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ANTIBACTERIAL ACTIVITY OF SOME LACTIC ACID BACTERIA ISOLATED FROM A LOCAL FERMENTED MILK PRODUCT (*PENDIDAM*) IN NGAOUNDERE, CAMEROON

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ABSTRACT

Fermented milk products are known to have potential activity against gastro-intestinal infections, food-borne pathogens and food-borne spoilage micro-organisms. The objectives of this study were to isolate lactic acid bacteria (LAB) as ferments present in pendidam (a locally fermented milk product), evaluate the antibacterial activity of the isolates against *Escherichia coli* and *Staphylococcus aureus* and partially characterise substances of the cell-free supernatant responsible for antibacterial activity. Twenty (20) strains of lactic acid bacteria (LAB) were isolated from 3 of 5 samples of pendidam collected from the locality of Wakwa (Ngaoundere), were identified based on their morphological, physiological and biochemical characteristics and were screened for their antibacterial activity using the well diffusion method on agar medium. A total of 16 *Lactobacillus* spp. and 4 *Streptococcus* species were identified. All the lactic acid bacteria (LAB) strains were active against *Staphylococcus aureus* whereas 6 of them were not active against *Escherichia coli*. The most active strains against both pathogens were L18 for *E. coli* and L19 for *S. aureus,* all belonging to *Lactobacillus* species and having a maximum inhibition zone of (4.5±0.1) mm. The inhibitory effect of neutralised cell-free supernatants of 10 isolates and thermal sensitivity of the supernatants of five active strains were studied. The results obtained did not show the complete loss of antibacterial activity of the extracts. This is an indication that the tested lactic acid bacterial strains secreted substances responsible for the antagonistic action observed on the pathogens and which will be further identified. This preliminarily work shows the potential application of autochthonous lactic acid bacteria or related substances to improve foods preservation.

Key words: Fermented milk, lactic acid bacteria, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli.*

INTRODUCTION

Micro-organisms are used in the production of fermented food products such as yoghurt (*Streptococcus* spp. and *Lactobacillus* spp.) cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.), gari, beer, wine, cocoa, coffee and sausage. Among them are lactic acid bacteria (LAB) which form a phylogenetically diverse group and are defined as Gram-positive, non-spore forming, catalase-negative, devoid of cytochromes, of anaerobic habit but aero-tolerant, fastidious, acid-tolerant, and strictly fermentative bacteria that secrete lactic acid as the major end product of sugar fermentation (Axelsson, 1998). In some food ecosystems, lactic acid bacteria (LAB) constitutes the dominant microflora. These organisms are able to produce antimicrobial compounds against competing flora, including pathogenic and food-borne spoilage bacteria (Daeschel, 1989; Davidson and Hoover, 1993). The primary antimicrobial effect exerted by lactic acid bacteria (LAB) is the production of lactic acid and reduction of pH (Daeschel, 1989). In addition, lactic acid bacteria (LAB) produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), organic acids, uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocins (Piard and Desmazeaud 1991, Ouwehand, 1998). It is the acidification process which is one of the most desirable side-effects of their growth. The pH may drop to as low as 4.0, low enough to inhibit the growth of most other microorganisms including the most common human enteric pathogens like *E. coli*, *Staphylococcus* and *Salmonella* species; hence their antibacterial effect which render foods prolonged shelf life. The search for new antimicrobial agents is a field of utmost importance since the prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide (Singer *et al*., 2003). Gastro-enteritis is equally a major public health problem in the world especially among developing countries with diarrhoea being one of the leading causes of mortality and morbidity (Bern *et al*., 1992; Kosek *et al*., 2003). Lactic acid bacteria (LAB) have been evaluated in research studies in animals, in humans and on foods such that it is possible to use lactic acid bacteria (LAB) for the treatment of different gastrointestinal diseases **(**Ljungh and Wadstrom, 2009) and for foods preservation (Labioui *et al*., 2005; Cocolin *et al*., 2007; Dortu and Thonart, 2009; Kouakou and Thonart, 2011) due to their antibacterial properties. Pendidam, a local fermented milk product, is highly consumed by the indigenes in the

northern part of Cameroon. It was noticed from a survey that in the Adamawa region, children suffering from stomach problems become well after consuming pendidam. Nevertheless, its capacity in combating gastro-invasive pathogens is yet to be established and made known to the consumers.

The main objective of this work is the microbiological valorisation of pendidam. Specifically, this project was aimed to isolate lactic acid bacteria (LAB) as ferments present in pendidam, evaluate of the antibacterial activity of isolated lactic acid bacteria (LAB) strains against the growth of *Escherichia coli* and *Staphylococcus aureus* and finally to characterise partially some substances responsible for antimicrobial activity. Hypothetically, we have to verify that pendidam lactic acid bacteria (LAB) exerts antibacterial effects and that the antibacterial activity of pendidam lactic acid bacteria (LAB)'s is due to its acidity and other active substances.

MATERIALS AND METHODS

This study was conducted from March 2009 to November 2010 in the Laboratory of Microbiology and Biotechnology of the National School of Agro-Industrial Sciences of the University of Ngaoundere (Cameroon).

