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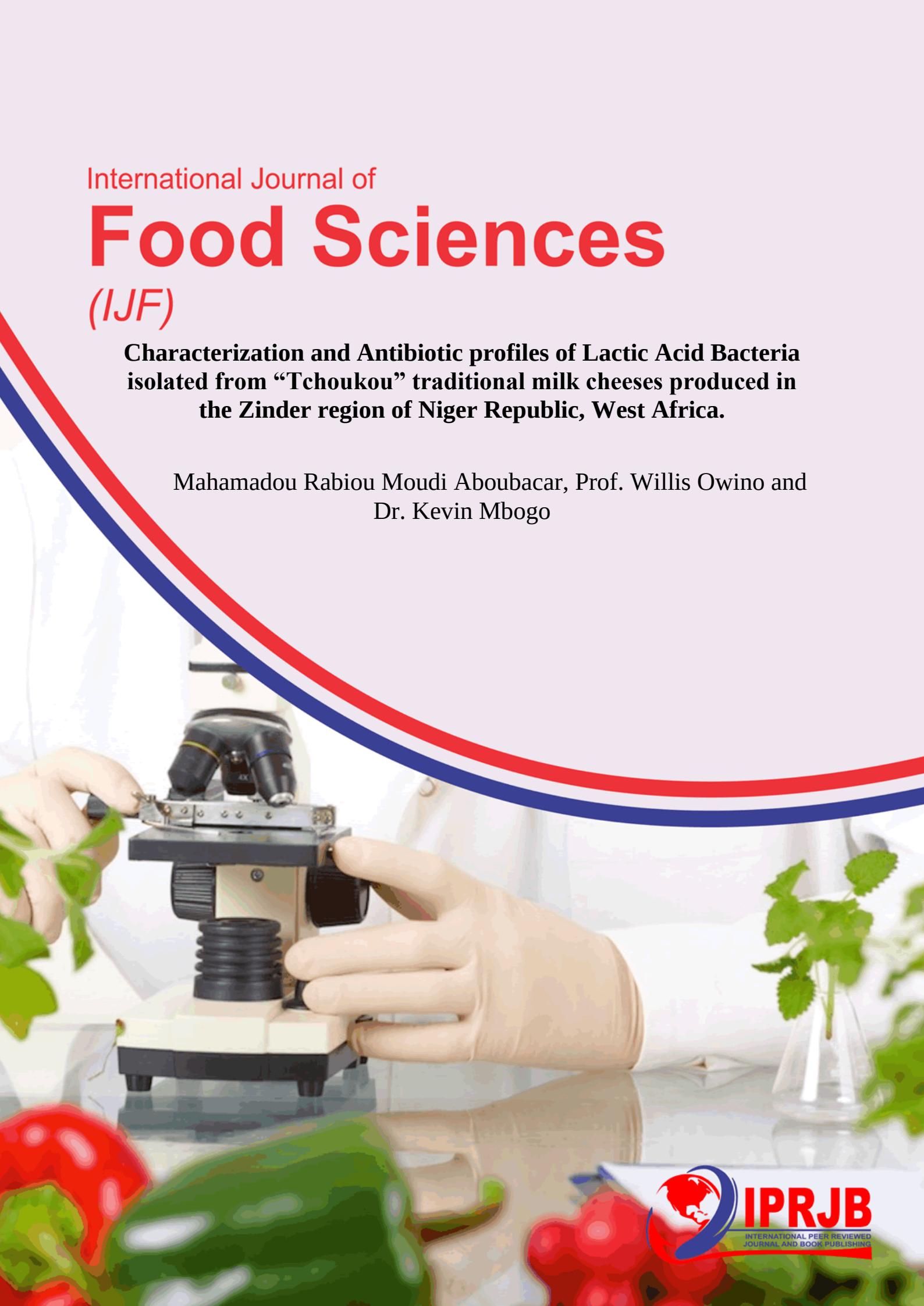

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Characterization and Antibiotic profiles of Lactic Acid Bacteria isolated from "Tchoukou" traditional milk cheeses produced in the Zinder region of Niger Republic, West Africa.

