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Antibiotic Susceptibility and Plasmid Profile of *Lactobacillus* Species Isolated from Yoghurt and Cheese retailed in Ado Ekiti. Ekiti state, Nigeria

Oluyege, Adekemi O., [1]Ogunmoroti, Oluwole S., [2]Akoja, Sunday O. and [3]Adeniyi Olusola B.
*[12]Department of Microbiology, Faculty of Science, Ekiti State University, Ekiti State. Nigeria.
[3]Department of Microbiology, Faculty of Science, Federal Polytechnic, Ado Ekiti, Ekiti State
*Corresponding author: Ogunmoroti, O. S., Email: oluwole1olade2@gmail.com
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ABSTRACT:
Lactobacillus species are involved in the production of cheese and yoghurt and are consumed normally along with the food. However, they have the potential of harboring antibiotic resistant genes which can lead to ineffective treatment of infections when such antibiotics are prescribed. The aim of this study was to determine the prevalence of antibiotic susceptibility of *Lactobacillus* isolated from yoghurt and cheese retailed in Ado- Ekiti, and the role of plasmids when resistance to multiple antibiotics is detected. Commercially prepared yoghurt (three) and cheese (three) retailed in Ado- Ekiti were analyzed in this study. Isolation of *Lactobacillus* sp. was carried out on De Man Rogosa Sharpe (MRS) agar using pour plate method. Microbial characterization was carried out using cultural and biochemical test as described by Bergey's manual of Systematic Bacteriology. Susceptibility to 8 antibiotics was done using disc diffusion method. Plasmid detection for ten isolates with multiple antibiotic resistances was determined by the alkaline lysis method. Plasmid curing was also done using acridine orange. Four *Lactobacillus* spp was isolated, namely: L. *brevis* (6), *L. fermentum* (8), *L. plantarum* (3), and *L. casei* (6). Average resistance to the 8 antibiotics among all species of *Lactobacillus* isolated from both cheese and yoghurt ranged between 64% - 100%. Resistance to ≥ 2 classes of antibiotics was observed in 12 out 23 isolates of *Lactobacillus*. Percentage resistance after plasmid curing was ranged from 0% - 63%. This study shows that plasmid is one of the major factors recognized for the rapid spread of antimicrobial resistance among *Lactobacillus* spp.

Keyword: *Antibiotic Susceptibility, Prevalence, yoghurt, cheese, Nigeria*

INTRODUCTION:

Lactic Acid Bacteria (LAB) is a broad group of non-sporing rods, non-motile, Gram- positive, usually facultative anaerobes that ferment carbohydrates to form lactic acid as the end-product [1]. LAB are grouped into *Lactobacillus, Leuconostoc, Lactococcus, Pediococcus, Bifidobacterium,* and *Streptococcus* based on their morphological, and biochemical characters. LAB is widely distributed in intestinal tracts of various animals where they live as normal flora. *Lactobacillus* spp. is useful microorganisms among the Lactic Acid Bacteria due to their fermentable capability of sugar to lactic acid. They have been found to synthetic capacity of nutrients such as supplying of vitamins, calcium, pyrimidines, purines, proteins, and decreased incidence of ulcer, cancer, diarrhea, constipation, and lactose intolerance. *Lactobacillus* is the largest genus in this order which contains almost 80 species and is used in differentiation products such as cheese, yogurt, sauerkraut, sausage, pickle, wine, beer, and juices [2].

Cheese as one of milk products or derivatives, is the curd or hard substance formed by the coagulation of milk of certain mammals by rennet or similar enzymes in the presence of lactic acid [3]. Manufacture of most cheese varieties involves combining 4 ingredients; salt, milk, rennet, and microorganisms. Subsequent processing and variations in ingredient blends have led to the evolution of all these cheese varieties. The association of bacteria in cheese can be classified based on their biochemical types, temperature, response and ability to cause infection and to promote health benefit [4;5]. Four genera of lactic acid bacilli that have been isolated in cheese were: *Lactobacillus, Leuconostoc, Lactococcus,* and *Pediococcus*[5]. Lactic acid bacteria play an important role in preventing the growth of undesirable bacteria like coliform and also improve the quality of cheese [6].

Yoghurt is high in calcium and protein, and people with lactose intolerance make it easier to digest because of its active cultures. Yoghurt is made by the addition of healthy and fermentable bacteria and live cultures to milk. These bacteria Ferment lactose in the milk medium to produce lactic acid, which acts on milk protein to give yogurt its texture and its characteristics [7]. *Lactobacillus delbrueckii* subsp. *bulgaricus* is commonly used alongside *Streptococcus thermophilus* as a starter for making yoghurt. The two species work in synergy with *Lactobacillus delbrueckii* subsp. *Bulgaricus* producing amino acid from milk proteins which are then used by *Streptococcus thermophilus* [8;9]. Both species produce lactic acid which gives yoghurt its tart flavour and acts as a preservative. The resulting decrease in pH also partially coagulates the milk proteins, such as casein, resulting in yogurt thickness [10].