Sampling: Five samples of pendidam and a fresh cow's milk sample were collected in sterile tubes from local producers at Wakwa Ngaoundere (Cameroon) and stored in a cooler during the transport. Samples which were not analysed immediately were kept in the refrigerator at 5ºC.

Origin of test micro-organisms: For testing antibacterial activity, food-borne pathogens *Staphylococcus aureus* CIP7625 and *Escherichia coli* provided by the Laboratory of Microbiology and Biotechnology of the National School of Agro-industrial Sciences (ENSAI, University of Ngaoundere, Cameroon) were used.

Determination of the acidity of pendidam samples: Dornic acidity was determined by titrating 10 ml of pendidam samples against 0.1N NaOH in a burette by addition of 3 drops of 0.5% phenolphthalein indicator and the determination of pH was done by dipping a pH metre (pH^{cp} ® H198128 by HANNA) in 5ml each of pendidam samples until readings were stable.

Preliminary antibacterial test: This was carried out by a modification of the well diffusion technique as used by Mbawala *et al*. (2009) on agar medium. It was aimed at selecting the most active samples prior to lactic acid bacteria assay as follows: to 15 ml of TSA-YE medium (Trypticase Soja Agar-Yeast Extract, Liofilchem Diagnostici, Italy) supplemented with tween-80 (1 g/l), were added 15 µl each of an 18 hrs pre-culture *E. coli* and *Staphylococcus aureus* obtained in TSB-YE (Trypticase Soja Broth-Yeast Extract, Liofilchem Diagnostici, Italy) after successive purification by streaking on their respective culture media (E.M.B and Mannitol Salt Agar medium (Chapman) (Liofilchem Diagnostici, Italy) respectively, each pathogen were mixed separately and thoroughly. They were poured into sterile Petri dishes and after solidification of the entire medium, six wells of 6 mm diameter each were created with the aid of Pasteur pipettes. Five of the wells were loaded with 25 µl of pendidam samples (Previously heated in a water bath at 40ºC so as to melt fats globules, then cooled to about 25ºC) and the remaining ones with 25 µl of fresh cow's milk, as control using a micropipette. The dishes were kept in the refrigerator at 5ºC for 24 hrs after which they were removed and incubated at 37ºC for 18 hrs. The diameter (in mm) of the zone of growth inhibition around each well were evaluated considering total zone minus the diameter of the well (6 mm). An inhibitory zone of less than 6 mm diameter correspond to lack of activity of the sample. Statistical analysis of results was done by using STATGRAPHICS Plus 5.0. The factorial design used was 5x2 comprised of 5 samples and 2 test strains.

Isolation of lactic acid bacteria from pendidam: Aseptically, 1 ml of each sample were added into 9 ml of sterile 0.9% NaCl solution (diluent) and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-7}) were performed and 0.1 ml aliquots of the appropriate dilutions were surface-plated in triplicate on MRS agar (Liofilchem Diagnostici, Italy) (De Man *et al.*, 1960), incubated anaerobically at 42ºC for 48 hrs for isolation of thermophilic Lactobacilli and Streptococci and other LAB strains. MRS agar plates were equally incubated anaerobically at 35ºC for 48 hrs for isolation of mesophilic Lactobacilli and Leuconostoc. Colonies from plates of MRS (35ºC) and MRS (42ºC) were randomly picked and isolates cultivated in MRS broth (Liofilchem Diagnostici, Italy) separately (that is, each of the isolates were cultivated in MRS broth at 37ºC separately). Purity was checked by streaking on MRS agar. The pure isolates were cultivated in MRS broth at 37ºC for 18 hrs and used in the experiment. Each of the isolates were inoculated in three slants without reloading the platinum handle (inoculation needle) in 3 tubes containing inclined MRS agar and incubated at 37°C for 48 hrs then kept at 5°C in the refrigerator for subsequent use if need be.

Partial identification of lactic acid bacteria isolated from pendidam: This was based on some biochemical characteristics. In this assay, Gram-positive, catalase-negative, peroxidase-negative and oxidase-negative isolates from MRS agar were partially assigned to a genus on basis of some key characteristics and tests (Weiss, 1992). Growth temperature of the isolates was determined at 10, 37 and 45ºC in 5 ml MRS broth by visual turbidity after 72 hrs incubation. The ability of the isolated LAB strains to tolerate salt were examined in 5 ml MRS broth, containing 6.5% (w/v) NaCl dispensed in

test tubes with incubation period of 4 days at 37ºC by visual turbidity if growth occurs , or otherwise. Homo-hetero fermentation tests were done using Sperber and Swans' test (Larpent, 1997). Homofermentative and heterofermentative strains were differentiated using the ability of heterofermentative bacteria to produce carbon dioxide in an appropriate medium (Mac Cleskey medium, Liofilchem Diagnostici, Italy) and accumulate it in Durham' tube after a treatment of 5 min at 80°C. Positive growth in acetate agar were used to distinguish the Lactobacilli from others genus. Sugar fermentation test was done on specific sugar as melibiose.