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Abstract

Purpose: The current study's aim is to identify Lactic Acid Bacteria (LAB) isolated from " Tchoukou" cheeses made using cow, camel, and goat milk sampled from Zinder region of Niger Republic.

Methodology: Nine samples were collected aseptically from cheesemakers in Zinder Region and the isolation of LAB isolates was carried out using selective media. The isolates were identified based on their phenotypic, biochemical and genotypic characteristics.

Findings: A total of 13 strains of Lactic Acid Bacteria (LAB) were isolated and morphologically and biochemically characterized. Cell morphology analysis identified 12 isolates as rods shaped while 1 isolate was cocci. All isolates were gram positive, Triple Sugar Ion Agar tests (TSIA) positive, and tested negative for catalase. The isolates were also found to be able to grow in a temperature range between 15 and 45 C.. The isolates' 16S rRNA gene was amplified using bacterial universal primers 27F and 1492R. Based on 16S rRNA gene analyses, the 13 LAB isolates were grouped into the genera Lactobacillus, and Weissella, traditionally known to occur in raw milk and milk products. The genus Lactobacillus was dominant with 76.92% of the LAB isolated. Most of the isolated strains were susceptible to eight antibiotics. Therefore, 5 (38.46%), 3(23.08%), and 4 (30.77%) isolates showed resistance respectively against Kanamycin, Streptomycin, and Co-Trimoxazole. One same isolate (7.69%) was discovered resistant to Sulphamethoxazole and Ampicillin.

Unique contribution to theory, practice and policy: this study was performed to characterize the LAB isolates found in homemade cheeses that could serve as the potential source for the industries and commercial applications.

Key words: *Tchoukou, Lactic Acid Bacteria, morphological and biochemical characteristics, 16S rRNA. Niger Republic.*

1.0 INTRODUCTION

Tchoukou is a typical Niger hard cheese prepared from raw milk from cows, goats, and camels. Tchoukou is a milk form used to preserve milk during the dry winter season. It is often created by drying in the sun, which allows it to dry fast without becoming acidic due to microbic flora. Fermented dairy foods are widely consumed all over the world, and demand has risen dramatically in recent years, with market trends indicating that this trend will continue [1]. The popularity of fermented dairy products among consumers is growing due to their nutritional and health benefits, as their influence on the bacterial flora of the gut improves digestion [2]. Cheese is generally defined as a product with a whey protein to casein ratio equal to or less than that of milk [3]. It is the generic name for a set of fermented milk-based food items that include a diverse microbial community (microbiota) that changes over time and varies depending on the kind of cheese, starter and adjunct cultures used [4]. These microbes are important in defining the final product's flavor, quality, and safety. The considerable interspecies variability in milk composition from which the cheese is generated, which can be from cows, camels, goats, or sheep [5], is another reason of diversity in cheese qualities.

Several cheese components are derived from the metabolic action of lactic acid bacteria (LAB), which play an essential part in the cheese-making process and assist to improve the texture and flavor of the finished product. Lactic acid bacteria hydrolyze lactose during fermentation, producing large levels of lactic acid and other organic acids [6].

According to the literature review, isolation and characterization of Lactic Acid Bacteria (LAB) from cow's milk, camel's and goat's milk cheese in the Republic of Niger is yet to be accomplished and thus necessitates these findings.

The objective of this research was to isolate LAB from traditional cheeses produced from goats' milk, cows' milk and camels' milk to identify those microorganisms based on the phenotypical, biochemical, and molecular characteristics.

2.0 MATERIALS AND METHODS

Sampling and isolation of LAB

Three types of cheeses that were sourced from the milk of goat, cow, and camel were sampled and collected under sterilized conditions from different cheesemakers in the region of Zinder in Niger Republic, West Africa. The purposive sampling method was used due to the sample size being investigated was quite small. Therefore, there were nine samples of cheeses utilized in this study whereby three from each of the three sources mentioned above. All the samples, in their dried state, were covered with aluminum foil and transported from Niger Republic to Kenya.

