accordingly. After autoclaving, 1% (v/v) overnight grown culture of the respective isolates was introduced into the cooled MRS broth aseptically and incubated at 37 °C for 24 h. After, the growth pattern were monitored by comparing the turbidity with uninoculated broth used as control and the ability of the *Lactobacillus* isolates to grow under different pH values were recorded (Authority EFS (2012) [11].

Bile Salt tolerance Test

The modified method of Gilliland *et al*., 1984 was used to determine the ability of the respective isolate to tolerate salt. MRS broth were prepared to obtain varying bile concentrations from 0.5, 1, 1.5, 2 and 2.5%. Pure culture of the isolates was introduced aseptically after autoclaving and cooling. The set-up was incubated for 24 hours at 37^0C and the growth measured afterwards by comparing the turbidity against uninoculated MRS broth. Bacterial growth was observed by measuring absorbance at 600 nm after 18–24 h of incubation at 37 °C. Bile salt-free MRS broth was used as control for this experiment (Pooja *et al*., 2014) [12].

Sodium Chloride (NaCl) Tolerance Test

All the isolates under test were grown at different sodium Chloride concentrations ranging from 1-6% in MRS broth. After autoclaving the broth mixtures, it was inoculated with an overnight culture of the respective pure isolates aseptically. The set-up was thereafter incubated at 37^0C for 24 hours. Afterwards, the bacteria growth was observed by comparing the turbidity with uninoculated MRS broth (Pooja *et al*., 2014) [12].

Antibiotic Susceptibility Test

The susceptibility test of the isolate to selected antibiotics was performed using agar disk diffusion method with some modifications. Bacteria lawn was made with the suspension of each LAB isolate using sterile swab on Sterile MRS agar. The antibiotics used included Tetracycline-30 µg), ampicillin (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), genta micin (10 µg), ciprofloxacin (5 µg), cephalotin (30 µg), cotri moxazole (25 µg), ceftriaxone (30 µg), amoxicillin (10 µg). The set-up was incubated for 24 hours at 37^0C. After the incubation time, the various zones of inhibitions were recorded and used to prepare data of either resistance or susceptible according to the Clinical Laboratory Standard Institute (CLSI, 2016).

Determination of the Antimicrobial and Antagonistic Activity of Isolates against Zoonotic Pathogens

The agar well diffusion method was used to determine the capacity of the respective isolate to antagonize the growth of some indicator zoonotic bacteria pathogen (Bulik *et al.*, 2010). The selection of the test pathogens was based on their common reported association with veterinary clinical diseases that are transmittable to humans in Nigeria. They were; *Salmonella sp., Escherichia coli, and Staphylococcus aureus* and were obtained at the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria and maintained on blood agar slants after authentication protocol. The cell-free supernatant of each LAB isolate was made by centrifuging overnight grown cultures of the cultures. A 50–100 µl of supernatant was used to fill in 6mm diameter well in Nutrient agar on which the test organisms were laid (lawn). The set-up (in triplicates) was incubated after allowing an hour for the supernatant to diffused into the wells. After incubation at 37^0C for 24 hours, the diameters of zones of inhibitions were measured and recorded (Sarita *et al.*, 2019).

Determination of minimum inhibitory concentration

The swabs of the respective clear zone of the test organism were streaked on to sterile nutrient agar plates to determine growth. From the growth responses, bacteriostatic and bacteriocidal status were classified. Where there was growth of the test organism, the inhibitory activity was interpreted as being bacteriostatic, but where there was no growth, it was referred to as being bactericidal (Andrews, 2001).

Results

Isolation and Identification of LAB Isolates

Whey and Nunu (Locally fermented dairy products) were collected from vendors in Giri and were used for the isolation of Lactic Acid Bacteria (LAB). Out of the total of 18 samples (10 whey and 8 Nunu), 53 discrete pure bacteria cultures were obtained from which 42 were morphologically and biochemically identified to be LAB strains (Table1). That is the results clearly showed Gram-positive, rod shaped, non-motile and catalase negative. However, after further screening for antimicrobial production ability and loss of some cultures, four (4) strains were finally maintained which were designated as; LB01, LB02, LB03 and LB04 and were preserved and used for further applications.

Table1: Origin and Number of isolates from the Dairy sample After Screening

Source of dairy Product	No. of Isolates	No. of LAB sp.
Giri North	13	10
Giri Gwags	10	07
Giri Bridge	05	03
UniAbuja	25	22
Total	53	42

Fig 1: Single Screened and Pure Colonies on MRS Agar.

Table 2: Morphology and Biochemical Characterization of Isolates

Selected Isolate	Morphology/ Cultural Xtics	Gram Rxn	Motility Test	Catalase Test	CHO Glucose	Fermentation Lactose	Test Sucrose	Acid Gas
LB01	small, whitish Smooth and round	+ Bacilli	-	-	+	-	+	+
LB02	Smooth shiny circular	+ Bacilli	-	-	+	-	+	+
LB03	Whitish, shiny round	+ Bacilli	-	-	+	+	+	+
LB04	Grey white Smooth-round	+ Bacilli	-	-	+	-	+	+
+=Positive; - =Negative								

Screening and Characterization of the Isolated *Lactobacillus* Strains

Tolerance Test at different pHs

All the isolated *Lactobacillus* spp. showed growth only at between pH3-5 (Table 3).

