International Journal of Microbiology and Biotechnology
2019; 4(3): 72-86
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20190403.13
ISSN: 2578-9678 (Print); ISSN: 2578-9686 (Online)

In Vitro Antimicrobial Characterization of *Lactobacillus* Isolates Towards Their Use as Probiotic Alternatives to Antibiotic Growth Promoters

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To cite this article:
Raoul Emeric Guetiya Wadoum, Fonteh Anyangwe Florence, Kaktcham Pierre Marie, Ulrich Landry Bemmo Kamdem, Chancel Hector Momo Kenfack, Foko Kouam Edith-Marius, Nathalie Nzekwa, Evina Horpa, Vittorio Colizzi, François Zambou Ngoufack. *In Vitro* Antimicrobial Characterization of *Lactobacillus* Isolates Towards Their Use as Probiotic Alternatives to Antibiotic Growth Promoters. *International Journal of Microbiology and Biotechnology.* Vol. 4, No. 3, 2019, pp. 72-86. doi: 10.11648/j.ijmb.20190403.13

Received: June 16, 2019; **Accepted**: July 16, 2019; **Published**: August 5, 2019

Abstract: In the present study, the probiotic potential of *Lactobacillus* isolates selected from fecal samples of farmyard chickens and ducks was scientifically validated for their use as alternatives to antibiotics in poultry. A total of 129 *Lactobacillus* isolates were characterized of which four produced inhibitory substances with antimicrobial activities. They were further identified on the basis of their carbohydrate fermentation profile and High-Resolution Melting analysis as *Lactobacillus paracasei MW-37CGZ*, *Lactobacillus paracasei MW-38CGZ*, *Lactobacillus plantarum MW-48CGZ* and *Lactobacillus plantarum MW-18CGZ*. The obtained results revealed that *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* showed strong antagonistic activities against human (nine) and zoonotic pathogens (eleven). The antimicrobial substance produced by *L. plantarum MW-18CGZ* was found to be proteinaceous, thus indicating that this substance may belong to a group of potent antimicrobial peptides produced by some microorganisms including lactic acid bacteria (LAB). Both viable and non-viable cells of the four isolates demonstrated good hydrophobicity in xylene with *L. plantarum MW-48CGZ* exhibiting higher hydrophobicity than other isolates (77.64±5.18%). They were susceptible to chloramphenicol, clindamycin, ampicilin and erythromycin with Minimum Inhibitory Concentration (MIC) below cut-off values established by the European Food Safety Authority (EFSA). Among the four *Lactobacillus*, *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* displayed high autoaggregation and coaggregation towards pathogens and all isolates survived in low-pH, high bile salt concentrations and none exhibited virulent factors. According to the obtained results, *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* could be considered as future biotherapeutic substitutes for antibiotics to reduce antibiotic residues in food derived from poultry as well as the generation and spread of antibiotic resistance.

Keywords: *Lactobacillus*, Probiotics, Antimicrobial Activity, Antibiotics Resistance, Public Health

1. Introduction

One of the major breakthroughs in the history of medicine is undoubtedly the discovery of antimicrobials. Their use in feed for food-producing animals to prevent diseases in animals and to improve the production performance in modern animal husbandry has led to healthier and more productive farm animals [1, 2]. However, the unreasonable use of antimicrobials has given rise to fear the development of resistant pathogens and the potential transfer of resistance

factors from animals to humans [3]. Facing the restriction of antibiotics in animal feeds as growth promoter, and due to rising levels of health consciousness and growing consumer awareness regarding gut health and the concept of preventive health care, various alternatives have been explored by scientists worldwide to replace antibiotics as growth promoter. The potential applications of lactobacilli as probiotics to improve human and animal health received increasing attention as scientific evidence continues to accumulate on the properties, functionality, and beneficial effects of probiotic microorganisms making them to be good candidates to replace antibiotics. Probiotics used as microbial feed additives could be biotechnological alternatives to antimicrobials used in livestock animals. Probiotics comprise beneficial microorganisms, of which bacteria of the genera *Lactobacillus* and *Bifidobacterium* are the most common types studied. These microorganisms are well known for their production of secondary metabolites with antimicrobial properties. They are generally regarded as safe (GRAS) and can therefore safely be used as probiotics for medical and veterinary applications. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4]. They are beneficial bacteria in that they favorably alter the intestinal microbiota balance, inhibit the growth of harmful bacteria, promote good digestion, boost the immune function and increase resistance to infection. [5]. In addition, the probiotic benefit and postbiotic metabolites produced by probiotic strains have been shown in various animal species including cattle, chickens and pigs suggesting a great potential of probiotics to be used as biotherapeutic and growth promoter for livestock animals. Presently, the selection and screening of probiotic properties of *Lactobacillus* strains isolated from fish, cow milk, soil and some fermented local beverages and foods in Cameroon have been extensively studied [6-9]. But to our knowledge, no information exists regarding the isolation and characterization of *Lactobacillus* strains from farmyard chickens and ducks to be used as probiotic feed additive. Accordingly, the aim of this study was to characterize *Lactobacillus* isolates from the fecal microbiota of farmyard chickens and ducks in the Western Highlands of Cameroon for potential use as antimicrobial probiotic feed supplement in aviculture. Various criteria for the selection of a probiotic were investigated and compared. Subsequently, a phenotypic and molecular identification of selected isolates was performed by evaluating their sugar fermentation profile and High-Resolution Melting (HRM) analysis, respectively.

2. Materials and Methods

2.1. Sample Origin and Collection

A total of 55 fecal samples were collected from farmyard chickens and ducks in the Western Highlands of Cameroon. All chickens and ducks were mature, and feces were collected directly from the cloacae. Fecal samples were placed in sterile plastic containers and stored at 4°C until delivery to the laboratory for the isolation of Lactic Acid Bacteria (LAB) on the same day at the Laboratory of Biochemistry, Food Science, and Nutrition (LABPMAN), Department of Biochemistry, Faculty of Science, University of Dschang, Cameroon. The experiment respected the rules formulated under the Animal Welfare Act by the United States Department of Agriculture (USDA) and by adopting ARRIVE guidelines [10].

2.2. Isolation of Lactic Acid Bacteria

One gram of each fecal samples was added to 100 mL of De Man-Rogosa-Sharp (MRS) broth (Biolife, Italy) as an initial solution. The sample solution was prepared in serial dilution up to 10^{-6}. Then, each dilution was plated on MRS agar supplemented with 0.05% cysteine-HCl and incubated under anaerobic conditions using a candle extinction jar with a moistened filter paper to provide a CO_2-enriched, water-vapor saturated atmosphere at 37°C for 48h. After incubation, single colonies were randomly selected, inoculated in MRS broth and incubated under the same conditions. The selected colonies were isolated by streaking on MRS agar. Finally, overnight cultures of all isolated colonies were stored at -20°C in MRS broth supplemented with 30% glycerol. Before being used, isolates were revived in MRS broth at 37°C for 24h under anaerobic conditions and subculture on MRS agar.

2.3. Determination of the LAB Isolates Antimicrobial Activity

The antimicrobial activity of each isolate was determined against the indicator strains/isolates, *Escherichia coli* ATCC 13706, *Salmonella enterica serovar Typhi* ATCC 6539, *Escherichia coli* and *Salmonella typhimurium* (poultry clinical isolate [3]) by modifying the agar overlay method described by Shokryazdan et al. [11]. Briefly, a loop-full ($\approx 10^8$ CFU. $spot^{-1}$) of a 6h cultured isolate was spotted on MRS agar and the plates were incubated anaerobically at 37°C for 48h to allow the formation of antimicrobial compounds. Cell suspensions of the indicator microorganisms were prepared as follows: each 24h cultured indicator strain on Mueller Hinton (MH) agar slant was suspended in sterile physiological saline (NaCl, 0.9%) to obtain a turbidity comparable to that of McFarland standard 0.5 ($\approx 10^8$ CFU. mL^{-1}). Of this cell suspension, 50 µL was inoculated in 5 mL of Mueller Hinton soft agar (0.75%) and overlaid on MRS agar plates containing grown isolates in spot form (≈5 mm diameter). After solidification of the overlaid agar medium, plates were incubated at 37°C for 24h. The zone diameter of inhibition (ZDI) values obtained were measured and interpreted following Shokryazdan et al. [11]. ZDI values > 20 mm, 10 – 20 mm and < 10 mm was considered as strong, intermediate and weak inhibitions, respectively.

2.4. Identification of Lactobacillus Isolates

2.4.1. Preliminary Identification of Isolates

All isolates were tested for catalase and oxidase activity, Gram stain, cell morphology, motility, CO_2 production from glucose and spore formation.

2.4.2. Identification Using Sugar Fermentation Profiles

Isolates were identified based on carbohydrate fermentation profile by using API 50 CH kit according to the manufacturer's instruction. The carbohydrate fermentation profile was then analyzed using the APILAB Plus software version 3.3.3 (BioMerieux, Marcy l'Etoile, France) to identify the species of the best isolates.