The samples were crushed into fine powder and 1g of each sample was diluted in 9ml quarter-strength Ringer's solution and vortexed to extract the characteristic LAB associated with dried cheese. 10 µl aliquots were spread onto prepared De Man, Rogosa, and Sharpe Agar (MRS agar, M641) and M17 (M929) agar plates. Plates were incubated at 30°C for 24-48 hours under aerobic conditions according to the method described by Stoll *et al.*, [7] with a slight modification. Colonies were chosen at random from the highest dilution agar plates for further analysis. The strains were then streaked to verify purity after being cultivated aerobically in MRS broth at 30°C Ibinabo, et al., [8]. All of the media was acquired through

Himedia (Mumbai, India). The isolates' stock cultures were kept at -80°C in MRS broth with 20% glycerol according to the method reported by Abdou *et al.*, [9]. The isolates' morphological and biochemical characteristics were examined, and their identification was verified by 16S rRNA gene sequencing.

2.1 Phenotypic characterization

The colony form was noticed after establishing a pure culture in a petri dish. Visual observations included shape, colour, edges, and the elevation of bacterial colonies were performed with slightly modified method described by Ismail *et al.*, [9]. The phase-contrast microscopy at 100x magnification was used to identify phenotypically Lactic acid bacteria (Shimadzu CX41, Japan), as well as standard tests such as catalase activity, gas generation from glucose in MRS broth, and growth at various temperatures (15 and 45°C).

2.1.1 Determination of cell morphology and Gram status

Overnight cultures were placed on microscopic slides and observed under a 100x magnification light microscope and then 3 % KOH was used to assess Gram status as described by Mulaw et al., [10] slightly modified. For the catalase test, as stated by Mulaw et al., [10] overnight cultures were placed on a tiny glass slide and two drops of 3 percent hydrogen peroxide were added and properly blended. A catalase test that is positive results in the production of gas bubbles. indicating that the test bacteria is generating catalase enzyme. A negative catalase test is indicated by the absence of gas bubbles.

2.1.2Growth at different temperatures

Bacilli are typically categorized using growth temperatures between 15 and 45°C. MRS broth was used to determine growth at various temperatures. 50µl of overnight cultures was inoculated into 9 ml test medium, incubated at 15 and 45°C, and colour and growth were monitored for five days times. A negative control was a 9ml test tube containing broth but with no LAB cultures Ibinabo *et al.*, [11].

2.2. Lactic Acid Bacteria under biochemical tests

Gas generation from glucose fermentation

This test was designed to determine if LAB isolates were homofermentative or heterofermentative. Inverted Durham tubes with 1 percent glucose were used to monitor Carbon (IV) oxide (CO2) generation from glucose in modified MRS broth. Separately, 50µl of LAB culture was put to 9 ml MRS broth in separate tubes containing 1 percent glucose and inverted Durham tubes. The test tubes were then incubated at 30°C for 5 days. Gas bubbles emerged in Durham tubes over the course of five days, showing that the isolates produced CO2 via glucose fermentation as described by [10].

Growth test on different concentrations of salt

One colony of bacteria was injected into the de Mann Rogosa Sharpe broth (MRS broth) medium at 5%, 6.5%, and 10% NaCl concentrations. The cultures were subsequently incubated at 37°C for one week. The sediment formation in the medium showed the growth of the bacterium Ismail *et al.*, [9].

Triple Sugar Iron Agar (TSIA) tests

The TSIA test was used to determine if bacteria could ferment glucose, lactose, or sucrose. The objective of this experiment was to find out if a particular isolated bacterium could digest glucose, lactose, and sucrose as applied in research by Ismail *et al.*, [9].