Susceptibility tests

Activity of isolated lactic acid bacteria on pathogens: To 15 ml of TSA-YE medium (Trypticase Soja Agar-Yeast Extract, Liofilchem Diagnostici, Italy) + tween- 80 (1g/l) were added 15 µl each of an 18 hrs pre-culture *E. coli* and *Staphylococcus aureus* obtained in TSB-YE (Trypticase Soja Broth-Yeast Extract, Liofilchem Diagnostici, Italy) by means of sterile 25 ml tubes. Each of the pathogens were separately and thoroughly mixed using a vortex and poured into sterile Petri dishes in triplicates. After solidification of the entire medium, six wells of 6 mm diameter each were created using Pasteur pipettes. Five of the wells were loaded with 25 µl, each with the isolated LAB strains (coded L1 to L20) and the remaining ones with 25 µl un-inoculated MRS broth, as control using a micropipette. The dishes were kept in the refrigerator at 5ºC for 24 hrs after which they were removed and incubated at 37ºC for 18 hrs. The diameter (in mm) of the zone of growth inhibition around each well were evaluated considering total zone minus the diameter of the well (6 mm). An inhibitory zone of less than 6 mm diameter correspond to lack of activity of the LAB strain.

Activity of cell- free supernatant on pathogens: This was done by partially characterising inhibitory substances in supernatant as follows: ten of the 20 isolated LAB strains possessing best of activity were selected and grown in MRS broth for 18 to 24 hrs at 37ºC and the cell-free supernatants (CFS) were obtained by filtration using membrane filters (Sartorius, 0.45 µm). Some filtrates were neutralised with 3N NaOH to pH 7.0 with the aid of filter strips so as to eliminate the effect of organic acid. The filtrates which were neutralised and un-neutralised (pH = 4.5, measured using a pH metre) were then used for the antibacterial activity using the agar well diffusion method as previously described. In this respect, 15 µl each of an 18 hrs pre-culture obtained in TSB-YE (Trypticase Soja Broth-Yeast Extract, Liofilchem Diagnostici, Italy) by means of sterile 25 ml tubes, of the pathogenic strain with the following concentrations: *E. coli*= $2.5x10^4$CFU/ml, and *S. aureus*= $63x10^4$CFU/ml were suspended in 15 ml of TSA-YE medium (Trypticase Soja Agar-Yeast Extract, Liofilchem Diagnostici, Italy) + tween 80 (1g/l), well stirred , poured into Petri dishes, wells created, then incubated at 37ºC for 18 hrs after solidification and introduction of 25 µl of the mentioned CFS to determine the zone of inhibition.

Activity of heat-treated cell-free supernatant (CFS) on pathogens: Thermal sensitivity of the LAB strains were accessed by heating 5 ml aliquots of cell-free supernatant (CFS) at 30, 60, 80 and 100ºC for 15 min prior to antibacterial activity evaluation in a water bath. The heated CFS were assessed against pre-cultured pathogens in TSB-YE using the well diffusion method previously described, after which inhibition zones were measured, results recorded and analysed statistically using STATGRAPHICS Plus5.0 and MS-Excel.

RESULTS AND DISCUSSION

Acidity of pendidam samples: Results obtained for the acidity of our different samples are represented in Figure 1. It can be deduced from these results that the pH vary from 4.01± 0.07 (sample 4) to 4.47 ± 0.11 (for sample 2). ANOVA showed that there is a significant ($P<0.05$) difference between these pH at 95% confidence level ($P = 0.05$). It can equally be deduced from the results that the values of titratable acidity vary from 94.3 ± 12.7▫D, for sample 2 to 109.7 ± 6.4▫D for sample 4. The samples showed an inverse relationship between titratable acidity and pH.

These results are different from that of Libouga *et al*. (2005) who worked on the quality of some Cameroonian fermented milk. Their samples were collected at pilot centres based in Ngaoundere, and they obtained titratable acidities ranging from 60 – 90 °D. The high acidity of our samples may probably be linked to the production process. Productions were solely traditional with little or no control. The starter cultures (ferment) used for fermentation were essentially old (over-aged) pendidam, whereas in pilot centres, commercial ferments are mostly used and the process is standardised. Also, Jiwoua (1990) postulated that the age of any fermented milk product will affect its acidity, that is, the more aged the product, the higher its acidity.

Preliminary antibacterial test of pendidam against pathogens: This test was performed in order to evaluate the potential of the fermented milk product (pendidam) to inhibit *Staphylococcus aureus* ($63x10^4$ CFU/ml) and *Escherichia coli* ($2.5x10^4$CFU/ml) and the consequent selection of more active ones for the isolation of lactic acid bacteria. The results showed an average inhibition zone of *Staphylococcus aureus* ranging from 2.5 mm (sample P1) to 4.5 mm (samples P4 and P5) and *Escherichia coli* ranging from 2.5 mm for sample P1 to 5.7 mm for sample P2. Statistical analyses by comparing analysis of variance with one-way ANOVA using

STATGRAHICS Plus 5.0 showed a statistically significant differences (P<0.05) between each pair of means at 95.0% confidence level between the means of the zones of inhibition of *Staphylococcus aureus*. On the other hand, the same analyses showed no significant difference between the means of *Escherichia coli* at the 95.0% confidence level (P<0.05). Figure 2 gives the inhibition zones samples against the test pathogens, with their corresponding P–values indicating significance differences at 95.0% confidence level.