2.4.3. Identification Using High-Resolution Melting (HRM) Analysis

i. *DNA Extraction from Pure Cultures*

To avoid bias, isolates were randomly assigned code prior to DNA extraction and High-Resolution Melting (HRM) analysis. Two milliliters of a 48h culture in MRS broth were centrifuged at 13,000g for 10min at 4°C to pellet the cells, which were then subjected to DNA extraction using MagCore® HF16 Automated Nucleic Acids extractor loaded with MagCore® Genomic DNA Bacterial kit (RBCBioscience) according to the manufacturer's specification. Initially, cells were treated with 20 mg. mL^{-1} of lysozyme (Vivantis) in lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM EDTA, pH 8.0; 1% Triton X-100) for 30min at 37°C. DNA concentration and quality were determined using µDrop™ Spectrophotometer (Multiskan GO, Thermo Scientific).

ii. *High-Resolution Melting (HRM) Analysis*

The PCR amplification reaction was performed in a 25 µL solution containing 0.875 µL of each primer, 12.5 µL (0.7 µM) of 2x HRM PCR Master Mix, 8.25 µL RNase-free water and 2.5 µL of isolated DNA. The PCR products were generated on Rotor-Gene Q (Qiagen, USA) using the Type-it HRM PCR Kit (Qiagen, USA). Universal primers (forward, 5′-TCC TAC GGG AGG CAG CAG T-3′; reverse, 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′) targeting the bacterial 16S rRNA gene were used (0.7 µM). The PCR conditions were denaturation 5min at 95°C, followed by 45 cycles of denaturation at 95°C for 10s, annealing at 55°C for 30s and extension at 72°C for 10s. After amplification, HRM analysis was performed from 65°C to 90°C at 0. 1°C. $step^{-1}$ with 2s holding time at each step. The Rotor-Gene Q series software version 2.0.3 (Qiagen, USA) was used to analyze HRM data.

2.5. Tolerance and Adhesion in Gastro-Intestinal Tract

2.5.1. Acid Tolerance

Isolates were preliminarily selected using a rapid test according to the method described by Pelinescu et al. [12]. For real assessment of acid tolerance, a 24h culture of each isolate in MRS broth was centrifuged at 3000rpm for 10min and the resulting pellet was suspended in a citrate buffer of pH 3 (10^8 CFU. mL^{-1}) for 4h at 37°C as described by Zambou et al. [13]. The suspensions were then centrifuged at 3000rpm for 10min at 4°C and washed twice in sterile saline solution to eliminate citrate buffer. Cells were suspended in a physiological NaCl solution and a series of ten-fold dilutions (10^{-2} to 10^{-10}) in 0.1% peptone water was prepared. A given amount of each dilution (100 µL) was plated on MRS agar and incubated anaerobically in GasPak anaerobic jar (Genbox anaer; BioMérieux, France) at 37°C for 48h. The percentage of viable bacteria was calculated.

2.5.2. Bile Salt Tolerance

Isolates were cultured on MRS agar medium for 24h at 37°C. Colonies were collected and suspended in 0.5 M phosphate buffer pH 7 supplemented with bile salts (Oxgall; Sigma, St Louis, USA) at different concentrations (0.1%; 0.3%; 0.5%) and in 0.5 M phosphate buffer pH 7 supplemented with bile salt N. 3 (Oxoid, Basingstoke, Hampshire, UK) at different concentrations (0.05%; 0.1%; 0.2%). The turbidity of suspensions was adjusted to McFarland standard 0.5 (10^8 CFU. mL^{-1}). The resulting suspensions were then incubated for 24h at 37°C followed by centrifugation at 3000rpm for 10min at 4°C and washed twice in sterile saline solution. Cells were again suspended in physiological solution and serially diluted (10^{-2} to 10^{-10}) in 0.1% peptone water. From each dilution, 100 µL was streaked on MRS agar and incubated anaerobically in GasPak anaerobic jar (Genbox anaer; BioMérieux, France) at 37°C for 48h. Results were expressed as the percent (log CFU) of resistant cell.

2.5.3. Qualitative Assay for Deconjugation of Bile Salts

Isolates were screened for bile salt hydrolase (BSH) activity by spotting 10 µL aliquots of overnight cultures on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurocholate (Sigma, USA) and 0.37 g. L^{-1} of $CaCl_2$ [14]. Plates were incubated anaerobically at 37°C for 72h. The precipitation zone surrounding the colonies indicated the bile salt hydrolase activity of bacteria. Isolates were grouped into one of the three arbitrary classes based on the diameter of the precipitation zones on BSH screening medium according to Mathara et al. [15]: a class with low BSH activity when the strain demonstrated a precipitation zone up to 10 mm; a class with medium BSH activity when the isolate demonstrated a precipitation zone of 11 to 15 mm; a class with high BSH activity when the isolate demonstrated a precipitation zone greater than 15 mm.

2.5.4. Cell Surface Hydrophobicity of Isolates

Hydrophobicity of the cell surface was assessed based on bacterial adherence to hydrocarbons (BATH). A modification of previous methods reported for cell surface hydrophobicity was used [16]. Bacteria cells grown aerobically in MRS broth for 18h at 37°C were harvested by centrifugation at 5000rpm for 10min. The pellet was washed twice in PBS (pH 7.2) and re-suspended in PBS before adjusting the turbidity to an absorbance of 0.25±0.05 at 600nm ($A_{initial}$), which corresponds to 10^7-10^8 CFU. mL^{-1}. A mixture comprising 3 mL of the bacteria suspension and 1 mL of xylene or chloroform ($A_{initial}$) or equal volumes of bacteria suspension and xylene (1: 1) was thoroughly mixed by vortexing vigorously for 5min in sterile test tubes and allowed to stand without agitation. The sample separates into two phases. The aqueous phase was collected into a cuvette after 1h to measure the absorbance at 600 nm (A_{final}). The influence of bacterial viability on the hydrophobicity abilities was

analyzed. For this purpose, bacterial suspensions were heat-inactivated by keeping them at 98°C for 10min and the BATH test was carried out as described above. The hydrophobicity was calculated from two replicates as the percentage change in absorbance of the original bacterial suspension due to cells partitioning into a hydrocarbon layer according to equations

$$100 \times (A_{initial} - A_{final}) / A_{initial}$$

2.5.5. Spectrophotometric Autoaggregation Assay

The *Lactobacillus* isolates were tested for their capacity to form self-aggregate using spectrophotometric assay. The method was modified and adapted from the descriptions of Reniero et al. [17] and Collado et al. [16]. The bacteria were grown aerobically for 18h in MRS broth at 37°C. They were harvested by centrifugation at 12000g for 10min; washed twice with phosphate buffered saline (pH 7.2) at 5000g for 10min and re-suspended in PBS at a concentration of approximately 10^7-10^8 CFU. mL^{-1} corresponding to an OD of 0.25±0.05 measured at 600 nm. The bacteria suspension was vortexed for 10s and then incubated at 37°C without agitation. After various times (2, 16 and 20h), 1 mL was gently collected from the top of the suspension without stirring it. A volume of the same bacteria in another tube was properly mixed before taking 1 mL suspension of the bacteria. The absorbance (OD) of either the top clear suspension or mixed suspension was measured at 600 nm using a spectrophotometer (UV-1601, SHIMADZU Japan). Autoaggregation was also observed 12 and 20h under a light microscope after Gram staining. The autoaggregation percentage was expressed $(1 - A_{supernatant}/A_{total\ bacterial\ suspension}) \times 100$.

2.5.6. Spectrophotometric Coaggregation Assay

The coaggregation between potential probiotics and zoonotic pathogens, which were aerobically grown for 18h at 37°C in MRS broth and Tryptic Soy broth (TSB) was tested spectrophotometrically using the method described by Collado et al. [16] with slight modifications. The bacteria cells were processed after harvesting by centrifugation at 12000g for 10min; washed twice with phosphate buffered saline (pH7.2) at 5000g for 10min and re-suspended in PBS at a concentration of approximately 10^7-10^8 CFU. mL^{-1} with an OD of 0.25±0.05 measured at 600 nm. A suspension of potential probiotics and pathogens at 1: 1 ratio was made in a sterile tube and vortexed for 10s before incubation at 37°C without shaking. Equal volumes of each potential probiotic or pathogen were also prepared and incubated under the same conditions. After various times (2, 16 and 20h), 1 mL sample was removed from the top of the tubes containing the mixed or individual bacteria without mixing to measure the absorbance (OD) at 600 nm using a spectrophotometer (UV1601, SHIMADZU, Japan). The coaggregation percentage was calculated using the following equation $[(A_{pat} + A_{probio})/2 - (A_{mix})/(A_{pa}t + A_{probio})/2] \times 100$, where A_{pat} and A_{probio} represent at 600nm of the separate bacterial suspensions in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at different times tested [18]. The assay was carried out in two independent experiments.