3.0 Antibiotic susceptibility tests

Eight antibiotic discs in a form of one ring were tested against the LAB isolates. To assess the antibiotic susceptibility against the isolates, spread plate technique was used to spread sample evenly onto the prepared media (MRS Agar) plates. This method is most often applied for microbial analysis of food or any related sample. Thus, after the inoculated plates were dried, a disc containing 8 different antibiotics including Gentamicin (Gen) Sulphamethoxazole (SX); Chloramphenicol (C) Ampicillin (AMP); Tetracycline (TE); Co-Trimoxazole (COT) Streptomycin (K); Kanamycin (K) were aseptically applied onto them. The rings were deposited in the middle of the plate using sterile forceps (F150S, U.S.A). After the accomplishment of rings deposit, all the inoculated plates were incubated immediately at 37°C and examined after 48 hours (Figure 1). The experiment was performed in duplicate. The zones showing complete inhibition were measured using ruler and diameters recorded in millimeter (Table 2). The initial diameter of each antibiotic disc was 7 mm.

4.0 Genotypic Identification

Genomic bacterial DNA was isolated from overnight cell cultures grown in MRS broth using AddPrep Bacterial Genomic DNA extraction Kit (AddBio Inc, Korea) according to the manufacturer's instructions. The DNA purity and concentration were measured using a NanoDrop spectrophotometer (PCRmax Lambda, Staffordshire, U. K), and DNA quality was verified using a 1 percent agarose gel electrophoresis. According to the method of Ibinabo *et al.*,[11] PCR amplification of 16S rRNA gene for presumptive LAB strains was carried out using bacterial universal primers 27 F:5-AGA GTT TGA TCC TGG CTC AG-3 and 1492 R:5-GGT TAC CTT GTT ACG ACT T-3. PCR was carried out in a 50µl reaction comprising 25 µl One Taq® 2X Master Mix with standard buffer (New England Biolabs, (M0482S)), 1 µl forward primer, 1 µl reverse primer, and 20 l RNase free water (BioConcept, Cat.N0:3-07F04-1, Switzerland). The mixture was distributed into 47 µl in a sterile PCR tube, and 3 µl of gDNA was added and utilized as a template. The amplified gene fragment was subjected to the following conditions: initial denaturation of the target DNA at 94°C for 1 minute, followed by 30 cycles at 94°C for 30 seconds, primer annealing at 52°C for 30 seconds, and primer extension at 68°C for 5 minutes. The PCR was terminated with a 10 minutes elongation at 68°C followed by a 4 minutes cooling period. The reactions were performed in a thermal cycler (ProFlex PCR systems). Electrophoresis using 1% (w/v) of agarose gel stained with Gel Red and observed using the Uvitec Cambridge gel documentation system (Uvitec, UK) confirmed the size of the PCR products for the 16S rRNA gene. According to the manufacturer guidelines, PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Germany). The Gene RulerTM 1 kb plus DNA Ladder was the molecular marker utilized (Thermo Fisher Scientific, US). At Human Genomics Macrogen Europe (Macrogen Europe B.V, Amsterdam, Netherlands), the purified amplicons were Sanger sequenced.

Phylogenetic analysis

The 16S rRNA gene sequences of the LAB isolates were checked for quality and edited using BioEdit Sequence Alignment Editor 7.2.5.0 Software package (https://bioedit.software.informer.com/download/). They were then compared to standard sequences of bacteria lineages in the public nucleotide sequence databases of the National Centre for Biotechnology Information (NCBI) using nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to discover closely related bacterial 16S rRNA gene sequences (Table 2).

- **Statistical analysis**

Using two-way ANOVA, SPSS statistics version 25, (https://www.updatestar.com/en/directdownload/ibm-spss-statistics/2321346), the zone of inhibition findings were represented as the mean and standard deviation of duplicate experiments.

5.0. RESULTS AND DISCUSSION

5.1. Results

5.1.1 Morphological and biochemical properties of the isolates

All isolates were identified based on their physical and biochemical properties (Table 1), as well as their capacity to grow at salt concentrations of 5% and 6.5 % NaCl. Only five isolates were shown to be viable at 10% NaCl concentration. When isolates were screened for their capacity to grow at 15°C and 45°C, it was revealed that all isolates could grow at 15°C, with only eight isolates growing at 45°C. Ten of the thirteen isolates produced gas from glucose.