LAB has been used increasingly in food production as probiotics over the last decades and has raised safety issues, one of which is the distribution and nature of acquiring of antimicrobial resistance [11]. Antimicrobial resistance is an increasing problem worldwide, with the effective treatment of bacterial infections being compromised. However, the development of bacterial resistance to the available antibiotics and increasing popularity of traditional medicine has led researchers to investigate the antimicrobial activities of both cheese and yoghurt. Some of them have been seen to have antimicrobial effect which automatically has been conferred to the soft cheese [6].

The antibiotics used extensively in treatment of both human and animal diseases have created a selective pressure for acquisition of resistance phenotypes that can be transmitted via food. The evolution of antibiotic resistant foodborne pathogens has been widely reported [12;13]. Recently, several studies have investigated the role of commensal bacteria [such as LAB] as reservoirs of antibiotic resistance genes similar to those found among human pathogens [14;15]. The main threat associated with these bacteria is that they could transfer resistance genes to pathogenic bacteria [16]. LAB often harbours plasmids of different sizes and some antibiotic

resistance determinants located on plasmids have been reported to occur in *Lc. lactis* and various *Lactobacillus* and *Enterococcus* species [17]. Finally, the misuse of antibiotics in the treatment of cattle and other animal producing milk had led to the presence of resistant genes in various bacterial species in milk and milk products such as cheese and yoghurt. Therefore, fermented dairy products that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria since fermentable bacteria are consumed directly with the milk products.

The objectives of the research are to: characterize the antibiotic susceptibility pattern of Lactobacillus spp. in yoghurt and cheese; examine the resistant pattern of the bacterial isolates; and carry out plasmid profile of the resistant strains of the bacterial isolates.

MATERIALS AND METHODS

Sample collection

The study was conducted on three samples each of cheese and yoghurts in Ekiti State University' Ado Ekiti, Nigeria. Local cheese and two different brand names of yoghurt (Hollandia and Freshyo) with batch numbers (2110715B24 and 1519 L2 respectively), dates of manufacture (11/07/2015 and 11/07/2015 respectively) and expiry date (10/01/2016 and 10/04/ 2016 respectively) were purchased from retailers outlets in Ado-Ekiti at two different locations. All samples were collected using sterile polythene bags and transported to the laboratory immediately after the hour of purchasing at room temperature at the point of retail. Samples were analyzed individually, and swabbed with 70% ethanol before opening. Culture media were rehydrated according to the manufacturer's instructions.

Microbiological analyses and Characterization of the samples

Microbiological analyses of the samples were carried out as described by Parvathy and Puthuvallil [18]. Colonies that developed on the plates were grouped on the bases of their cultural characteristics. Pure cultures of all bacterial isolates were obtained by repeated streaking on MRS agar plates. The following morphological characteristics of each isolate were determined: Gram-staining, spore stain, and motility test. Biochemical tests were also performed on cultural characteristics of the isolates. These include catalase, indole, oxidase and sugar utilization (glucose, lactose and sucrose sorbitol, xylose, ribose, and fructose). The isolates were incubated anaerobically at 37^{o}c for 24-48h in a air tight incubator.

Characterization of isolates

Colonies that developed on the plates were grouped on the bases of their cultural characteristics. Pure cultures of all bacterial isolates were obtained by repeated streaking on MRS agar plates. The following morphological characteristics of each isolate were determined: Gram-staining, and motility test. Biochemical tests were also performed on cultural characteristics of the isolates. These include catalase, indole, oxidase and sugar utilization (glucose, lactose and sucrose sorbitol, xylose, ribose, and fructose)

Carbohydrate fermentation pattern of the isolates

For this, *Lactobacilli* cultures were subjected to sugar fermentation reactions using MRS broth medium. MRS medium was used as basal medium. Four milliliter of the medium was taken in each tube and sterilized by autoclaving. One sugar disc was aseptically added to each tube. Each tube was inoculated with 0.1 mL of inoculum, incubated at 37 °C for 24–72h and the results of colour change were recorded as positive or negative. A control using 0.1 mL sterile water as inoculum was used to compare the color change. Sugars used to determine the fermentation profile of *Lactobacilli* isolates were, glucose, lactose, sorbitol, sucrose and fructose. The cultures were identified based on the pattern of sugar utilization [18]

Gas from glucose:

Sterile test tubes of 10 mL glucose broth containing Durham's tube (inverted and dipped), were inoculated with *Lactobacilli* cultures at the at 1% and incubated at 37 °C for 24–72 h. Gas production that appeared in the form of a hollow space in Durham's tube was recorded as a positive result.

Sodium chloride (NaCl) tolerance:

For determination of NaCl tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of NaCl (4 %). The broths were inoculated with 100 µl overnight culture of the isolates and incubated anaerobically at 37°C for 24 to 48hours. After 48 hour incubation, growth was determined using a spectrophotometer, reading the optical density at 600 nm [19].