2.6. Antagonistic Activities

2.6.1. Antimicrobial Activity Against Pathogens

Twenty strains that are pathogenic to human (nine) and animal (eleven) were used as test pathogens to investigate the antimicrobial activity of the *Lactobacillus* isolates. Human pathogens consisted of cultures such as *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 10541, *Escherichia coli* ATCC 13706, *Salmonella typhi* ATCC 6539, *Pseudomonas aeruginosa* ATCC 20027, *Candida albicans* ATCC 10261; *Streptococcus mutans* DSM 20523 and *Klebsiella pneumonia* (a clinical isolate from our laboratory). Eleven zoonotic pathogens isolated from poultry fecal samples on selective and semi-selective media during outbreaks in the study area were further used as test pathogens from animal. They were identified as *Clostridium* sp., *Escherichia vulneris*, *Proteus vulgaris*, *Proteus mirabilis*, *Providencia rettgevi*, *Pseudomonas aeruginosa*, *Staphylococcus sciuri*, *Staphylococcus epidermidis*, *Salmonella* sp., *Listeria* sp. and *Shigella* sp. [3]. Identification was performed using API 20 E, API Staph and API 20 NE.

The antagonistic activities of the isolates against the 20 pathogenic strains were evaluated by a modified agar overlay method described by Shokryazdan et al. [11]. Briefly, a loop-full (≈10^8 CFU. $spot^{-1}$) of a 6h culture of each *Lactobacillus* strain was spotted on MRS agar and plates were incubated anaerobically at 37°C for 18h in anaerobic jars containing gaspack to allow exhibition of antimicrobial compounds. After colony development on MRS agar, the plates were overlaid with 10 mL of soft (0.75% agar) microorganism-specific medium, seeded with 1% (v/v) of an active overnight culture of the target pathogenic strain (≈10^8 CFU. mL^{-1}), and incubated aerobically at 37°C. Only media seeded with *Candida albicans* were incubated at 24°C. The microorganism-specific media were Sabouraud Dextrose broth (SDB) for *Candida albicans*, Clostridial Differential Broth (CDB) for *Clostridium*, Trypticase Soy broth (TSB) for *Staphylococcus* sp. and *Enterococcus faecalis*, Brain Heart Infusion (BHI) for *Listeria* sp. and Nutrient broth (NB) for other pathogenic strains (all media from Sigma). The zone diameter of inhibition (ZDI) were measured and interpreted as described before (2.3).

2.6.2. Characterization of Antimicrobial Substances

The *Lactobacillus* isolates were assayed for the production of substances with antimicrobial properties such as bacteriocin, hydrogen peroxide, and organic acids using the agar well diffusion method described by Toure et al. [19] with modifications. The bacterial isolates were grown in 25 mL of MRS broth at 37°C overnight, after which the cultures were centrifuged at 4000g for 10min at 4°C. The supernatant of each strain was divided into equal portions for different assays. For bacteriocin assay, the supernatant (5 mL) was treated with trypsin (Fluka Biochemika, Switzerland), lipase (Sigma-Aldrich, Germany) and lysozyme (Fluka Biochemika,

Switzerland) at 1 mg. mL^{-1}. For organic acids assay, the supernatant (5 mL) was adjusted to pH 6.5 using 1 N NaOH, and for hydrogen peroxide assay, the supernatant (5 mL) was treated with 0.5 mg. mL^{-1} catalase (Sigma, Milan, Italy). Treated supernatants were filter sterilized through 0.22 µm pore-size filters (Schleicher & Schuell, Roma, Italy), and 100 µL was placed into wells (7 mm diameter) of MRS agar plates, overlaid with 10 mL of soft Nutrient agar inoculated with 1% (v/v) of the sensitive indicator strain *Escherichia coli* ATCC 13706. The plates were kept at 4°C for 3h for better diffusion of the treated supernatant and then incubated at 37°C. After 48h the inhibition zone diameters were measured.

2.7. Evaluation of Virulence Factor Expression

2.7.1. Antibiotic Susceptibility Test

Antibiotic susceptibility testing of the isolates was carried out using the broth microdilution method according to the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) [20]. The following antibiotics obtained from Oxoid and Fluka were tested: ampicillin, tetracycline, streptomycin, gentamicin, chloramphenicol, kanamycin, erythromycin, clindamycin. For preparing stock solutions (1280 µg. mL^{-1}) all antibiotics were dissolved in water, phosphate buffer or solvent. The stock solutions were diluted to obtain solutions with concentrations of 0.25 to 128 µg. mL^{-1}. For the preparation of bacterial inocula, colonies from overnight cultures of each *Lactobacillus* isolates were suspended in 5 mL 0.85% NaCl solution, adjusted to a turbidity of 0.2 ± 0.02 (620 nm), and diluted 1: 500 in LAB susceptibility test medium (LSM) broth (Oxoid). Then, 50 µL of each diluted inoculum was added to each well of 96-well microdilution plates containing 50 µL of an antibiotic solution, resulting in a bacterial concentration of about 10^4 CFU. $well^{-1}$ and antibiotic concentrations in the range of 0.12 to 64 µg. mL^{-1}. Inoculated plates were incubated anaerobically at 37°C for 48h. After incubation, a stereomicroscope (Carl Zeiss, 2x) was used to determine the minimum inhibitory concentration (MIC) values defined as the lowest concentration of an antibiotic in which visible growth was inhibited. To classify the isolates as susceptible or resistant, MIC values were compared with the MIC breakpoints recommended by EFSA.

2.7.2. Hemolytic Activity

Hemolytic activity was investigated as described by Gerhardt et al. [21] using *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 20027 as positive controls. A 16h broth culture was streaked onto sterile blood agar plates. Plates were incubated anaerobically at 30°C for 48h. The hemolytic reaction was recorded by observation of a clear zone around the colonies (β-hemolysis), a partial hydrolysis and greening zone (α-hemolysis) or no reaction (γ-hemolysis).

2.7.3. Gelatinase Activity

Gelatinase activity was investigated as described by Harrigan and Mc Cance [22]. A 16h culture was streaked onto nutrient gelatin agar (Oxoid, Basingstoke, Hampshire, UK). The plates were incubated anaerobically for 48h at 30°C afterwards they were flooded with a saturated ammonium sulphate solution and observed for clear zones surrounding the colonies.

2.7.4. CAMP-Like Factor Expression

The CAMP-like factor was evidenced by streaking the tested isolates at 8 mm distance from the beta-hemolysis producing *Staphylococcus aureus* ATCC 25923 on 5% sheep blood agar plates and incubated aerobically at 37°C for 24h. The synergetic clear hemolysis, often noticed on the junction of both microorganisms with an arrow-like appearance, indicated the production of a CAMP-like factor.

2.7.5. Proteolytic Activity

Caseinase activity was determined using an agar with 15% soluble casein as substrate. The isolates were spotted on this medium. After incubation at 37°C for 24h, the proteolysis of casein was indicated as a clear zone surrounding the colonies.

2.7.6. Inter-Antagonism (Co-Existence) Assay

The ability of strain to coexist was tested by a cross-streak method [23]. Active colonies of strains grown at 37°C for 16h were suspended in saline to a density of 10^8 CFU. mL^{-1}. A swab stick of each bacterial suspension was streaked in horizontal and vertical forms across each other on MRS agar plate. The plates were incubated aerobically at 37°C for 48h to observe antagonism.

2.8. Cumulative Probiotic Potential

The probiotic potential of the *Lactobacillus* strains was assessed using 11 point scores, and the cumulative probiotic potential (CPP) calculated according to following formula: CPP = Maximum score/Observed score x 100 [24].

2.9. Statistical Analyses

The computer program GraphPad InStat version 7.04 was used for the one-way analysis of variance (ANOVA). Tukey's means comparison test was used with statistical significance pre-set at 0.05.

3. Results

3.1. Identification of Isolates with Antimicrobial Activities

Results of the antimicrobial activity screening showed that four out of 129 isolates could inhibit *Salmonella typhimurium* (poultry clinical isolate), *Salmonella enterica serovar Typhi* ATCC 6539, *Escherichia coli* ATCC 13706 and *Escherichia coli* (poultry clinical isolate). Selected isolates *MW-48CGZ, MW-37CGZ, MW-18CGZ and MW-38CGZ* were then identified and evaluated for their probiotic properties.

They were Gram-positive, rod shaped, oxidase and catalase negative as well as weren't spore forming and didn't produced CO_2 from glucose. Most phenotypic characteristics suggested that these isolates could belong to the genus *Lactobacillus*. Selected isolates were further identified based

on their biochemical sugar fermentative profile and High-Resolution Melt (HRM) analysis.

An HRM assay was used in order to identify isolates presenting antimicrobial activity. HRM curves were able to distinguish between *L. plantarum* and *L. paracasei* separating melting peaks of 16S rDNA amplicons of each species (Table 1).