Table 3: Tolerance Test of Isolates at Different pHs

Isolate	pH range						
	2	3	4	5	6	7	8
LB01	-	+	+	+	-	-	-
LB02	-	+	+	+	-	-	-
LB03	-	+	+	+	-	-	-
LB04	-	+	+	+	-	-	-

Table 4: Tolerance to Bile Salt and Sodium Chloride (NaCl)

Test	Isolate			
	LB01	LB02	LB03	LB04
Bile salt				
(0.3%)	+	+	+	+
NaCl				
3%	+	+	+	+
5%	+	+	+	+
7%	+	+	+	+
+=Positive; -+ Negative				

The selected isolates were able to tolerate thereby producing growth in 03% bile concentration and 3%-7% sodium concentrations respectively (Table 4)

Antibiotic Susceptibility Test

The results of the antibiotic susceptibility pattern of the selected LAB isolates against ten (10) major antibiotics were used. All the isolates were sensitive to all the tested antibiotics viz; Tetracycline, Amoxyclav, Cephalothin, Erythromycin, Sulfamethizole, Clindamycin, Gentamycin, Vancomycin, Chloramphenicaol and ciprofloxacin.

Antimicrobial Activity Against the Test Pathogens

The antagonistic activity of the cell-free supernatants of the LAB isolates against the test zoonotic pathogens (*Salmonella sp., Escherichia coli, and Staphylococcus aureus)* using the modified agar-well diffusion method showed that all the LAB isolates antagonistic activities though at varying degrees (Fig.2). As presented in Fig.3, the average range of inhibition was between 24-31 mm. In the overall, this value is higher than most reported cases in the literature.

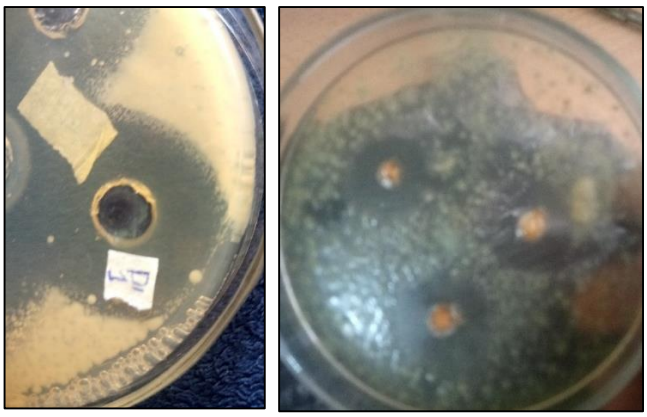

Fig 2: The LAB Cell-free Supernatant Creating Zones of Inhibition against Test Pathogens in Agar Well

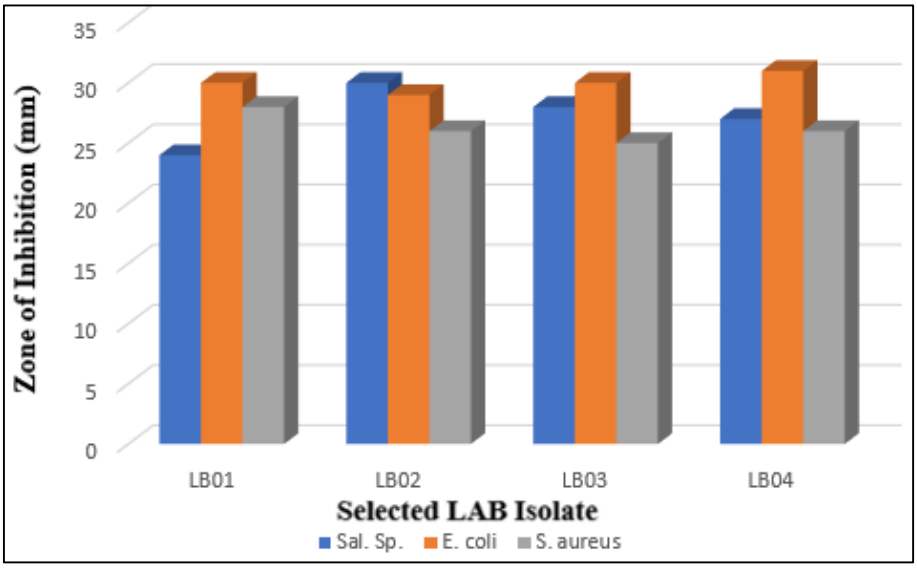

Fig 3: The Antagonistic Activity of the Selected LAB Isolates against the Test Zoonotic Pathogens

Minimal Inhibitory Concentration of Cell-Free Supernatant (CFS) of LAB Isolates

The results of the MIC show that MIC for LB03 isolate was 50 µl against *S. aureus and E. coli*, 100 µl against *Salmonella sp.*; LB01 isolate showed MIC of 60 µl against all the test bacteria; LB04 isolate of 60 µl against E. coli, 50 µl against *Salmonella and S. aureus* and LB02 showed 50 µl against *E. coli and* 80 µl against *Salmonella sp*. and *S. aureus* respectively (Fig.4).