Physiological characterization of isolates.

After confirming the purity of culture, each isolate was further assessed for growth at a certain temperatures. Growth of isolates at (10 °C). The isolates were tested for their ability to grow in MRS broth at 10 ± 1 °C for 7 days by incubating for 24–48 h. For this, 10 mL of MRS broth tubes were inoculated at 1% of *Lactobacilli* cultures. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at 10 °C and results were noted as positive or negative.

A**ntibiotics susceptibility testing**

The Kirby- Bauer diffusion method was used to determine the antibiotic susceptibility profiles of the bacterial isolates. Pure culture of organisms were enriched and activated in 5mls of nutrient broth and incubated at 37°C to a turbidity of 0.5 Mac Farland standards, the turbidity was adjusted to match with 10^{-5}CFU/ml. The Muller Hinton (MH) agar was inoculated by streaking using sterile cotton swab of each of the cultures. The antibiotics disks were applied using sterile forceps and sufficiently separated from each other for about 2.5cm distance in order to prevent over lapping of the zones of inhibition. The agar plates were left on the bench for 30minutes to allow for diffusion of the antibiotic and the plates were incubated inverted at 37°C for 24hours. Results were recorded by measuring the zone of inhibition with ruler in (mm) and comparing with Clinical and Laboratory Standards Institute [20] interpretative performance standard for antimicrobial disk susceptibility testing. The following common antibiotics were used: Augmentin (30µg), Ciprofloxacillin (5µg), Ceftriaxone (30µg), Penicillin (100 I. µ), Tetracycline (30µg), Ampicillin (10µg), Cefixime (30µg), Nalidixic Acid (30µg).

Protocol for Gram positive bacteria plasmid isolation for Gram +ve organisms

Overnight culture (1.5ml) was Spinned for 1 minute in a micro-centrifuge to pellet cells. Cells were suspended in 200µl of solution A (100 mM glucose, 50 mM Tris hydrochloride (pH 8), 10 mM EDTA) containing 10 mg of lysozyme per ml and 5ug mutanolysin, and incubated for 30 min at 37°C. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2N NaOH and the samples was mixed by inverting tubes. 300µl of a 30% potassium acetate solution (pH 4.8) was added and mixed by vortexing. After incubating on ice for 5 minutes, debris was removed by a 5-minute centrifugation in a centrifuge. The supernatant and extract was transferred once with a phenol-chloroform mixture (1:1). The precipitate plasmid DNA with

an equal volume of isopropanol was allowed to dry and dissolved the plasmid DNA with TE buffer.

Agarose gel electrophoresis

Agarose Gel Electrophoresis is a separation method that can be used to separate DNA based on their molecular weight. The concentration of Agarose used is dependent on the size of DNA to be separated but basically they can be used as follows: Plasmid DNA – 0.8% Agarose. 1X TBE buffer (or 1X TAE Buffer) (Tris Boric acid (or Acetic acid) EDTA, Agarose powder, (λ-DNA HIND III digested), 0.5-10µl micropipette and tips, Ethidium bromide (1mg/ml), Loading dye.

PROCEDURE:

Agarose powder (0.8g) for plasmid DNA was weighed out and 150mls of 1X TBE buffer was added to it. It was dissolved by boiling using a magnetic stirrer or microwave oven. It was allowed to cool to about 60°C, then 10µl of ethidium bromide was added and mixed by swirling gently. It was poured into an electrophoresis tank with the comb in place and a gel thickness of about 4-5mm was obtained and bubbles were avoided. It was allowed to solidify (about 20minutes) and the comb was removed. Then, the tray was placed in the electrophoresis tank. 1X TBE buffer was poured into the tank and the surface of the gel was covered by the buffer. 15µl of sample was mixed with 2µl of the loading dye. Samples were carefully loaded into the wells created by the combs. (Marker was loaded on lane 1 followed by the controls). The electrodes were connected to the power pack in such a way that the negative terminal was at the end where the sample has been loaded. Electrophoresis ran at 60-100V until the loading dye has migrated about three-quarter of the gel. Electrodes were turned-off and disconnected. The gel was observed under a Ultra-Violet transilluminator. [21, 22, 23].

Curing of plasmids by treatment with acridine orange

Bacteria cells were grown in nutrient or Mueller Hinton broth overnight. 5mls of Nutrient broth supplemented with 1mg/ml acridine orange was prepared, and the organisms was sub-culture into Nutrient Broth containing the acridine orange, and Incubated at 37ºC from 48 hours to about one week. Cured organism was plate out on MRS agar.

Post-Plasmid curing Test

The plasmid curing isolates was confirmed by loss of plasmid and antibiotic susceptibility testing using antibiotics to which organisms were resistant follow similar procedure in antibiotic susceptibility testing.