Table 1. *Identification of Lactobacillus isolates using API 50CHL and 16S rDNA amplicon (HRMA).*

Lactobacillus Isolates	API 5OCH Identification (% similarity) *	HRM analysis (Tm °C) **	Final identification
MW-37CGZ	*L. paracasei (99.9)*	82.45	*L. paracasei MW-37CGZ*
MW-38CGZ	*L. paracasei (99.9)*	82.47	*L. paracasei MW-38CGZ*
MW-18CGZ	*L. plantarum (99.9)*	80.92	*L. plantarum MW-18CGZ*
MW-48CGZ	*L. plantarum (99.9)*	81.03	*L. plantarum MW-48CGZ*

* The percentages of similarities; ** Melting Temperature; reference strains: *L. plantarum* (80.88) and *L. paracasei* (82.50).

Results showed that the sugar fermentative profile and HRM curve of isolates *MW-18CGZ* and *MW-48CGZ* were closely related to that of *L. plantarum* while those of *MW-37CGZ* and *MW-38CGZ* were related to *L. paracasei* (Figure 1). Therefore, isolates *MW-48CGZ* and *MW-18CGZ* were called respectively, *L. plantarum MW-48CGZ* and *L. plantarum MW-18CGZ* while on the same note, isolates *MW-37CGZ* and *MW-38CGZ* were called *L. paracasei MW-37CGZ* and *L. paracasei MW-38CGZ* respectively, in this study (Figure 2).

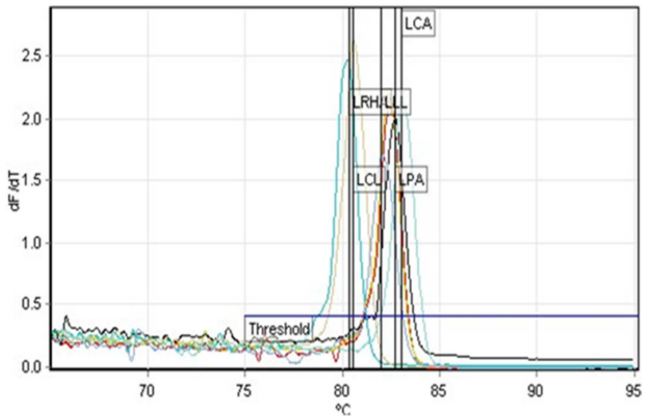

Figure 1. *HRM melting curve profiles of isolates MW-37CGZ and MW-38CGZ as well as reference strains L. casei (LCA), L. paracasei (LPA), L. rhamnosus (LRH), L. lactis (LLL) and L. curvatus (LCU).*

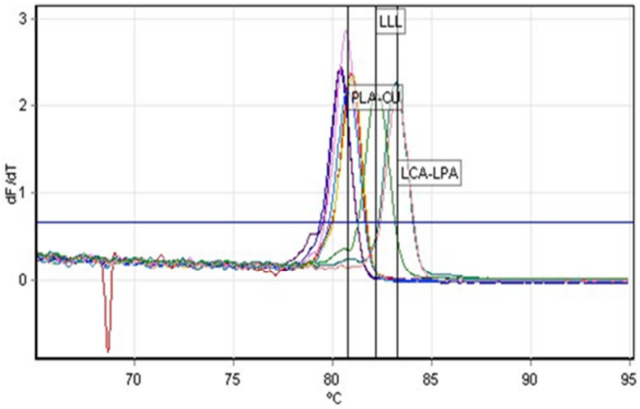

Figure 2. *HRM melting curve profiles of isolates MW-48CGZ and MW-18CGZ as well as reference strains L. casei (LCA), L. paracasei (LPA), L. rhamnosus (LRH), L. lactis (LLL) and L. curvatus (LCU).*

3.2. Tolerance and Adhesion in Gastro-Intestinal Tract

Table 2 shows the viability of the four *Lactobacillus* isolates selected for probiotic characterization at pH 3. All *Lactobacillus* isolates showed good tolerance to acid (pH 3), however the level of tolerance varied among the isolates. Of these four *Lactobacillus* isolates, two (*L. plantarum MW-18CGZ* and *L. plantarum MW-48CGZ*) showed significant higher ($p < 0.05$) acid tolerance with loss in cell viability of only 0.107 and 0.111 log units respectively. Furthermore, *L. paracasei MW-37CGZ* and *L. paracasei MW-38CGZ* presented comparable ($p > 0.05$) viability loss of about 0.140 log units.

Table 2. *Viability of Lactobacillus isolates after 3h exposure to pH 3.*

Cell viability (log CFU. mL^{-1}) *			
***Lactobacillus* strains**	**pH 7.3 (control)**	**pH 3**	**Reduction of viability (log units)**
L. paracasei MW-37CGZ	8.937 ± 0.02	8.796 ± 0.01	0.141^{b}
L. paracasei MW-38CGZ	8.993 ± 0.03	8.853 ± 0.02	0.140^{b}
L. plantarum MW-18CGZ	8.982 ± 0.02	8.875 ± 0.03	0.107^{a}
L. plantarum MW-48CGZ	8.924 ± 0.04	8.813 ± 0.02	0.111^{a}

*Values are means±SD of two independent experiments, each with duplicate. Number in column having the same letter are not significantly different ($P > 0.05$).

The results of bile tolerance for *Lactobacillus* isolates are shown in Table 3 and 4. All four *Lactobacillus* isolates exhibited tolerance to bile salts N. 3 and bile salts (oxgall). However, the degrees of tolerance varied among isolates. *L. paracasei MW-38CGZ* showed the highest ($p < 0.05$) tolerance to bile salt with cell viability not affected when challenge with 0.1% bile salts and a comprehensive stable tolerance at 0.122 log units reduction at 0.3 and 0.5% bile salts respectively. Moreover, this isolate presented a similar tolerance profile when challenged with bile salt N. 3 at various concentration by exhibiting the lowest reduction of cell viability in contrast to other isolates. *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* showed comparable tolerance profiles in both bile salts used. Taking everything into accounts, the *strain L. plantarum MW-48CGZ* exhibited highest sensitivity to bile salts and bile salts N. 3. Interestingly, the bile salts tolerance profile of these isolates correlated with their bile salt hydrolase activity

displayed by a precipitation zone around colonies on plate assay. The precipitation diameter of all isolates was between 15 to 20 mm with *L. paracasei MW-38CGZ* exhibiting the highest BSH activity (20 mm).

Table 3. *Growth of Lactobacillus isolates in MRS broth (control) and MRS broth supplemented with various concentration of Bile.*

Cell viability in Bile salt (log CFU. mL^{-1}) *							
***Lactobacillus* strain**	**MRS**	**MRS + 0.1%**	**Viability (+/-) ****	**MRS + 0.3%**	**Viability (+/-)**	**MRS + 0.5%**	**Viability (+/-)**
L. paracasei MW-37CGZ	8.91 ± 0.01	7.77 ± 0.03	-1.14^{a}	7.72 ± 0.02	-1.19^{a}	5.94 ± 0.02	-2.97^{a}
L. paracasei MW-38CGZ	7.22 ± 0.04	7.21 ± 0.02	- 0.01^{b}	6.0 ± 0.03	-1.22^{a}	6.0 ± 0.03	-1.22^{b}
L. plantarum MW-18CGZ	9.01 ± 0.07	7.47 ± 0.04	-1.54^{c}	7.41 ± 0.01	-1.60^{b}	7.48 ± 0.04	-1.53^{c}
L. plantarum MW-48CGZ	8.63 ± 0.02	7.17 ± 0.01	- 1.46^{d}	5.75 ± 0.04	-2.88^{c}	5.78 ± 0.01	-2.85^{d}

Table 4. *Growth of Lactobacillus isolates in MRS broth (control) and MRS broth supplemented with various concentration of Bile salts N. 3.*

Cell viability in Bile salt N. 3 (log CFU. mL^{-1}) *							
***Lactobacillus* strain**	**MRS**	**MRS + 0.05%**	**Viability (+/-) ****	**MRS + 0.1%**	**Viability (+/-)**	**MRS + 0.2%**	**Viability (+/-)**
L. paracasei MW-37CGZ	8.91 ± 0.01	5.95 ± 0.02	-2.96^{a}	5.95 ± 0.02	-2.96^{a}	4.41 ± 0.02	- 4.5^{a}
L. paracasei MW-38CGZ	7.22 ± 0.04	6.05 ± 0.03	-1.17^{b}	4.80 ± 0.03	-2.42^{b}	3.62 ± 0.03	-3.6^{b}
L. plantarum MW-18CGZ	9.01 ± 0.07	7.45 ± 0.01	-1.56^{c}	6.01 ± 0.01	-3.00^{a}	4.50 ± 0.01	- 4.5^{a}
L. plantarum MW-48CGZ	8.63 ± 0.02	5.50 ± 0.04	-3.13^{d}	5.35 ± 0.04	-3.28^{c}	4.40 ± 0.04	- 4.23^{c}

* Values are means ± SD of two independent experiments, each with duplicate; Number in column having the same letter are not significantly different (P > 0.05).