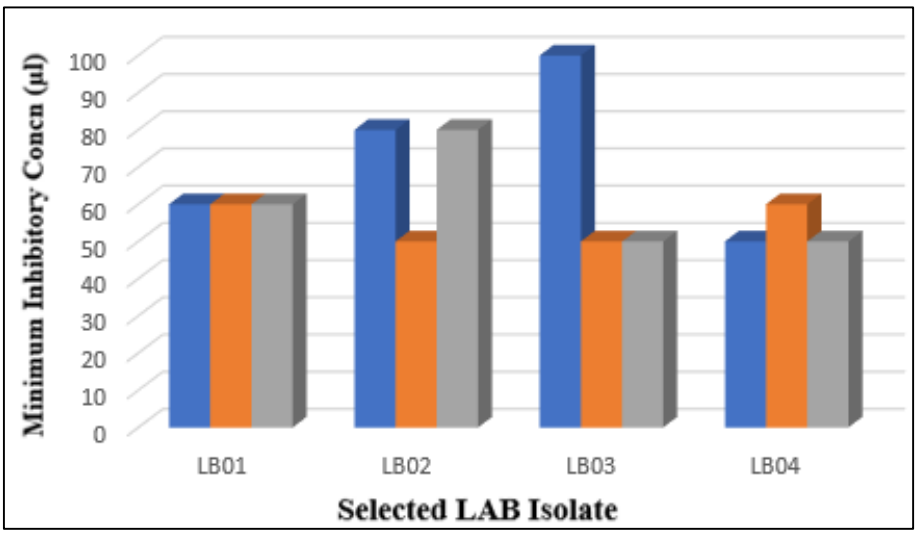

Fig 4: The Minimal Inhibitory Concentration of Cell-Free Supernatant

Discussion

Giving the aim of this research which was to isolate some indigenous Lactic Acid bacteria with antimicrobial activity against some Zoonotic bacteria pathogens, 42 LAB isolates were identified out of the 53 discrete bacteria isolates that appeared on MRS agar after culturing the dairy products based on morphological and biochemical characterization. The bacteria also passed the basic functional and technological criteria of Lactic acid bacteria which include their tolerance towards bile salt, Sodium chloride concentration as well as high pH which mimic the intestinal environment. This is in agreement with several reports including those of Anita *et al*., 2014 in the research on Identification and Characterization of Lactic Acid Bacteria in a Commercial Probiotic Culture. However, in our work, four isolates that exhibited additional high qualities on the based of antimicrobial production tendency were selected for further applications. To be an effective and efficient producer of antimicrobial metabolites which may include low pH and other organic acids, the candidate isolate must be able to pass *in vitro* low pH tests as reported by researchers including Mehmet *et al.*, 2015 in the work titled '*In Vitro* Properties of Potential Probiotic Indigenous Lactic Acid Bacteria Originating from Traditional Pickles'.

As the results in this experiment depicts, all the collected *Lactobacillus* spp. showed high tolerance to the range of pHs and grew well in acidic condition. Bile salts also constitute an important huddle factor which are used to characterize Lactic acid bacteria. The isolate in this result survived the 0.3% bile for more than 6 hours. These combined effects means that the isolated bacteria might be able to out compete many intestinal pathogens in that about 0.3% bile salts available in the human intestinal tract of healthy humans. All the isolated cultures were also able to tolerate 5% of NaCl and this observation is in accord with the work of Sherifah and Efemena, 2018).

Antimicrobial activity is one of the most important selection criteria for effective and novel probiotics status of LAB. It is documented that most bacteria isolates are able to secure their habitats against other organisms by being able to create unconducive environment through metabolite production (Mduduzi, 2017) [20]. Some acquire the antimicrobial effects by producing some substances, such as organic acids (lactic, acetic, propionic acids, succinic acid, etc.), hydrogen peroxide, low-molecular weight antimicrobial substances and bacteriocins. This might be the major metabolite the isolates in this research used against the test organisms as experienced in the course of research by Mokoena *et al.*, 2016).

Conclusion and Recommendations

The set of Lactic acid bacteria strains isolated from whey and Nunu in this work all have *in vitro* antimicrobial activity thereby making them potential candidates for the control of some zoonotic diseases. The emergence and re-emergence of zoonotic pathogen globally call for basic review of the potencies of the conventional antibiotics with a view to shifting towards the discovery and application of novel bio-active components of microbial origins. However, since the supernatants were tested at in vitro scale, it should also be considered for in vivo assay and documentation. This result also suggest and support more ground work for the formulation of novel antimicrobial agents using both conventional and bio-active constituents for better results.

Conflict of Interest

There is none regarding this work.

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