Table 1: Colony morphologies and biochemical characteristics of lactic acid bacteria strains

Isolates ID	Gram stain	Shape	KOH	Form	Catalase	15°C / 45°C	Gas	TSIA
ZC1	+	rods	-	circular	-	+/-	+	+
ZC2	+	rods	-	circular	-	+/-	-	+
ZC3	+	rods	-	circular	-	+/+	+	+
ZC4	+	rods	-	circular	-	+/-	-	+
ZCa1	+	rods	-	circular	-	+/+	+	+
ZCa2	+	rods	-	circular	-	+/+	+	+
ZCa3	+	rods	-	circular	-	+/+	+	+
ZCa4	+	rods	-	circular	-	+/+	+	+
ZG1	+	cocci	-	circular	-	+/+	+	+
ZG2	+	rods	-	circular	-	+/+	+	+
ZG3	+	rods	-	circular	-	+/+	+	+
ZG4	+	rods	-	circular	-	+/-	-	+

ZG5	**+**	**rods**	**-**	**circular**	**-**	**+/-**	**+**	**+**

+=positive test, -= negative test, +/- = growth at 15°C not at 45°C, +/+ = growth at both 15 and 45°C

5.1.2. Antibiotic susceptibility tests

The results obtained from antibiotic susceptibility tests against the isolated strains (Figure 1), indicated that all strains were susceptible to most of the antibiotics including Gentamicin (Gen) Sulphamethoxazole (SX); Chloramphenicol (C) Ampicillin (AMP); Tetracycline (TE); and Co-Trimoxazole (COT). However, some strains strongly expressed resistance: ZC2 (7.25±0.35mm), ZC3(7.65±0.92mm), ZC2(7.5±0.71mm), ZC1(7.5±0.71mm), ZG2(7±0.00mm), ZG3(7±0.00mm), and ZG2(7.55±0.78mm) respectively to Ampicillin, Chloramphenicol, Tetracycline, Co-Trimoxazole, Streptomycin, and Kanamycin. Therefore, the isolate ZCa3 (39.75±1.18mm) was the most susceptible against Amp while ZC4 (35.75±0.35mm) showed the highest zone of inhibition against Chloramphenicol. The isolates ZC1(30.5 ±2.12mm), ZCa2(32.75±0.96mm), ZG5(31.85±0.21 mm), ZCa4(20.4±2.26mm), ZG1 (20.75±1.77mm), and ZG3(11.5±2.12 mm) were discovered to be strongly sensitive to respectively Gentamicin, Sulphamethoxazole, Tetracycline, Co-Trimoxazole, Streptomycin and Kanamycin (Table 2).