Cell-surface hydrophobicity was performed in order to study the potential of selected isolates to adhere to the intestinal mucus. Hydrophobic cell surface was denoted by high adherence to xylene, an apolar solvent. The adhesion percentages of viable and non-viable *Lactobacillus* isolates to xylene and chloroform are shown in Table 5. It is apparent that the cell surface activity of viable isolates in chloroform was higher than in xylene, but the trend is not consistent with non-viable cells.

While the highest percentage in the two ratios of xylene 3: 1 and 1: 1 for viable *L. paracasei MW-37CGZ* were 44.06% and 43.82% respectively, for *L. plantarum MW-48CGZ*, it was 44.30% and 52.7%. The least percentage value in chloroform 3: 1 was 52.13% for viable cells of *L. paracasei MW-37CGZ* against 55.18%, 75.25% and 77.64% *L. paracasei MW-38CGZ*, *L. plantarum MW-18CGZ* and *L. plantarum MW-48CGZ* respectively. *L. plantarum MW-48CGZ* presented the highest ($p < 0.05$) viable percentage in chloroform followed by *L. plantarum MW-18CGZ* with value ranging from 77.64% to 75.25% respectively. However, no difference ($p > 0.05$) was observed between adhesion percentages of all viable isolates in xylene 3: 1. Despite the variability and strain specificity in the assay ratio, non-viable cells of *L. paracasei MW-38CGZ* and *L. paracasei MW-37 CGZ* appears to give the lowest values in chloroform 3: 1.

3.3. Antagonistic Activities and Preliminary Characterization of Antimicrobial Substances

The antagonistic properties of *Lactobacillus* isolates against human and zoonotic pathogenic strains are shown in Table 6. The results showed that all the isolated *Lactobacillus* exhibited inhibition on the growth of pathogens. However, this inhibition varied among isolates. *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* showed strong antagonistic activities against human and zoonotic species of *Salmonella* used in this study with zone diameters of inhibition more than 20 mm.

Table 5. *Cell surface hydrophobicity of viable and non-viable Lactobacillus isolates.*

% adhesion to hydrocarbons				**% adhesion to hydrocarbons**		
Viable (mean ± SD) *	**Xylene 3: 1**	**Xylene 1: 1**	**Chloroform 3: 1**	**Non-Viable (mean ± SD) ***		
				Xylene 3: 1	**Xylene 1: 1**	**Chloroform 3: 1**
L. paracasei MW-37CGZ	44.06 ± 3.83^{a}	43.82 ± 4.67^{a}	52.13 ± 6.32^{a}	35.90 ± 0.6^{a}	30.62 ± 9.7^{a}	34.13 ± 6.32^{a}
L. paracasei MW-38CGZ	43.89 ± 3.70^{a}	44.93 ± 1.58^{a}	55.18 ± 5.12^{a}	35.80 ± 3.7^{a}	29.36 ± 2.9^{a}	30.21 ± 4.1^{a}
L. plantarum MW-18CGZ	43.90 ± 3.62^{a}	55.59 ± 3.73^{b}	75.25 ± 5.26^{b}	39.5 ± 2.4^{a}	48.9 ± 3.5^{b}	63.8 ± 2.6^{b}
L. plantarum MW-48CGZ	44.30 ± 2.87^{a}	52.7 ± 1.28^{b}	77.64 ± 5.18^{b}	40.20 ± 1.6^{a}	50.01± 1.1^{b}	65 ± 6.1^{b}

* Values are means ± SD of two independent experiments, each with duplicate. Number in column having the same letter are not significantly different (P > 0.05).

Moreover, these isolates exhibited good inhibition against 19 other pathogens. However, *L. plantarum MW-48CGZ* and *L. paracasei MW-38CGZ* showed moderate inhibitory activities against all pathogens with zone diameters of inhibition less than 15 mm. Among the four isolates, *L. plantarum MW-18CGZ* was the most effective strain in inhibiting the growth of the test pathogens. It showed the highest inhibitory activity against all test pathogens. In contrast, *L. paracasei MW-38CGZ* was the least effective isolate, showing the lowest inhibitory activities against 7 of 20 tested pathogens.

Table 6. *Antagonistic activity of Lactobacillus isolates against human and zoonotic.*

Lactobacillus isolates				
	L. paracasei MW-37CGZ	***L. paracasei MW-38CGZ***	***L. plantarum MW-18CGZ****	***L. plantarum MW-48CGZ***
Human Pathogen	ZDI (mm) ± SD**			
Listeria innocua ATCC 33090	16.93 ± 3.67^{abc}	12.68 ± 1.42^{ad}	20.48 ± 4.26^{b}	11.70 ± 0.68^{cd}
Staphylococcus aureus ATCC 25923	10.65 ± 2.74^{a}	11.10 ± 2.64^{a}	15.70 ± 1.07^{bd}	19.33 ± 1.61^{cd}
Streptococcus mutans DSM 20523	27.58 ± 2.69^{a}	09.78 ± 1.32^{b}	25.85 ± 1.56^{a}	10.03 ± 0.53^{bc}
Enterococcus faecalis ATCC10541	24.33 ± 0.44^{a}	14.60 ± 0.48^{b}	16.20 ± 1.27^{bc}	14.70 ± 2.65^{bd}
Escherichia coli ATCC 13706	13.80 ± 2.93^{ab}	10.30 ± 0.21^{a}	18.48 ± 1.71^{b}	10.85 ± 1.37^{a}
Salmonella typhi ATCC 6539	21.28 ± 2.4^{a}	08.05 ± 0.68^{b}	27.83 ± 2.40^{c}	12.50 ± 2.05^{b}
Pseudomonas aeruginosa ATCC 20027	14.15 ± 0.78^{a}	12.30 ± 3.03^{a}	20.85 ± 0.58^{b}	13.93 ± 1.66^{a}
Candida albicans ATCC 10261	20.30 ± 0.58^{ac}	13.33 ± 2.00^{b}	23.85 ± 3.33^{a}	14.85 ± 0.28^{bc}
Klebsiella pneumonia (clinical isolate)	13.70 ± 2.84^{ab}	11.98 ± 3.38^{a}	19.15 ± 1.56^{b}	12.53 ± 1.61^{a}
Zoonotic Pathogen	ZDI (mm) ± SD**			
Clostridium sp.	17.18 ± 0.73^{a}	09.15 ± 2.25^{bd}	26.13 ± 0.83^{c}	11.83 ± 1.02^{ad}
Escherichia vulneris	18.30 ± 1.95^{a}	07.30 ± 1.66^{bc}	16.13 ± 2.98^{ad}	11.20 ± 2.35^{cd}
Proteus vulgaris	12.40 ± 1.27^{a}	5.5 ± 1.66^{bd}	28.33 ± 0.73^{c}	11.14 ± 0.47^{ad}
Proteus mirabilis	18.68 ± 1.32^{a}	12.28 ± 0.63^{b}	27.15 ± 1.95^{c}	16.15 ± 1.76^{a}
Providencia rettgevi	13.58 ± 1.51^{a}	10.90 ± 0.88^{a}	$24.751.56^{b}$	10.70 ± 0.48^{a}
Pseudomonas aeruginosa	13.30 ± 1.46^{a}	05.08 ± 1.12^{b}	11.70 ± 1.07^{a}	15.45 ± 0.97^{a}
Staphylococcus sciuri	20.48 ± 3.47^{ab}	14.45 ± 2.15^{ac}	22.53 ± 0.83^{b}	13.55 ± 1.95^{c}
Staphylococcus epidermidis	17.10 ± 3.72^{a}	05.20 ± 0.88^{b}	25.93 ± 1.61^{c}	13.25 ± 3.52^{a}
Salmonella sp.	23.83 ± 0.34^{a}	13.85 ± 1.37^{bc}	27.70 ± 1.46^{a}	12.85 ± 2.54^{c}
Listeria sp.	15.80 ± 2.64^{a}	11.28 ± 1.32^{a}	27.1 ± 1.95^{b}	14.43 ± 1.02^{a}
Shigella sp.	20.23 ± 2.00^{a}	12.05 ± 2.05^{bc}	22.65 ± 0.97^{a}	10.05 ± 1.95^{c}

* Showed the highest antimicrobial activity; ∗∗ Values are means ± SD of two independent experiments, each with duplicate, Number (s) in row having the same letter are not significantly different (P > 0.05). ZDI: zone diameter of inhibition.

The antimicrobial substance, produced by the *Lactobacillus* isolates, was characterized by the agar well diffusion assay against the indicator strain *Escherichia coli* ATCC 13706. The results showed that culture supernatants of all selected *Lactobacillus* isolates treated with lipase (1 mg. mL^{-1}) or lysozyme (1 mg. mL^{-1}) did not affect their inhibitory activities against the indicator strain, thus confirming that this substances are not or doesn't contain a fat or carbohydrate moiety. In addition, culture supernatants treated with catalase also did not affect the inhibitory activities of isolates against the indicator strain. This showed that inhibition by the isolates was not due to hydrogen peroxide production.