Though there was no statistical significant difference among the samples on the inhibition of *E. coli*, inhibition of *S. aureus* varied significantly for some samples. Samples P2 and P4 were the most active against *S. aureus* since they showed higher inhibitory effects, while sample P1 was the least active. Based on their respective activities, the less active samples (P1 and P3) were dropped, whereas the more active samples (P2, P4 and P5) were selected for the isolation of strains of Lactic Acid Bacteria. Figure 3 is a photograph of the inhibitory effect of pendidam samples against *Escherichia coli* and *Staphylococcus aureus.*

Isolation of lactic acid bacteria from pendidam: After inoculation of appropriate dilutions of samples P2, P4 and P5 on MRS agar media, a total of 20 different LAB strains were isolated. Isolation was based on the catalase, peroxidase, oxidase, gram staining tests, and their microscopic shapes. The specific results are shown in Table 1. It was observed that 3 LAB strains (L9, L10, L11) from sample P2 where obtained at 35°C and 4 (L12, L13, L14 L15) at 42°C. From sample P4, five (L16, L17, L18, L19, L20) were isolated at 35°C and none at 42°C. With respect to sample P5, five (L4, L5, L6, L7, L8) LAB strains were isolated at 35°C and three (L1, L2, L3) at 42°C, making a total of 13 LAB isolated at 35°C and 7 LAB at 42°C. From Table 1, all the strains (L1 to L20) were catalase negative, peroxidase negative, oxidase negative and gram positive. Sixteen of the 20 strains were rod-shaped of varying lengths while four were cocci (L12, L13, L14 and L15). The potential of milk as LAB substrate had been demonstrated in an earlier study on fermented milk in Burkina Faso which reported 98 LAB strains over 100 strains isolated (Aly *et al*., 2004). In this present study, LAB isolates from MRS agar were classified as *Lactobacillus* spp. (16 strains) based on its rod shape whereas four can be classified as *Streptococcus* spp., all based on their spherical shape. In accordance with Savadogo *et al.* (2004), who worked on fermented milk of Burkina Faso, lactic acid bacteria are said to be gram positive, catalase negative, oxidase negative, peroxidase negative cocci, cocco-bacilli or rod shaped isolates with characteristic cell arrangements when grown on MRS growth media and that bacteria with these characteristics are said to be Lactic Acid Bacteria. From the works of Bottazzi (1998), lactobacilli and other LAB genera can be well found and isolated from milk and dairy products, be it fermented milk products. These findings were equally inline with the observations of others who worked on fermented milk, Almaz *et al.* (1999) in Ethiopia, Ayad *et al.* (2004) in Egypt. In addition to the cell morphology, gram stain, colony size and others biochemical characteristics of the isolated LAB strains are mentioned in table 1. We can, from the simplified dichotomous key (Federighi, 2005), together with the other characteristics appreciate 16 strains of bacilli and 4 strains of cocci (Table 1). To be more explicit, none of the isolates grew at 10ºC; all grew at 37ºC and varied growths were observed at 45ºC. Since no growth was observed at 10ºC but at 45ºC, the homofermentative and chained nature of the cocci were tentatively grouped to belong to the genus Streptococci (L12, L13, L14 and L15) whereas the rods were Lactobacilli (16). Lactobacilli which showed growth in acetate agar were further classified into subgroups based on their growth temperatures, gas production and growth in 6.5% sodium chloride as belonging to group I Lactobacilli (thermobacterium) which are homofermentative thermophiles (L17, L18, L19, L20); group II (streptobacterium) which are homofermentative mesophiles (Bottazzi, 1998) represented here by L6, L7 and group III Lactobacilli (beta-bacterium) which are either heterofermentative mesophiles or thermophiles (Kandler and Weiss, 1986) being L1, L2, L3, L4, L5, L8, L9, L10, L11 and L16.

Susceptibility tests

Lactic acid bacteria strains against test micro-organisms*:* Figure 4 shows the inhibitory activity of the 20 identified Lactic acid bacterial strains against *Staphylococcus aureus* and *Escherichia coli*. For both *S. aureus* and *E. coli*, inhibition by some strains were statistically different (P<0.05).

According to the Figure 4, the most active strain against *S. aureus* was L19 with an average inhibition zone of (4.5±0.1) mm, followed by L20 with an average inhibition zone of (4.0±0.0) mm and L4, L13 and L18 with average inhibition zones of 3.5±0.1, 3.5±0.1 and (3.5±0.0) mm respectively. The least active strains against *S. aureus* were L2, L6, L10, L14 and L15, which showed an equal inhibitory potential with an average inhibition zone of (1.0±0.0) mm. For *E. coli*, the most active strain was L18 with an average inhibition zone of (4.5±0.1) mm, followed by L19 with an average inhibition zone of (4.0±0.0) mm and L9 with an average inhibition zone of (3.5±0.3) mm. Lactic acid bacteria strains L1, L2, L3, L4, L14 and L15 showed no inhibitory effect against *E. coli*. *Escherichia coli* strains were not sensitive to inhibitory substances produced by the lactic acid bacteria as compared to the other indicator strains. The resistance of Gram negative bacteria is attributed to the particular nature of their cellular envelop, the