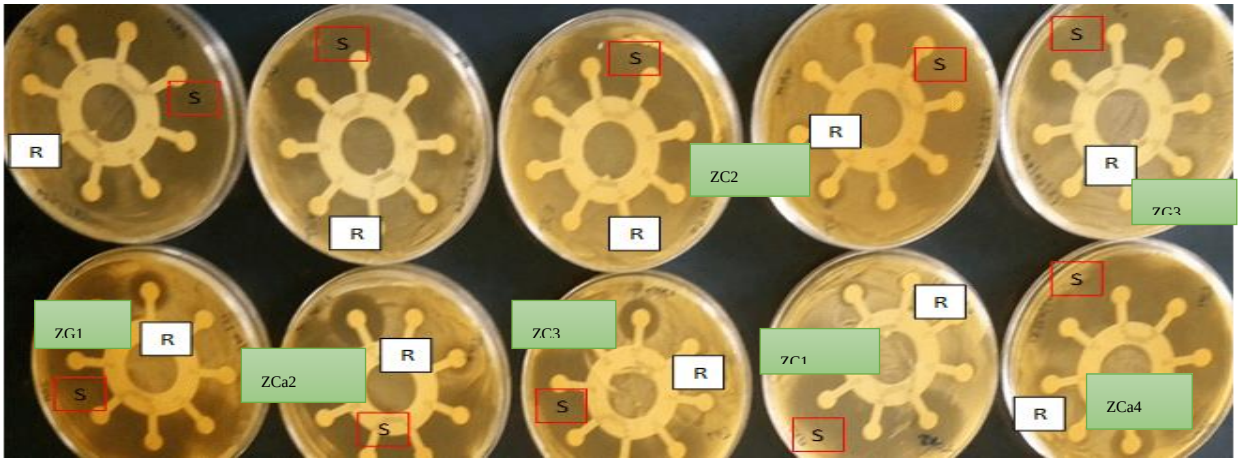


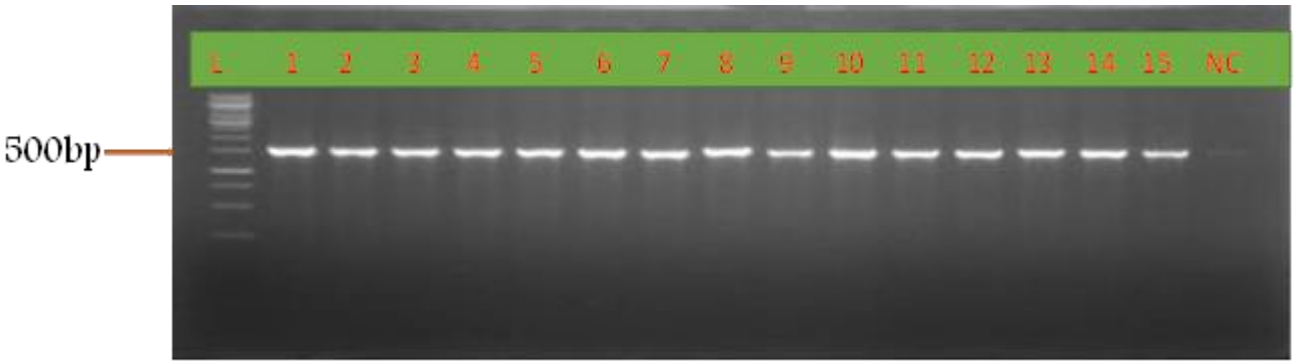
Figure 1. susceptibility (S) and resistance (R) of some Lactic Acid Bacteria

Table2: antibiotic susceptibility test

Isolates	Gentamicin	Sulphamethoxazole	Chloramphenicol	Ampicillin	Tetracycline	C0-Trimoxazole	Streptomycin	Kanamycin
ZG1	30.5±2.12	24.6±0.85	21.4±0.85	29.45±0.78	26.65±1.91	8.5±2.12	20.75±1.77	7.8±1.13
ZG2	27.65±0.49	25.5±0.71	33.3±0.99	29±1.41	29.7±3.25	7±0.00	7.75±0.35	7.55±0.78
ZG3	9.5±0.71	23.5±0.71	24.7±1.84	21.9±2.97	30.5±2.12	7.75±1.06	7±0.00	11.5±2.12
ZG4	20.6±1.98	9.85±4.03	23.65±3.32	23.45±0.78	28.9±1.56	21.35±3.75	9±2.83	8±1.41
ZG5	27.5±0.71	8.8±2.55	27.7±3,25	28.5±2.12	31.85±0.21	8.75±2.47	9.5±3.54	7.65±0.49
ZCa1	14.25±1.06	23.25±1.77	22.9±1.56	37.25±1.06	26.4±2.26	19.25±1.06	11.25±1.06	9.7±3.82
ZCa2	17.15±1.20	32.75±0.96	31.35±0.92	30.5±0.71	26.3±2.40	27.4±0.85	13.25±1.06	8±1.41
ZCa3	17.5±0.71	25.65±0.49	24.35±2.33	39.75±1.18	27±1.41	18.5±2.12	14.85±0.21	7.5±0.71
ZCa4	16.25±1.06	25.5±0.71	22.8±1.70	29.25±1.06	25.5±2.12	20.4±2.26	14.5±0.71	7.95±0.07
ZC1	16.5±9.19	10±4.24	8.5±2.12	9.75±3.89	7.5±0.71	7.25±0.35	7.5±0.71	8±1.41
ZC2	13.9±0.14	7.25±0.35	11±1.41	7.5±0.71	27.25±1.06	9.3±0.99	9.05±0.49	10.35±0.92
ZC3	15.8±0.28	30.75±1.06	7.65±0.92	27.45±0.78	29.6±3.39	8.15±1.63	8.5±2.12	7.9±2.27
ZC4	13.6±0.57	8.9±2.69	35.75±0.35	36.5±2.12	25.8±3.11	9.35±3.32	8.45±2.05	8.7±2.40