RESULTS

Table1 indicates the average number and percentage distribution of the Lactobacillus isolated from the cheese and yoghurt samples collected in Ado- Ekiti. It was therefore observed at dilution 10^{-3} that: Highest value of Average Numbers of Lactobacillus Count (AVLC) in cheese with respect of percentage value was recorded as Ch_1 86(35.98), Ch_2 73(30.54), Ch_3 80(33.47), similarly, In Freshyo and Hollandia AVLC values and their percentage values was recorded as F_1 41(35.96), F_2 37(32.47), F3 36(31.59) and H_1 32(32.32), H_2 37(36.36), H_3 31(31.31). However, the value for colony forming unit per gram in Cheese was recorded as Ch1 (8.6×10^4CFU/g), Ch_2 (7.3×10^4CFU/g), Ch3 (8.0×10^4CFU/g), and the range value in CFU/ml was recorded in yoghurt as 3.7×10^4CFU/ml to 3.1×10^4CFU/ml. From the biochemical results in table (2) below, it is noted that at 4%NaCl, *L. plantarum*, *L. casei* and *L. fermentum* showed positive growth that indicate they can tolerate NaCl for their growth. Conversely, only *L. brevis* showed negative result which indicated they are not probiotic organisms. Figure 1 shows the most occurrence bacteria was *L. brevis* (4) in cheese, *L. casei* (4) in freshyo, and *L. fermentum* (4) in hollandia samples. The lowest occurrence in all selected samples was *L. plantarum*, (1) in freshyo, (2) in hollandia, and (0) in cheese samples.

Table1: Average Number and Percentage Distribution of *Lactobacillus* spp. Isolated from Cheese and Yoghurt Retailed in Ado-Ekiti.

Samples	No of Samples	Code	Average No. of Lactobacillus Count	% D	AVLC in CFU/g (cheese) and CFU/ml (yoghurt)
Cheese	3	Ch_1	86	35.98	8.6×10^4
		Ch_2	73	30.54	7.3×10^4
		Ch_3	80	33.47	8.0×10^4
Freshyo	3	F_1	41	35.96	4.1×10^4
		F_2	37	32.47	3.7×10^4
		F_3	36	31.59	3.6×10^4
Hollandia	3	H_1	32	32.32	3.2×10^4
		H_2	36	36.36	3.6×10^4
		H_2	31	31.31	3.1×10^4

Key: Ch = Cheese, F = Freshyo, H = Hollandia, %D = Percentage Distribution, AVLC = Average Numbers of Lactobacillus Count

From the table 3, it was observed that, all *L. brevis* isolated from cheese developed 100 percent resistance to tetracycline and penicillin but were sensitive to ciprofloxacin. Thus all *L. casei* isolated from cheese were developed 100% resistance to ampicillin and penicillin but sensitive to ciprofloxacin, tetracycline, ceftriaxone, cefixime and nalidixic acid. *L. fermentum* isolated from cheese sample showed 100% resistance to ciprofloxacin but sensitive to nalidixic acid. In Freshyo, all *L. brevis* were fully resistant to augmentin and tetracycline, and penicillin but sensitive to ampicillin. *L. casei* also developed multiple resistance to augmentin, ampicillin, penicillin and cefixime with 75% respectively but sensitive to ciprofloxacin, tetracycline, and nalidixic acid. *L. fermentum* showed resistance to ampicillin and nalidixic acid with 100% respectively while sensitive to ciprofloxacin. *L. plantarum* isolated from freshyo also developed resistance to augmentin, penicillin and cefixime with 100% each. Similarly in hollandia, *L. brevis* developed 100% resistance to the following antibiotics: augmentin, ampicillin, tetracycline, and cefixime but sensitive to ciprofloxacin, ceftriaxone, penicillin, and nalidixic acid. Also, *L. fermentum* isolated from hollandia sample developed resistance to all prescribed antibiotics in this study. Finally, in exception of ciprofloxacin, *L. plantarum* was

found resistant to augmentin, ampicillin, penicillin, ceftriaxone, and cefixime.

TABLE2: Biochemical Activities of the Lactobacillus Isolated from cheese and yoghurt retail in Ado Eki

Samples	s/n	Code	Gram RT	Catalase	Motility	Indole	Citrate	Oxidase	MR	VP	Xylose	Glucose	Sucrose	Fructose	Ribose	Lactose	Sorbitol		Growth at 4% NaCl	Organisms
Cheese	1	C5	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	2	C10	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	3	C4	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	4	C7	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	5	C3	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*L. casei*
	6	C8	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	7	C1	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
Freshyo	8	F3	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	9	F10	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*L. casei*
	10	F7	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*L. casei*
	11	F4	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	12	F2	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L. brevis*
	13	F6	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*L. casei*
	14	F1	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	15	F5	GPB	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*L. plantarum*
	16	F8	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*L. casei*
Hollandia	17	H3	GPB	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*L. plantarum*
	18	H6	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	19	H10	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	20	H7	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	21	H8	GPB	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*L. plantarum*
	22	H1	GPB	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	*L. fermentum*
	23	H5	GPB	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	*L.brevis*

Key: C = Cheese, H = Hollandia, F = Freshyo, MR= Methyl Red test, VP = Voge Proskauer test

Table3: Percentage Distributions of Antibiotic Resistant *Lactobacillus* spp. isolated from both cheese and yoghurt samples.