mechanisms of action described for bacteriocin bringing in phenomenon of adsorption. According to Bhunia *et al*. (1991) the pediocin (bacteriocin produced by *Pediococcus acidolactis*) interact with lipoteichoic acids that are absent in Gram-negative bacteria. As the results indicate, the zones of inhibition were varied ranging between 1 to 4.5 mm. This revealed that the LAB inhibited pathogenic bacteria tested according to Schillinger and Lucke (1989) who mentioned that inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 0.5 mm or larger. Similar studies were carried out by Al-Allaf *et al*. (2009) who worked on the antimicrobial activity of lactic acid bacteria against some pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp. etc). Their results showed that the LAB could inhibit these pathogens by developing a zone around the wells which contains these lactic acid bacteria with the zone of inhibition ranging between 0.6 to 4 mm. This inhibition is possibly due to the fact that Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins in the course of their metabolism Oyetayo *et al*. (2003). Considering both microbes, lactic acid bacteria strains L18, L19 and L20 were relatively more active than the other strains.

The effects of un-neutralised and neutralised cell-free supernatant (CFS) against *Staphylococcus aureus* and *Escherichia coli*: From preliminary susceptibility tests with the 20 strains, cell-free supernatant obtained after filtration of 10 active strains were tested against *S. aureus* and *E. coli*. The supernatants were tested on one hand at their normal acidic pH (4.5) and on the other hand at a neutral pH. The results obtained are represented in Figure 5. It shows that there are statistically significant differences ($P<0.05$) between the mean neutralised and un-neutralised CFS on *S. aureus* and *E. coli* from one level of LAB Strain to another at the 95.0% confidence level. The average inhibitory zones of the un-neutralised CFS (at pH 4.5) for both pathogens ranged from 0 to 4 mm and 0 to 3.5 mm at neutral pH. Un-neutralised CFS of LAB strains L9 and L19 showed the extreme inhibitory zone ranging from 2.5 ± 0.1 to (4.0 ± 0.0) mm respectively, for *S. aureus* while those of L4 and L19 strains showed the values of 0 to (3.5 ± 0.1) mm respectively, for *E. coli*.

At neutral pH, both *Staphylococcus aureus* and *Escherichia coli* were never inhibited by the CFS of L4, L8 and L11 whereas there were slide reduction of antibacterial activity in the rest of the LAB strains with L19 exhibiting the highest inhibitory effect of (3.5 ± 0.1) mm against *S. aureus* and (3.0± 0.3) mm against *E. coli*. The fact that no inhibition was noticed by L4, L8 and L11 against both pathogens is an indication that their initial activities were due to organic acid secretion such as lactic acid. On the other hand, the reduction of activity in the rest of the strains is an indication of the presence of other antibacterial substances such as peroxides, diacetyls and bacteriocins. These observations are in agreement with those reported by Ogunbanwo *et al*. (2003) who showed that *L. brevis* excreted other compounds such as bacteriocins that inhibited the growth of pathogens as well as Tatsadjieu *et al*. (2009) who showed that some LAB strains did inhibit the growth of *Salmonella enterica* CIP8132 and *Escherichia coli* CIP548.

Activity of heat-treated cell-free supernatant (CFS) on pathogens: Figure 6 shows the effect of treating the CFS of 5 selected lactic bacterial strains isolated from pendidam against *S. aureus* and *E. coli*. We can observe from it that an augmentation in temperature led to a decrease in antimicrobial activity of most of the CFS samples except that of L17 which do not varied significantly. Analysis of variance with ANOVA using STATGRAHICS Plus 5.0 revealed that there is a statistically significant difference between the means of *S. aureus* at 30, 60, 80 and 100°C from one level of LAB strain to another at the 95.0% confidence level. Multiple column tests were equally performed to determine which means are significantly different from one row to the other at the various temperatures. This indicated a significant reduction of activity from 30 to 100°C except for strain L17 both for *Staphylococcus aureus* and *Escherichia coli.*

The antagonistic effect for strain L17 (which was constant) is possibly due to the absence of bacteriocins or may contain a heat stable bacteriocin. Its antibacterial effect could thus be attributed to the presence of other antimicrobials like peroxides and organic acids. The observed reduction in antibacterial activity shown by the other LAB strains (L13, L17, L18, L19 and L20) against the test microbes suggest the presence of active molecules other than diacetyls, peroxides and organic acids called bacteriocins due to the notion that proteins are denatured at higher temperatures. It is also possible that the L17 strain produce bacteriocins which exhibit a thermal stability. This had been demonstrated for bacteriocin produced by *L. brevis* OG1 which showed thermal stability during 60 min at 100°C (Ogunbanwo *et al*., 2003). L13, L18, L19 and L20 therefore are probable to contain antibacterial agents other than low molecular mass (LMM) antimicrobials. Other LMM compounds that have been reported are often active at low pH and are heat stable with a broad spectrum of activity (Ouweland, 1998). However, it is not clear whether the antimicrobial effects are caused by these compounds or due to primary metabolites such as lactic and acetic acids, hydrogen peroxide, etc. Lortie *et al*. (1993) reported the production of LMM inhibitory substances by *Lb. casei* strains, the activity being possibly due to the effects of small antibiotics, peptides,

Table 1: Some morphological, physiological and biochemical characteristics of the isolated LAB strains