However, neutralized supernatants (pH 6.5) of *L. paracasei MW-37CGZ*, *L. paracasei MW-38CGZ* and *L. plantarum MW-48CGZ* did not have any inhibitory activity against the indicator strain, which implied that the inhibitory effects of these isolates were due to their organic acid productions (Table 7). Finally, the inhibitory substance produced by *L. plantarum MW-18CGZ* was fully inactivated by the proteolytic enzyme trypsin, thus confirming its proteinaceous nature.

Table 7. *Inhibitory activity of supernatants of Lactobacillus isolates against sensitive Escherichia coli ATCC 13706.*

Zone Diameter of Inhibition (mm) including 6mm well diameter*						
Lactobacillus Isolates	**Untreated Supernatant (control)**	**Neutralized Supernatant (pH 6.5)**	**Supernatant + catalase (0.5mg/mL)**	**Supernatant +trypsin (1mg/mL)**	**Supernatant + lipase (1mg/mL)**	**Supernatant + lysozyme (1mg/mL)**
Lactobacillus paracasei MW-37CGZ	14 ± 0.88	NI**	13.5 ± 0.68	12.9 ± 0.68	12.8 ± 1.27	13.9 ± 0.27
Lactobacillus paracasei MW-38CGZ	15 ± 0.29	NI	14.25 ± 0.58	13.4 ± 0.24	13.25 ± 0.83	13.8 ± 0.48
Lactobacillus plantarum MW-18CGZ	20.4 ± 1.2	18.5 ± 0.2	17.8 ± 0.5	NI	17.2 ± 0.9	17.7 ± 0.4
Lactobacillus plantarum MW-48CGZ	17.3 ± 1.4	NI	15.5 ± 1.2	16 ± 1.0	15.5 ± 0.8	17 ± 0.5

* Values are means ± SD of two independent experiments, each with duplicate; ** No inhibition.

3.4. Expression of Virulence Factors

The results of Minimum inhibitory concentrations (MIC) values for antibiotic susceptibility of the *Lactobacillus* isolates against eight tested antibiotics are shown in Table 8. When challenged with ampicillin, erythromycin, clindamycin, and chloramphenicol, all isolates exhibited MIC values lower than the MIC breakpoint values recommended for their respective species by the European Food Safety Authority (EFSA). However, we observed that *L. paracasei MW-37CGZ* and *L. paracasei MW-38CGZ* were resistant to the aminoglycosides (gentamicin, kanamycin and streptomycin) used. In addition, *L. plantarum MW-18CGZ* as well as *L. plantarum MW-48CGZ* were resistant to tetracycline. Vancomycin was not tested since susceptibility testing of *L. plantarum* and *L. paracasei* against vancomycin is not required.

Table 8. *Minimum inhibitory concentrations for antibiotic susceptibility of Lactobacillus isolates.*

Antibiotic [MIC (µg. mL^{-1})]								
***Lactobacillus* isolates**	**AMP**	**GEN**	**KAN**	**STR**	**ERY**	**CLI**	**TET**	**CAM**
Breakpoints*	4	32	64	64	1	4	4	4
Lactobacillus paracasei MW-37CGZ	2	> 32	> 64	> 64	0.25	0.5	4	0.5
Lactobacillus paracasei MW-38CGZ	1	> 32	> 64	> 64	0.25	0.5	4	0.25
Breakpoints*	2	16	64	NR	1	4	32	4
Lactobacillus plantarum MW-18CGZ	0.5	> 32	> 64	NR	0.5	0.125	> 32	0.5
Lactobacillus plantarum MW-48CGZ	0.25	> 32	> 64	NR	0.125	0.25	> 32	0.5

∗Values are provided by EFSA [20] for respective isolate; AMP: Ampicillin, CAM: Chloramphenicol, CLI: Clindamycin, ERY: Erythromycin, GEN: Gentamicin, KAN: Kanamycin, STR: Streptomycin, TET: Tetracycline. NR: not required.

L. plantarum MW-48CGZ, *L. plantarum MW-18CZ*, *L. paracasei MW-37CGZ* and *L. paracasei MW-38CGZ* had no clear transparent or greenish zone on the blood agar plates, surrounding their colonies, and thus were found to be γ-hemolytic or non-hemolytic. Moreover, none of the isolates showed caseinase and gelatinase activity as well as CAMP-like factor expression. The ability of isolates to coexist was tested by a cross-streak method. We observed no visible growth inhibition at the cross-section of isolates as shown in Figure 3.

The autoaggregation properties of isolates were further assessed over a period of 2, 16 and 20h at 37°C as shown in Table 9. In general, the isolates showed higher percentages of autoaggregation after 20h of incubation at 37°C and the most autoaggregative isolates were *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* with autoaggregation percentage of 68.71% and 65.27% respectively. In addition, *L. plantarum MW-48CGZ* showed the lowest autoaggregation percentages after 20h of incubation.

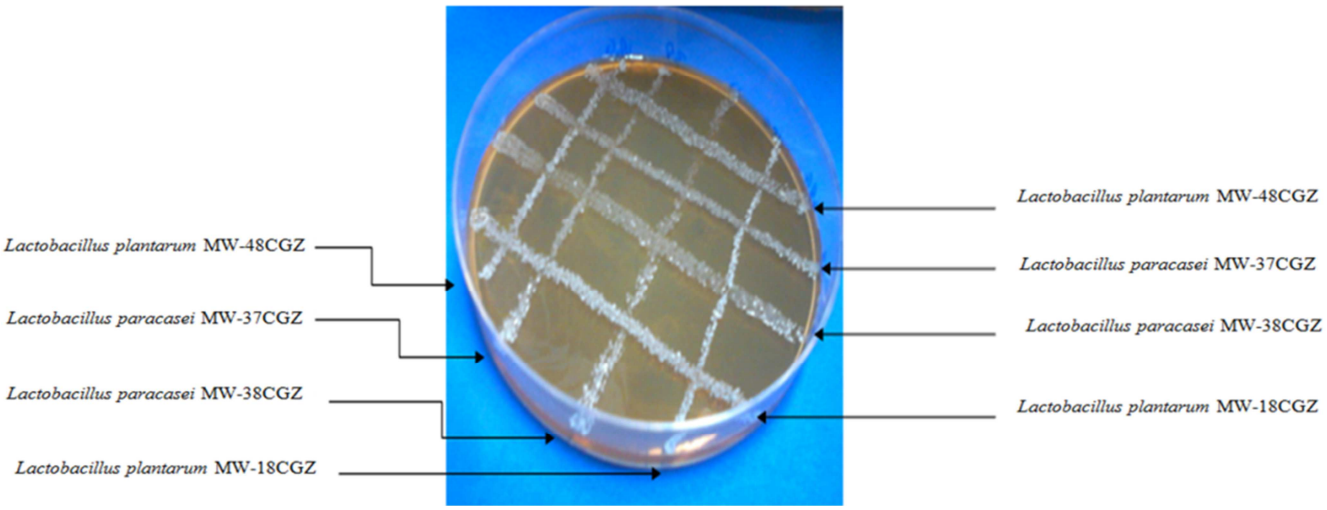

Figure 3. *Growth tolerance among Lactobacillus isolates.*

Table 9. *Autoaggregation properties of selected Lactobacillus isolates.*

% Autoaggregation at 37°C*			
	2h	**16h**	**20h**
***Lactobacillus* isolates**			
Lactobacillus paracasei MW-37CGZ	3.40 ± 2.35^{a}	24.10 ± 1.42^{a}	65.27 ± 5.78^{a}
Lactobacillus paracasei MW-38CGZ	2.8 ± 1.34^{a}	22.63 ± 1.78^{a}	46.10 ± 4.29^{b}
Lactobacillus plantarum MW-18CGZ	2.4 ± 0.42^{a}	43.20 ± 3.72^{b}	68.71 ± 3.83^{a}
Lactobacillus plantarum MW-48CGZ	2.3 ± 0.64^{a}	19.50 ± 1.89^{a}	32.45 ± 2.71^{c}

*Values are means ± SD of two independent experiments, each with duplicate, Number in column having the same letter are not significantly different (P > 0.05).

The coaggregation properties of selected isolates were analyzed at 37°C against eleven zoonotic pathogens at different times as shown in Table 10. All the potential probiotic isolates tested showed aggregation abilities with pathogens, however, the percentage of coaggregation was isolate-specific and dependent on time. The coaggregation properties were calculated according to the method described by Handley et al. [18]. Interestingly, *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* respectively, showed better ability to coaggregate with all pathogens tested after 20h of incubation. Isolates with least coaggregation percentages were *L. plantarum MW-48CGZ* and *L. paracasei MW-38CGZ*.

3.5. Cumulative Probiotic Potential

The individual Cumulative Probiotic Potential (CPP) was 100% for *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* while *L. plantarum MW-48CGZ* and *L. paracasei MW-38CGZ* had a CPP of 82% as described in Table 11.