L / A	*L. brevis*			*L. casei*			*L. fermentum*			*L. plantarum*		
	C (4)	**F (2)**	**H (1)**	**C (1)**	**F (4)**	**H (0)**	**C (2)**	**F (2)**	**H (4)**	**C (0)**	**F (1)**	**H (2)**
AMC	2(50)	2(100)	1(100)	1(100)	3(75)	0	1(50)	1(50)	1(25)	0	1(100)	2(100)
CIP	0	1(50)	0	0	0	0	2(100)	0	3(75)	0	0	0
AMP	2(50)	0	1(100)	1(100)	3(75)	0	1(50)	2(100)	4(100)	0	0	2(100)
TET	4(100)	2(100)	1(100)	0	0	0	1(50)	1(50)	4(100)	0	0	1(50)
CRO	2(50)	1(50)	0	0	2(50)	0	2(100)	1(50)	2(50)	0	0	2(100)
CXM	2(50)	1(50)	1(100)	0	3(75)	0	1(50)	1(50)	3(75)	0	1(100)	2(100)
NA	2(50)	2(100)	0	0	0	0	0	2(100)	3(75)	0	0	2(100)
PEN	4(100)	2(100)	0	1(100)	3(75)	0	1(50)	1(50)	4(100)	0	1(100)	2(100)
AVR	2.6(64)	1.6(79)	1(100)	1(100)	2.8(70)	0	1.3(64)	1.3(64)	3(75)	0	1(100)	1.9(93)

Key

AMC = augmentin, CIP = Ciprofloxacin, AMP = Ampicillin, TET = Tetracycline, CRO = Ceftriaxone, PEN = Penicillin, CXM = Cefixime, NA = Nalidixic acid, C = Cheese, H = Hollandia, F = Freshyo, A= Antibiotics, L = *Lactobacillus* spp., AVR = Average Resistance.

Table (4): Antibiotic resistant patterns of the isolated *Lactobacillus* spp.

Resistant Pattern	nRP	*Lacbacillus* spp.	Source
CRO-PEN	1	*L. brevis*	Cheese
AMC-AMP-PEN	1	*L. casei*	Cheese
CIP-AMP-PEN	1	*L. fermentum*	Cheese
AMP-CRO-PEN-CXM	1	*L. brevis*	Cheese
AMC-TET-PEN-NA	1	*L. brevis*	Cheese
AMC-CIP-AMP-TET-CRO-PEN-CXM	1	*L. fermentum*	Cheese
AMC-AMP-TET-PEN-CXM-NA	1	*L. brevis*	Cheese
AMP-PEN	1	*L. brevis*	Freshyo
AMC-AMP-CXM	1	*L. casei*	Freshyo
AMC-CIP-PEN	1	*L. casei*	Freshyo
AMC-PEN-CXM	1	*L. plantarum*	Freshyo
AMP-CRO-PEN-CXM	1	*L. casei*	Freshyo
AMC-AMP-TET-NA	1	*L. fermentum*	Freshyo
AMC-CRO-PEN-NA	1	*L. brevis*	Freshyo
AMC-CIP-TET-PEN-NA	1	*L. brevis*	Freshyo
AMP-CRO-PEN-CXM-NA	1	*L. fermentum*	Freshyo
AMC-AMP-CRO-PEN-CXM	1	*L. casei*	Freshyo
AMC-AMP-CXM	1	*L. brevis*	Hollandia
AMC-AMP-TET-PEN-CXM-NA	1	*L. fermentum*	Hollandia
AMP-TET-CRO-PEN-CXM-NA	1	*L. fermentum*	Hollandia
AMC-AMP-TET-CRO-PEN-CXM	1	*L. fermentum*	Hollandia
AMC-CIP-AMP-TET-CRO-PEN-CXM	1	*L. fermentum*	Hollandia
AMC-AMP-CRO-PEN-CXM-NA	1	*L. fermentum*	Hollandia

Key; nRP = Number of Resistant Pattern.

In the table (3) above, *L. plantarum* isolated from freshyo, *L. casei* isolated cheese and *L. brevis* isolated from hollandia exhibited highest percentage in Average Resistance (AVR) to antibiotics with 100%, follow by 93%, 79%, 75% and 70% of *L. plantarum*, *L. brevis*, *L. fermentum*, and *L. casei* isolated from hollandia and freshyo respectively.