5.1.3. Molecular identification of the isolates

Prior to sequencing, PCR amplicons were obtained and prepared for sequencing (Figure 2). The BLASTn search was used to compare isolates, which revealed 94 to 100% identity similarity. (Table 3). Based on the BLASTn search performed against the GenBank (Figure 2), the following ten isolates including ZC1 (KP889230.1), ZC2 (KJ784539.1), ZC3 (MG754569.1), ZC4 (MF357249.1), ZCa1 (KX538919.1), ZCa2 (MF369878.1), ZCa3 (KM495896.1), ZCa4 (KR135828.1), ZG4 (EU637399.1) and ZG5 KM4958.1) were found to belong to the genus of Lactobacillus. And only three isolates, ZG1 (HQ009757.1), ZG2 (KX156221.1), and ZG3 (KP137384.1) were affiliated with *Weisella cibaria.*



***L**= 1kb DNA Ladder, **NC**= negative control, **red number** = simple serial numbers*

Figure 2: PCR amplicons of some amplified bacterial DNA.

Table 3: molecular identities of LAB strains

S/N ID	Isolate	Closest relative	Accession number	% Identity
1	**ZC1**	*Lactobacillus plantarum* strain FJ005	KP889230.1	**100**
2	**ZC2**	*Lactobacillus plantarum.* SMG131	KJ784539.1	**97**
3	**ZC3**	*Lactobacillus plantarum* strain Sourdough_K3	MG754569.1	**99**
4	**ZC4**	*Lactobacillus paraplantarum* strain CAU4459	MF357249.1	**100**
5	**ZCa1**	*Lactobacillus plantarum* strain LP1-4	KX538919.1	**100**
6	**ZCa2**	*Lactobacillus plantarum* strain CAU:226	MF369878.1	**99**
7	**ZCa3**	*Lactobacillus plantarum* strain gp112	KM495896.1	**98**
8	**ZCa4**	*Lactobacillus plantarum.* PP3	KR135828.1	**97**
9	**ZG1**	*Weissella cibaria* strain HN79	HQ009757.1	**100**
10	**ZG2**	*Weissella cibaria* strain BPLP4	KX156221.1	**98**
11	**ZG3**	*Weissella cibaria* strain AT22	KP137384.1	**98**
12	**ZG4**	*Lactobacillus plantarum* strain Y-2-14	EU637399.1	**94**
13	**ZG5**	*Lactobacillus plantarum* strain gp112	KM495896.1	**100**

***ZC** indicates isolates obtained from cows' milk cheese; **ZCa** indicates isolates obtained from camels' milk cheese; **ZG** indicates isolates obtained from goats' milk cheese.*