Table 10. *Coaggregation properties of selected Lactobacillus isolates against pathogens.*

***Lactobacillus* isolates**			
	2h	**16h**	**20h**
Lactobacillus paracasei MW-37CGZ			
Clostridium sp.	6.10 ± 3.10	17.28 ± 2.45	37.84 ± 1.32
Escherichia vulneris	3.18 ± 2.45*	12.51 ± 2.12	36.12 ± 1.12
Proteus vulgaris	4.75 ± 3.55	18.05 ± 5.98	39.9 ± 3.67
Proteus mirabilis	6.43 ± 1.44	19.7 ± 2.88	38.66 ± 2.65
Providencia rettgevi	10.11 ± 4.33	11.30 ± 4.63	35.96 ± 2.20
Pseudomonas aeruginosa	7.36 ± 2.57	19.54 ± 0.87	32.61 ± 3.34
Staphylococcus sciuri	12.13 ± 2.88	18.15 ± 5.23	30.49 ± 4.48
Staphylococcus epidermidis	8.6 ± 3.64	22.08 ± 8.74	37.82 ± 2.34
Salmonella sp.	5.05 ± 1.84	24.34 ± 3.67	37.78 ± 2.67
Listeria sp.	3.78 ± 2.63	24.10 ± 3.70	35.63 ± 3.54
Shigella sp.	6.15 ± 2.97	26.46 ± 2.77	35.66 ± 1.32
Lactobacillus paracasei MW-38CGZ			
Clostridium sp.	6.35 ± 2.86	18.22 ± 2.65	10.72 ± 3.98
Escherichia vulneris	2.87 ± 2.55	17.62 ± 2.75	10.04 ± 2.66
Proteus vulgaris	1.30 ± 1.87*	11.15 ± 2.20	18.28 ± 3.56
Proteus mirabilis	3.65 ± 1.44	18.47 ± 1.88	16.44 ± 2.64
Providencia rettgevi	6.63 ± 1.89	19.82 ± 0.65	13.79 ± 4.86
Pseudomonas aeruginosa	5.60 ± 1.78	13.56 ± 1.54	18.11 ± 3.22
Staphylococcus sciuri	5.42 ± 1.67	17.11 ± 3.34	11.27 ± 1.01
Staphylococcus epidermidis	1.32 ± 1.65*	15.31 ± 2.89	15.34 ± 7.87
Salmonella sp.	7.32 ± 2.47	14.63 ± 2.78	14.45 ± 5.56
Listeria sp.	2.12 ± 1.65*	14.42 ± 1.89	17.29 ± 2.45
Shigella sp.	5.71 ± 1.54	17.10 ± 1.22	15.73 ± 4.98
Lactobacillus plantarum MW-18CGZ			
Clostridium sp.	10.32 ± 2.40	18.22 ± 2.03	51.34 ± 2.11
Escherichia vulneris	12.13 ± 1.14	13.12 ± 2.34	47.41 ± 1.33
Proteus vulgaris	13.27 ± 2.22	28.81 ± 1.07	55.17 ± 3.12
Proteus mirabilis	14.11 ± 1.56	19.65 ± 5.89	58.86 ± 4.14
Providencia rettgevi	19.22 ± 1.89	32.23 ± 2.34	53.17 ± 3.34
Pseudomonas aeruginosa	19.72 ± 2.67	26.01 ± 1.31	38.11 ± 2.21
Staphylococcus sciuri	14.22 ± 3.27	13.78 ± 4.41	37.14 ± 1.40
Staphylococcus epidermidis	23.81 ± 1.48	27.84 ± 2.23	54.01 ± 2.19
Salmonella sp.	11.82 ± 2.02	18.13 ± 3.01	54.43 ± 2.16
Listeria sp.	16.10 ± 1.78	26.44 ± 2.08	46.32 ± 3.15
Shigella sp.	18.11 ± 1.15	24.08 ± 1.34	45.13 ± 2.45
Lactobacillus plantarum MW-48CGZ			
Clostridium sp.	4.21 ± 1.08	16.11 ± 1.45	17.48 ± 3.22
Escherichia vulneris	6.15 ± 3.60	18.10 ± 2.42	12.32 ± 1.22
Proteus vulgaris	4.53 ± 2.43	14.12 ± 1.40	11.42 ± 3.34
Proteus mirabilis	3.82 ± 2.63	15.36 ± 1.56	14.30 ± 3.45
Providencia rettgevi	1.30 ± 1.80*	18.24 ± 1.56	14.14 ± 1.22
Pseudomonas aeruginosa	8.50 ± 2.34	13.34 ± 1.44	11.33 ± 2.45
Staphylococcus sciuri	2.32 ± 1.11*	19.59 ± 2.44	18.06 ± 2.24
Staphylococcus epidermidis	2.41 ± 1.24*	11.34 ± 2.32	10.12 ± 3.02
Salmonella sp.	5.21 ± 2.32	10.71 ± 1.90	14.03 ± 4.15
Listeria sp.	2.32 ± 2.24*	7.29 ± 2.43	10.76 ± 1.82
Shigella sp.	1.19 ± 1.12*	11.29 ± 2.11	16.22 ± 1.18

Values are means ± SD of two independent experiments, each with duplicate, *No significantly different ($P > 0.05$) from the control taken as 0% (no coaggregation).

Table 11. *Cumulative probiotic potential (CPP) score of selected Lactobacillus isolates.*

Individual Isolate Score					
	Indicator score	***L. paracasei MW-37CGZ***	***L. paracasei MW-38CGZ***	***L. plantarum MW-18CGZ***	***L. plantarum* MW-48CGZ**
Probiotic Characters					
Acidic pH tolerance	Resistant = 1 Sensitive = 0	1	1	1	1
Bile salt tolerance	Resistant = 1 Sensitive = 0	1	1	1	1

Individual Isolate Score					
	Indicator score	***L. paracasei* MW-37CGZ**	***L. paracasei* MW-38CGZ**	***L. plantarum* MW-18CGZ**	***L. plantarum* MW-48CGZ**
Bile salts deconjugation	Resistant = 1 Sensitive = 0	1	1	1	1
Cell surface Hydrophobicity	% < 35 = 0 % ≥ 35 = 1	1	1	1	1
Coaggregation	% < 35 = 0 % ≥ 35 = 1	1	0	1	0
Antimicrobial activity	DZI ≤ 15 mm = 0 DZI > 15 mm = 1	1	0	1	0
Antibiotic sensitivity	Intrinsic resistance/Sensitive = 1 Other resistance = 0	1	1	1	1
Haemolytic activity	β-haemolytic = 0 α-haemolytic = 0 γ-haemolytic = 1	1	1	1	1
Gelatinase activity	Activity = 0 No activity = 1	1	1	1	1
CAMP-like Factor expression	Expression = 0 No expression = 1	1	1	1	1
Caseinase activity	Activity = 0 No activity = 1	1	1	1	1
Total Score	11	11	9	11	9
Cumulative probiotic potential		100%	82%	100%	82%

4. Discussion

The emergence and spread of drug-resistant bacteria derived from food animal production in Low-and middle-incomes countries such as Cameroon is exacerbated by the irrational and poorly controlled use of antimicrobial in farms. Addressing this raising public health concern, a tripartite collaboration on antimicrobial resistance (AMR) composed of the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) was established aiming to collectively implement actions to reduce the emergence and spread of AMR [25]. The above concerns were evidenced in our previous study which showed that almost all zoonotic pathogens isolated from chickens' fecal samples in the Western Highlands of Cameroon were resistant to tested antibiotics [3].

In the present study, the probiotic potential of *Lactobacillus* isolates selected from fecal samples of farmyard chickens and ducks was evaluated for their potential use as alternatives to antibiotics in poultry farming. The isolation from farmyard animals was to limit the risk of selecting lactobacilli already resistant to antibiotics as these animals grow in a natural environment. Furthermore, the isolation of lactobacilli from various locally available natural sources is a reasonable way to discover new probiotic strains with outstanding properties than those already available.

The use of probiotic has been well documented, and their antibacterial and antifungal properties considered as an important attribute in selecting potential candidates [26]. In the current study, the antimicrobial activity of isolates was considered as a very important criterion for selection of candidates as they may produce natural substances exhibiting antagonistic properties similar to antibiotics. Based on this, four *Lactobacillus* isolates showed antagonistic activity against 20 strains which are pathogenic to humans and animals. The obtained results revealed that *Lactobacillus plantarum MW-18CGZ* and *Lactobacillus paracasei MW-37CGZ* showed stronger antimicrobial activity than the two other isolates. Interestingly, both isolates exhibited antagonistic activities against all zoonotic pathogens isolated previously from poultry and proved to be non-susceptible to most of the antibiotic tested, thus making them to be good candidates [3]. In addition, *Salmonella* spp., *Listeria* spp. and *Escherichia* spp. known as the most important poultry zoonotic pathogens were inhibited. According to the Centers for Disease Control and Prevention (CDC), *Salmonella* spp. are one of the major bacterial causes of foodborne gastroenteritis in United States with approximately 40,000 confirmed cases of salmonellosis annually. Poultry, meat products, and eggs are most commonly identified as food sources responsible for outbreaks of salmonellosis; however, many other foodstuffs such as ice cream, vegetables and fruits have also been vehicles of large outbreaks [27]. The treatment of salmonellosis infection in both human and animal may not be effective if the causative strains are already resistant, thus the use of probiotic as substitute to antibiotics may be considered. Abdel-Daim et al. [28] had reported that out of 32 strains of *L. plantarum* used in their study, 12 could inhibit the growth of *Salmonella* isolates recovered from stool specimens of patients. Similarly, our results showed that the most effective strain with the highest inhibition activity was *Lactobacillus plantarum MW-18CGZ.*

The concept of antagonistic activity of lactobacilli against pathogenic strains has been well documented in a review by Suskovic et al. [26]. These inhibitory activities are linked to the production of primary and secondary metabolites possessing antimicrobial properties which together with the mechanism of competitive exclusion would prevent colonization of the gastrointestinal tract (GIT) by pathogens [29]. Among the antimicrobial substances, organic acids (especially lactic and acetic acids), hydrogen peroxide, and bacteriocins are the most common antimicrobial substances that have been reported to be produced by probiotic strains.