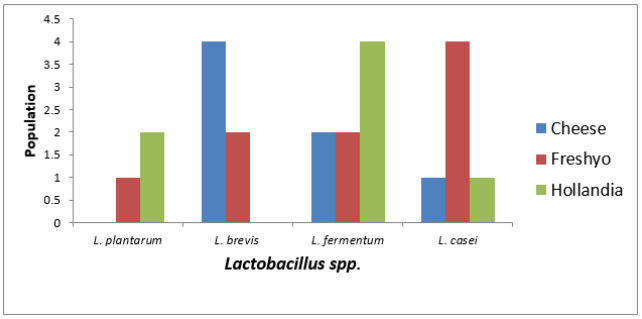

Figure1: A bar graph illustrates the population of Lactobacillus spp. isolated from Cheese, freshyo, and hollandia.

From the graph above, it shows that *L. brevis*(4) dominated the bacterial population in Cheese, *L. fermentum* (4) dominated in holladia and *L. casei* (4) dominated in freshyo. Also *L. plantarum* was scanty in both holladia and freshyo and totally absent in cheese.

Table (4) indicates that *L. brevis* isolated from and *L. casei* isolated from freshyo showed the same multiple drug resistant patterns on ampicillin, ceftriaxone, penicillin, and cefixime**.** Similarly, *L. brevis* isolated from cheese and *L. fermentum* isolated from hollandia showed the same multiple drug resistance pattern on augmentin, ampicillin, tetracycline, penicillin, cefixime, and nalidixic acid. Finally, *L. casei* isolated from freshyo and *L. brevis* isolated from hollandia developed the same resistant pattern to augmentin, ampicillin, and cefixime**.** Observation from the table 4 showed that *L. fermentum* isolated from the cheese and hollandia samples exhibited highest resistant pattern to seven antibiotics.

Table (5) showed that Percentage Resistant (PR) values from before-to-after of the plasmid isolates was compared as follow: Augmentin (83-33%), Ciprofloxacin (50-17%), Ampicillin (83-33%), Tetracycline (67-33%), Ceftriaxone (83-33%), Cefixime, (83-17%), Nalidixic acid (67-00%) and Penicillin (100-67%). Similarly, the PR values of *Lactobacillus* also reduced from left to right across the table (5) below. However, *L. fermentum* isolated from hollandia sample had penicillin resistant genes on its chromosome; while *L. fermentum* isolated from freshyo (F4) showed that resistant genes are located on plasmids, this indicated that plasmid could be a potential source for multiple resistance to various drugs. *L. fermentum* isolated from cheese also had both ampicillin and cefixime resistant genes on their plasmids, while resistance to other used antibiotics are mediated by chromosomal genes.

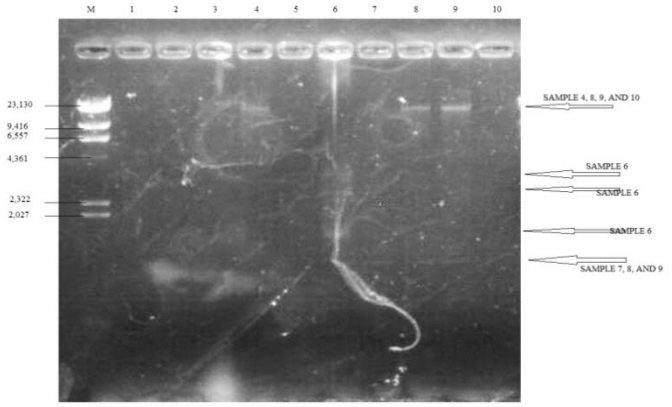

Plate 1: Agarose gel photograph of plasmid DNA of multiple drug resistant bacteria

1(H3) = *L. plantarum*, 2(H6) = *L. fermentum*, 3(H8) = *L. plantarum*, 4(H10) = *L. fermentum*
5(H7) = *L. fermentum*, 6(F4) = *L. fermentum*, 7(F8) = *L. casei*, 8(F3) = *L. brevis*,
9(C8) = *L. fermentum*, 10(C7) = *L. brevis*
Plate (1) revealed that among 10 isolates, only six isolate, 4, 6, 7, 8, 9 and 10 showed the presence of plasmids. Figure2 shows the agarose gel photograph, isolate 6 showed with 3 plasmids corresponding to the molecular weights of 4,361, 2322, and 2,027 bp, respectively. Also observed other isolates with their respective base paired: 4(23,130bp), 7(4,361bp), 8(9,416bp), and 9(6,557bp).

L. brevis isolated from freshyo (F8) showed that multiple antibiotic resistances are mediated by chromosome, while *L. brevis* isolated from the cheese sample (C7) showed that multiple resistance to antibiotics is by plasmid. In the other hand, ampicillin resistant *L. brevis* (C7) is mediated by chromosomal genes. Moreover, *L. casei* isolated from Freshyo also showed that augmentin, ciprofloxacin, ceftriaxone, and nalidixic acid resistant genes are located on the plasmid. Thus, it is indicated that both tetracycline and penicillin resistant genes is mediated by chromosome.