LAB strains	Pendi-dam sample	Tempera-ture of isolation (°C)	Colony size (mm)	Gram stain	Shape of LAB	CAT	PEROX	OX	Growth at 10ºC	Growth at 37ºC	Growth at 45ºC	Growth in 6.5% NaCl	Gas production from sugar	Growth in acetate agar	Genus
L1	P5	42	2	+	isolated, paired, short - chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L2	P5	42	1.5	+	straight-chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L3	P5	42	1.5	+	long chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L4	P5	35	2.5	+	short -straight chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L5	P5	35	1	+	very short single, chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L6	P5	35	2.5	+	short-chained rods	-	-	-	-	+	-	-	±	+	*Lactobacillus*
L7	P5	35	2.5	+	short rods	-	-	-	-	+	-	-	±	+	*Lactobacillus*
L8	P5	35	1.5	+	straight- long rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L9	P2	35	3	+	short-chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L10	P2	35	1	+	very sort chained rods, in clusters	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L11	P2	35	1.5	+	short chained rods, mostly isolated	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L12	P2	42	1	+	chained cocci , in clusters	-	-	-	-	+	+	-	-	ND	*Streptococcus*
L13	P2	42	0.5	+	chained cocci , in clusters	-	-	-	-	+	+	-	-	ND	*Streptococcus*
L14	P2	42	0.5	+	single, diploid, chained cocci	-	-	-	-	+	+	-	-	ND	*Streptococcus*
L15	P2	42	0.4	+	clustered chained cocci	-	-	-	-	+	+	-	-	ND	*Streptococcus*
L16	P4	35	3	+	short-chained rods	-	-	-	-	+	+	-	+	+	*Lactobacillus*
L17	P4	35	1.8	+	short rods in clusters	-	-	-	-	+	+	+	-	+	*Lactobacillus*
L18	P4	35	0.5	+	short-chained rods	-	-	-	-	+	+	+	-	+	*Lactobacillus*
L19	P4	35	1	+	long-chained rods	-	-	-	-	+	+	+	-	+	*Lactobacillus*
L20	P4	35	1	+	averagely long chained rods	-	-	-	-	+	+	+	-	+	*Lactobacillus*

LAB= lactic acid bacteria; CAT = Catalase; PEROX = Peroxidase; OX = Oxidase; + = positive; - = negative; ± = variable; ND = not determined in the medium
N.B.: Lactobaccilli were tested in MRS broth whereas cocci were tested in M17 for gas production from sugar (melibiose).

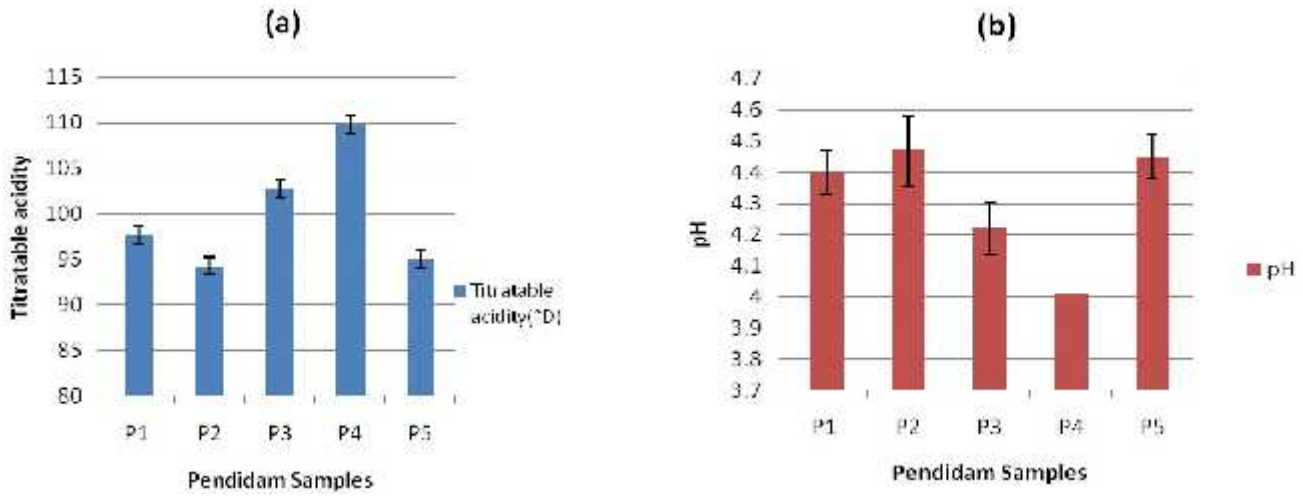


Figure 1: Evaluation of (a) titratable acidity and (b) pH of pendidam samples.