In the present study, the antagonistic substance produced by *L. plantarum MW-18CGZ* was found to be proteinaceous, thus indicating that this substance may belong to a group of potent antimicrobial peptides such as bacteriocin produced by some microorganisms including lactic acid bacteria. Recently, Li et al. [30] revealed that the cell free supernatant of *L. plantarum* LZ206 showed antimicrobial activity similar to that observed in this study for *L. plantarum MW-18CGZ.*

Bacteriocins are a group of potent antimicrobial peptides

primarily active against closely related organisms and differ in spectrum of activity, mode of action, molecular weight (MW), genetic origin and biochemical properties [31]. The ability of *L. plantarum MW-18CGZ* to produce bacteriocin could be an added value and further study should be conducted to characterize the antimicrobial peptide produced by this isolate. On the other hand, the antagonistic activities *of L. paracasei MW-37CGZ*, *L. plantarum MW-48CGZ* and *L. paracasei MW-38CGZ* were found to be due to organic acid production and not hydrogen peroxide or bacteriocin. Poppi et al. [32], had also found that the inhibitory effects of *L. reuteri* and *L. delbrueckii* subsp. *delbrueckii* on pathogenic *Escherichia coli* O157: H7 were due to their production of organic acids. Similarly, Wasfi et al. [33] reported that the antagonistic activity of *L. salivarius* on *Strep. mutans* was linked to peroxide.

The results of acid tolerance showed that all four selected *Lactobacillus* isolates exhibited good acid resistance at pH 3 for 4 h, with *L. plantarum MW-18CGZ* showing a better acid tolerance than the other. This indicates that selected isolates can survive in low pH which is considered fundamental for selection of probiotics candidates [34]. Similar studies indicated that cell viability of lactobacilli during low pH challenge, varies significantly among isolates [35-37]. Moreover, it is also apparent here that the acid tolerance of the isolates was not related to the source as the later may impact the tolerance profile of isolates [11].

In addition to acid tolerance, a potential probiotic candidate should be able to tolerate bile salts more importantly as bile acids are produced continuously in birds [24]. The bile salt N. 3 used alongside bile salts is a mixture of the two salts, sodium cholate and sodium deoxycholate, which are the sodium salts of cholic and deoxycholic acid respectively, thus making up the natural conjugated bile acids. In this study, exposure to bile salts had little adverse effect on the survival of the four selected *Lactobacillus* candidates. All four isolates showed good tolerance to bile salts, with slight variations in the degree of tolerance. Campana et al. [38] also found that the ability to tolerate bile salt was strain-specific among the tested *Lactobacillus* strains and Chen et al. [39] reported that a stress tolerant strain of *L. kefiranofaciens* M1 can adapt in the presence of bile salts. Interestingly, our results showed that the ability of the four *Lactobacillus* isolates to tolerate bile salts maybe linked to their ability to produce Bile Salt Hydrolase (BSH), an enzyme that plays a central role in bile salts deconjugation. Several studies indicated that *Lactobacillus* species were able to adapt in a bile rich environment via the production of BSH [40].

In our study, viable and non-viable cells of the four isolates showed significant hydrophobicity with xylene. Particularly, *L. plantarum MW-48CGZ* exhibited higher hydrophobicity than other isolates. These results suggest that these isolates had strong hydrophobic interaction and are expected to adhere to the mucus or epithelial cells through hydrophobic interaction necessary for microbial colonization even though studies have indicated that cell surface properties are not enough to scientifically explain it [41, 42]. For these reasons, the ability of the *Lactobacillus* isolates to adhere and colonize on epithelial cells and mucosal surface was further investigated by autoaggregation and coaggregation to evaluate the abilities of isolates to inhibit the colonization of cells surfaces by pathogenic microorganisms [43]. The abilities of *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* to autoaggregation and coaggregation is an advantage in achieving greater mass that is necessary for enhancing tolerance to the GIT system, exerting of certain health benefits and prevention of pathogen colonization. However, there is no consensus in regard to the ability of lactobacilli to autoaggregate or coaggregate as evidence by literature disparity [44-46].

In this study, a serial twofold broth microdilution method was used to assess the susceptibility of the *Lactobacillus* isolates to eight antibiotics suggested by EFSA [20]. The obtained results are in accordance with lactobacilli susceptibility profile reported by authors indicating that they are generally susceptible to antibiotics [47]. However, previous studies have reported the presence of acquired resistance genes in lactobacilli [48-50]. This implies that the resistance of two isolates evaluated here to tetracycline should be further investigated even though this resistance is intrinsic, thus not transferable as indicated by Feichtinger et al. [51] and Pavunc et al. [52]. Moreover, earlier studies reported discrepancy in the susceptibility profile of lactobacilli using various methods including agar dilution, broth microdilution, disk diffusion, and Etest [53, 54].

Results assessing the safety properties of the isolates, indicated that they couldn't exhibit virulence factors which is essential because gelatinase, caseinase and hemolytic activities as well as Camp-like factors would destroy the epithelial layer, thus providing channel for pathogens translocation form the GIT to bloodstream [55]. Similar results were obtained by Sieladie et al. [7] with *L. plantarum* 29V.

The API 50 CHL system from BioMeriéux was successfully used as first line identification method by testing the fermentation capabilities of *Lactobacillus* isolates. However, studies have reported mismatches and the inability of the API system to accurately identify lactobacilli [56-59]. Consequently, a molecular assay namely High-resolution melt (HRM) analysis which is a sensitive post-polymerase chain reaction (PCR) method was applied as a fast, accurate, and reproducible method to confirm the identity of selected isolates. The 16S rDNA gene of the four isolates was amplified by real-time PCR and the amplicons were used for subsequent HRM analysis. The different *Lactobacillus* isolates tested generated distinctive HRM profiles, allowing the discrimination and differentiation of each strain. Based on the results obtained in this study, HRM confirmed to be a potent tool for microbial identification. The added advantages of this method reside in the fact that it is simple, rapid, and inexpensive, even though it depends strongly on good PCR instruments and dyes. Moreover, there is no need to process the sample after the PCR reaction and this allows

increase sensitivity of the method as compared to traditional PCR. The results of the HRM analysis were consistent with those achieved while assessing the enzymatic fermentative profile of isolates, thus confirming the original identification of isolates. Despite this, we will further improve the taxonomy of these isolates by sequencing the 16S ribosomal DNA.

The illustration of cumulative probiotic potential (CPP) was used here to summarize the overall performance of each of the four isolates tested. The CPP has been successfully described in previous studies for probiotic assessment [24, 60]. In the present investigation, the four lactobacilli isolates had CPP values raging between 80 to 100%. Similar values were reported by authors [61-62].

5. Conclusion

The results of this *in vitro* study showed that *L. paracasei MW-37CGZ*, *L. paracasei MW-38CGZ*, *L. plantarum MW-18CGZ* and *L. plantarum MW-48CGZ* isolated from the intestinal microbiota of farmyard chickens and ducks presented some important probiotic properties with varying levels. We attempted to demonstrate that these isolates can survive in the GIT, attach to the epithelial cells and proved to be safe. To the best of our knowledge, this is the first study assessing the antagonistic activity of lactobacilli, isolated from unexplored native microbiota of farmyard chickens and ducks. From the above results and the CPP analysis, we suggest that *L. plantarum MW-18CGZ* and *L. paracasei MW-37CGZ* have *in vitro* probiotic properties comparable or higher to those that are already available. Since these two *Lactobacillus* isolates showed strong antagonistic activities against a wide range of human and zoonotic pathogens, they could be considered as effective biotherapeutic substitute for antibiotics as feed additive in the poultry industry, so as to reduce antibiotic residues from food animals and the generation and spread of antibiotic resistance. Finally, the antimicrobial peptide produced by *L. plantarum MW-18CGZ* will be further characterized and *in vivo* assay performed to confirm the beneficial effect in animals.

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