Table 5: Comparative outcomes of antibiotic susceptibility test of multiple resistant *Lactobacillus spp.* after plasmid curing.

Bacteria with plasmid	ID NO	Time	AMC	CIP	AMP	TET	CRO	CXM	NA	PEN	Percentage resistance	P.N.
L. fermentum	H10	After	S	S	S	S	S	S	S	R	1(13%)	4
		Before	R	R	R	R	R	R	R	R	8(100%)	
L. fermentums	F4	After	S	S	S	S	S	S	S	S	0(00%)	6
		Before	S	S	R	S	R	R	R	R	5(63%)	
L. brevis	F8	After	R	S	R	S	R	R	S	R	5(63%)	7
		Before	R	S	R	S	R	R	S	R	5(63%)	
L. casei	F3	After	S	S	S	R	S	S	S	R	2(25%)	8
		Before	R	R	S	R	R	S	R	R	6(75%)	
L. fermentum	C8	After	R	R	S	R	R	S	S	R	5(63%)	9
		Before	R	R	R	R	R	R	S	R	7(88%)	
L. brevis	C7	After	S	S	R	S	S	S	S	S	1(13%)	10
		Before	R	S	R	R	S	R	R	R	6(75%)	
Percentage Resistance	-	After	2(33%)	1(17%)	2(33%)	2(33%)	2(33%)	1(17%)	0(00%)	4(67%)	-	-
		before	5(83%)	3(50%)	5(83%)	4(67%)	5(83%)	5(83%)	4(67%)	6(100%)	-	

Key R = Resistance, S = Susceptibility, P.N. = Plasmid number

DISCUSSION

The goal of this research work was to isolate, characterize potential *Lactobacillus spp.*, and evaluate antibiotic susceptibility of *Lactobacillus* spp. present in commercially prepared cheese and yoghurt (hollandia and freshyo) retailed in Ado Ekiti, and to access the role plasmid profiles in multiple antibiotic resistances. Based on the morphological and biochemical characteristics, four (4) possible bacteria were identified as *Lactobacillus* spp. from the samples. Cultural characteristics and gram staining of the isolates were observed to be rough, smooth, rod shaped, convex (raised), irregular, circular, non-spore forming, gram positive, facultative anaerobic which indicate them to be the member of *Lactobacillus* spp, these are in agreement with the work of Abhijit *et al.* [24], also in agreement to Bergey's manual of Systematic Bacteriology. In accordance to the work of Dhanasekaran *et al.*,[25] ,the significant growth of the isolates at pH 6.5 on MRS – agar plates in anaerobic conditions further confirmed their identification as *Lactobacillus* spp. All of the isolates were Catalase, Oxidase, Motility, Citrate, Indole, Methyl Red, and Voge Proskauer negatives, the results are similar with the findings of Elizete and Carlos [26], and Abhijit *et al.*, [24].

Among the carbohydrates used in this study, all the bacteria except *L. plantarum* were able to ferment glucose (see Table 3). Thus all the bacteria were able to metabolize sucrose, xylose, ribose and lactose. In exception of *L. fermentum* all the bacterial isolates were able to metabolize sorbitol and fructose but *L. brevis* could not ferment sorbitol, this indicates that they are able to grow in variety of habitats utilizing different type of carbohydrates.

pH is an important factor which can dramatically affect bacterial growth. To be used as probiotic, organisms have to tolerate low pH of human gut. The isolated *Lactobacillus* spp. can tolerate a wide range of pH (1-9) and grow well at acidic pH (1-5). According to Abhijit *et al.* [24], growth of certain types of bacteria can be inhibited by sodium chloride (NaCl), and probiotic organisms have to withstand high salt concentration in human gut. The current results showed that *Lactobacillus* spp. isolated from cheese and yoghurt samples was able to tolerate 1-9% of NaCl and best growth was observed at 4% NaCl (table3), this agreed to the work of Abhijit *et al.* [24]. In this present study, 0.05-0.3% bile salt was

supplemented in the growth media, as it corresponded to that found in the human intestinal tract and 0.3% is the maximum concentration that is present in healthy men [19]. Therefore, before selection of probiotic bacteria for human consumption it must be endurable to 0.3% bile concentration [27].

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial effects of lactic acid bacteria are incurred by producing some substances such as organic acids [lactic, acetic, propionic acids], carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins [28;24]. Probiotic bacteria including *Lactobacillus*, Bifidobacterium and *Streptococcus* spp. are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against disease caused by an imbalance of the gut microflora, several experimental observations have showed a potential protective effect of probiotic bacteria against the development of colon tumors [29].