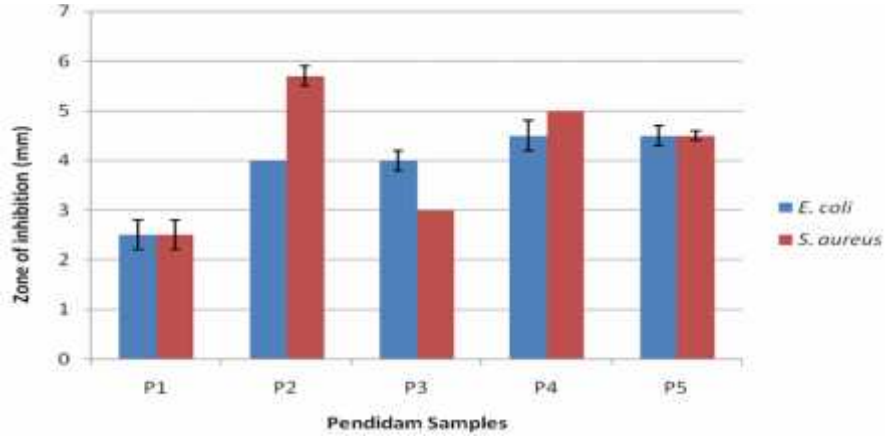
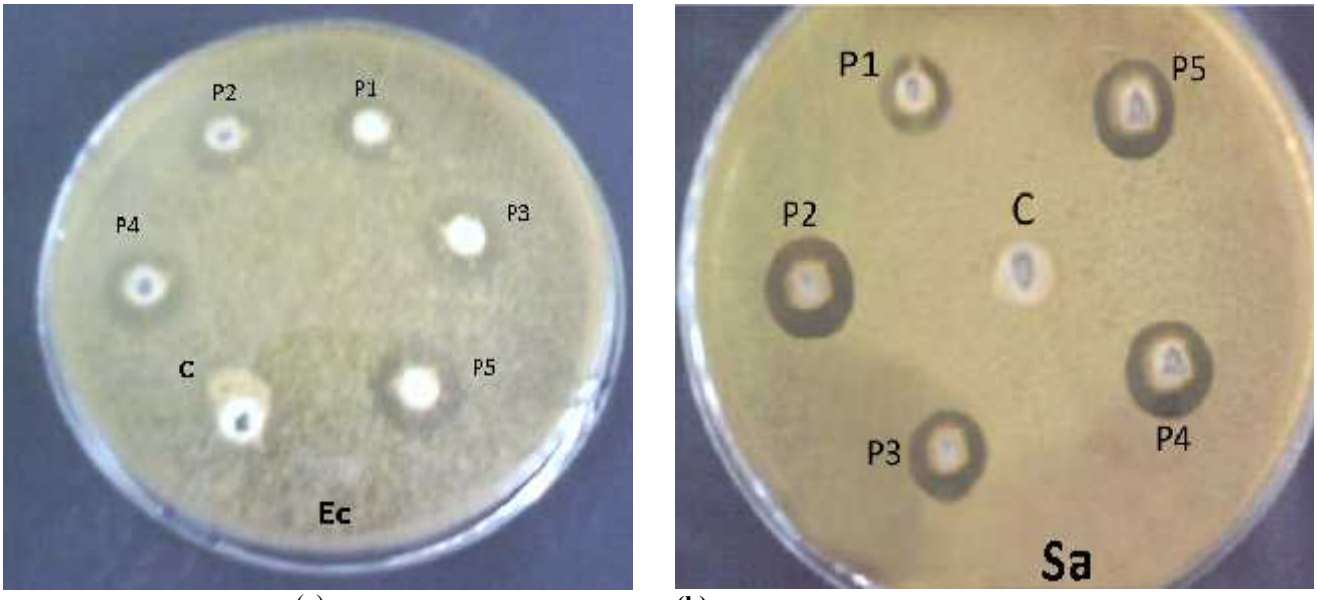


Figure 2: Effect of pendidam samples on the growth of *S. aureus* and *E. coli*.



(a) **(b)**

***Figure 3: Evaluation of inhibitory effect of pendidam samples against (a)* E. coli *and (b)* S. aureus.** *P1 to P5 = pendidam samples; C = control (fresh milk sample).*

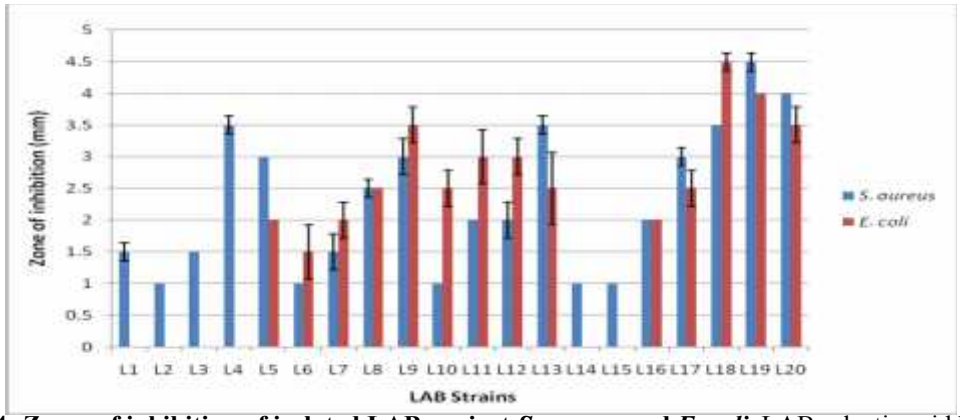


Figure 4: Zones of inhibition of isolated LAB against *S. aureus* and *E. coli.* LAB = lactic acid bacteria.