5.2. Discussion

Lactic acid bacteria are microorganisms that produce lactic acid as a result of the fermentation of carbohydrates. They are generally regarded micro- organisms with no harmful activity Ibinabo *et al.*, [11]. In the food and beverage industries, LABs are usually utilized as probiotic or functional starter microorganisms. Lactic acid is the main end product of carbohydrate fermentation in these starter microorganisms Smid *et al.*,[14]. They are widely used to process dairy and non-dairy food products such as yoghurt (*Streptococcus spp* and *Lactobacillus spp)*, cheese (*Lactobacillus spp and Lactococcus spp*), and Sauerkraut (*Leuconostoc spp*) Ouattara *et al.*, [15]. The study design was carried out to explore the

dominance of LAB (Table 2) involved in the production of artisanal cheese in the Zinder region (Niger Republic). The study's results and findings are in line with the findings of Mulaw *et al.,* [10] whereby they showed that most the LAB isolates belong to the genus of Lactobacillus and only 3 were found to belong to the genus of Weissella. Similar results were found by Jonathan *et al.,*[16] and Bansal *et al.,*[17]. The morphology of the LAB (Table 1) obtained in this study was in agreement with the findings reported by Husseini *et al.,*[18] and Ibinabo *et al.,*[11] where they isolated LAB from fermented vegetable amaranth. A carbohydrate utilization test using Triple Sugar Ion Agar was performed to determine whether the isolates can ferment sucrose, glucose, and galactose. All the isolated bacteria were found to be able to ferment the given carbohydrates. These findings are consistent with the research results of Husseini *et al.* [18]. When The isolates were put to the test and seeing if they could grow at 15 and 45°C, it was discovered that all of them could grow at 15°C, but only 8 could grow at 45°C. Similar results have been previously reported by Ibinabo *et al.,* [11]. The isolates showed their ability to grow at a salt concentration of 5% and 6.5% NaCl; but only 5 isolates were discovered to be growing at a NaCl concentration of 10%. This is closely in accordance with the study of Ismail *et al.,* [9]. The production of gas was investigated as a functioning parameter with the aim of classifying the isolates as homofermentative or heterofermentative. This categorization is useful for selecting the food matrix that would get the potentially probiotic microorganism. It was found that ten of the thirteen isolates generated gas from glucose. In contrast with da Silva *et al.,* [19] found only one LAB isolate that generated gas from glucose. Regarding to the antibiotic susceptibility tests, this study results showed that most of the LAB isolates from "Tchoukou" were susceptible against a panel of eight antibiotics somehow. These findings corroborated previous research report of Devirgiliis *et al.,* [20] who according to their previous laboratory report "phenotypic characterisation of tetracycline, erythromycin, and kanamycin resistance in 500 Lactic Acid Bacteria (LAB) isolated from raw materials and end products collected during the production process of a classic Italian cheese, Mozzarella Bufala Campana (MBC). Antibiotic resistant genes were almost exclusively found in bacteria isolated from raw, unprocessed substrates, whereas the final, marketed products did not contain phenotypically resistant Lactic Acid Bacteria, implying that the procedures used to make MBC operate as a negative selection against those components of the fermenting microflora that are most frequently found to harbour Antibiotic resistant genes".

6.0. Conclusion

The findings of this study revealed which strain of Lactic Acid Bacteria is present in handmade cheeses produced in Zinder region of the Republic of Niger. Lactic acid bacteria were obtained from cheese samples, morphologically and biochemically examined, and molecularly identified as *Lactobacillus plantarum* and *Weissella cibaria*. *L. plantarum* was found to be the most dominant LAB in these cheeses samples. Therefore, the cheeses that were sourced from camel's milk and goat's milk contain high Lactic Acid Bacteria with the good morphological and biochemical properties which could serve as the potential source for the industries and commercial applications. In this, we recommend the probiotic properties of the same isolates to be investigated in quality assurance methodologies for measuring dosage, viability, and structural and functional integrity of vital microbiome knowledge and translating it into emotional and meaningful advantages for policymakers.

Disclosure statement

The authors report no conflicts of interest. The authors are solely responsible for the content of the manuscript.

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Author Contributions

Mahamadou R., M., A., conceptualization & writing-Original Draft Preparation; Willis Owino and Kevin Mbogo: supervision & Writing-Review& Editing.

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