Antibiotic resistance of all the 23 isolates was examined by disc diffusion method and these isolates were found to be varied in their antibiotic resistance against 8 used antibiotics which belong to different groups. *L. fermentum* isolated from cheese and holladia was observed to have the same resistant pattern to augmentin, ciprofloxacin, ampicillin, tetracycline, ceftriaxone, penicillin, and cefixime before plasmid curing but was susceptible to the all 8 antibiotic used for this work and this can pose undesirable effects to mankind. In this study, higher drug resistance (before curing) among the *Lactobacillus* spp. was found against Penicillin G (100%), Augmentin (70%), Ampicillin (90%), and others are Tetracycline (70%), Ceftriaxone (70%) and Cefixime (90%). This is in accordance with the work of (30) where drug resistance among the isolates was found against ampicillin, ciprofloxacin, ceftriaxone, erythromycin, chloramphenicol, piperacciline, trimethoprime-sulfomethoxaxole, nalidixic acid and vancomycin. According to the findings of Pant *et al.* **[**31], multiple antibiotic resistances are thereby rendering the antibiotic treatment ineffective. Incidence and prevalence of antibiotic resistance in *Lactobacillus* present in milk products such as cheese, yoghurt, and other milk products is still high [30,32].

The resistance of *Lactobacillus* spp. just like other bacteria occurs by several mechanisms like efflux pump, production of enzymes that inactivate the drug and change the target of action of antibiotics [33]. The resistance mechanisms are transmitted through exchange of genetic materials between bacteria of the same species [34]. The mechanism of spreading antibiotic resistant genes in bacterial populations has been identified with plasmids [35; 36]. Table [9] also showed that *L. fermentum* isolated from holladia sample had penicillin resistant genes on its chromosome; while *L. fermentum* isolated from freshyo showed that resistant genes are located on plasmids, this indicated that plasmid could be a potential source for multiple resistance to various drugs. *L. fermentum* isolated from cheese also had both ampicillin and cefixime resistant genes on their plasmids, while resistance to other used antibiotics are mediated by chromosomal genes, this also agreed with the work of Shames *et al*. [37].

In this study, *L. brevis* isolated from freshyo showed that multiple antibiotic resistances are mediated by chromosome but not by plasmid, while *L. brevis* isolated from the cheese sample showed that multiple resistance to antibiotics is by plasmid, this was related to the study of Gevers *et al*., [17]. In the other hand, ampicillin resistant *L. brevis* [table 9] is mediated by chromosomal genes. According to the study of Roberts, [38], Tetracycline, for example, has more than 40 different genes conferring antibiotic resistance discovered on both plasmids and chromosome, and the number of tetracycline resistance genes continues to increase, moreover, in this study *L. casei* isolated from Freshyo also showed that augmentin, ciprofloxacin, ceftriaxone, and nalidixic acid resistant genes that are located on the plasmid, and thus indicated that both tetracycline and penicillin resistant genes is mediated by chromosome, also agree with the work of [39] which showed in a document that multiple resistance genes are harbored on Resistance plasmids, some of which are conjugative.

The unnecessary and improper use of antimicrobial drugs in veterinary and human medicine also promotes development of resistant strains with resistant plasmids. Rasko *et al.* [40] reported that both pathogenic and non-pathogenic strains resistant to drugs may be transported from animals to humans via food. Such strains act as an important source for *in vivo* transmission of resistant plasmids to drug sensitive strains in the animal bowel mainly through conjugation [41], this particular report confirmed the same way *Lactobacillus* spp. Harboured their resistant genes to various antibiotics. The use of plasmid profiles in many bacteria had given a wide understanding in DNA types [chromosomes or plasmids] responded for antibiotic resistance. In this study, determination of the possible linkage of multidrug resistance with plasmid DNA in the isolates and plasmid profile was conducted for isolate 4, 6, 7, 8, 9 and 10 as shown on the agarose gel photograph of plasmid DNA [Figure 1], as these was found to be resistant to more than two groups of antibiotics. The plasmid cured cells became sensitive to all previously resistant antibiotics except the isolate 7 and 9 on the plasmid plate which depict *L. brevis* isolated from freshyo yoghurt and *L. fermentum* from cheese; this revealed that antibiotic resistance marker genes were located on plasmid. However, further study such as transfer of plasmid into another suitable host is necessary to confirm the plasmid mediated antibiotic resistance.

CONCLUSION

A very considerable question which handlers should go about is “how safety the commercially prepared yoghurt and cheese consumed readily by people are”? Since *Lactobacillus* species involved in the production of cheese and yoghurt and are consumed normally along with the food and the potential transferability of these resistant genes poses a threat to food safety. However they have the potential of harboring antibiotic resistant genes which is a health risk that can lead to ineffective treatment of infections when such antibiotics are prescribed. Therefore in other to prevent the danger risk associated with consumption of any fermented product, commercially prepared food should be properly monitored, action must be taken to slow the rate of evolution and spread of antibiotic resistant genes. And make sure the food in question is free from resistant *Lactobacillus* and other organisms involved.

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