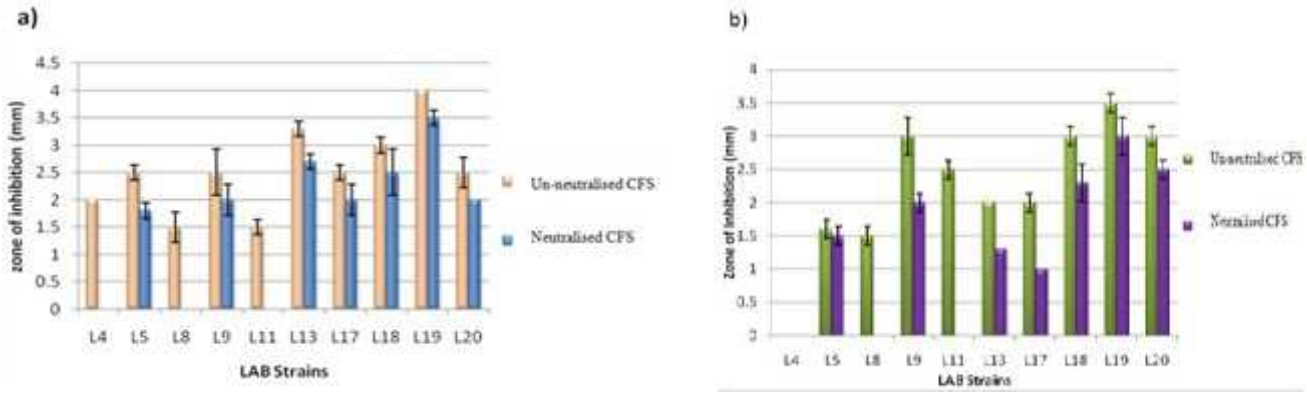


Figure 5: Effect of un-neutralised and neutralised Cell-Free Supernatant (CFS) against (a) *S. aureus* (b) *E. coli*. LAB = lactic acid bacteria.

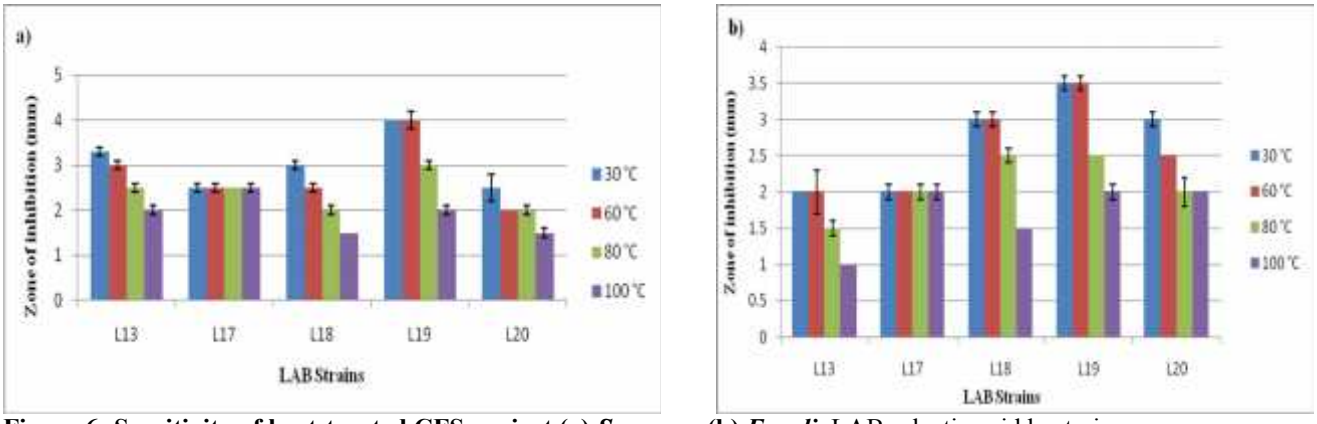


Figure 6: Sensitivity of heat-treated CFS against (a) *S. aureus* (b) *E. coli*. LAB = lactic acid bacteria.

short-chain fatty acids and lactic acid. Treatment of the cell-free supernatant with heat has thus revealed the presence of active peptide molecules which could be characterized as bacteriocins.

Conclusion: In the present study, the cultures from twenty strains of lactic acid bacteria isolated from pendidam exhibited antimicrobial activity against two indicator test strains (*Staphylococcus aureus* and *Escherichia coli*). The classification of the isolated strains according to genus level only showed that they were mainly Lactobacilli (16 strains) and few Streptococci (4 strains) with the most active strain against *S. aureus* being L19 while the least active were L2, L6, L10, L14 and L15. The most active strain of Lactic acid bacteria against *E. coli* was L18 while L1, L2, L3, L4, L14 and L15 strains showed no inhibitory effect on this pathogen. The cocci were therefore less active than their

corresponding rod-shaped counterparts. The inhibitory effect of neutralised cell-free supernatant of 10 isolates and thermal sensitivity of five selected strains did not result in the complete loss of the antibacterial activity following their assessment. This last result is an indication that the lactic acid bacteria strains secreted active substances other than organic acids responsible for the antagonistic action observed in the pathogens such as, peroxides, diacetyls and bacteriocins among others. From the results obtained, pendidam with its natural lactic acid bacteria or their related actives substances can be used as an antibacterial source of products.

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