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# *In vitro* Study of Potential Probiotic Lactic Acid Bacteria Isolated from The Gut of Chickens in Abeokuta, Nigeria

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## ABSTRACT

Abuse of antibiotics in Veterinary Practice and Animal Production results in the emergence of antibiotic-resistant bacteria and the presence of residual antibiotics in food animal production with attendant Public Health concerns. Probiotics have been reported to alleviate these challenges. In this study, 36 isolates of Lactic acid bacteria (LAB) were isolated from the gut of broilers on de Mann, Rogosa and Sharp (MRS) agar. The isolates were screened for their ability to grow at pH 3.0, 2.5 and 2.0 for 3 hours. Twenty-seven (75%) isolates demonstrated a survival percentage of ≥ 70 % after 3 hours of acid exposure at pH 3.0; 20 (55.55%) isolates at pH 2.5 showed a survival percentage of ≥ 50 % after 3 hours and 11 survived at pH 2.0 for at least 2 hours with a survival percentage of ≥ 50 %. These 11 isolates also showed resistance to 0.3 % and 0.5 % bile salt concentrations with survival percentages ranging from 68.74 – 89.98 % and 63.26 – 86.95 % respectively. The eleven potential probiotic LAB inhibited the growth of *Escherichia coli*, *Salmonella typhimurium*, *Salmonella arizonae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *E. coli* O157:H7 and *Staphylococcus aureus* with diameter of inhibition zones ranging from 9.5 – 17.0 mm. The eleven isolates were non-pathogenic (γ-haemolytic) and exhibited resistance to antibiotics erythromycin, gentamycin, streptomycin, amoxicillin, cotrimoxazole, ceftriaxone, ceftazidime, cefuroxime, cefixime and augmentin. isolates were susceptible to chloramphenicol and nitrofurantoin while eight and five isolates were susceptible to ciprofloxacin and ofloxacin respectively. All the isolates could tolerate salt (NaCl) concentrations of 2 and 4 % but not 8 and 10 %. The isolates differed in growth patterns at temperatures 10 ºC, 15 ºC and 45 ºC and in their sugar fermentation patterns. The 16S rRNA gene sequence homology confirmed these isolates as *Enterococcus. faecium*, *E. faecalis*, *E. durans*, *Lactobacillus acidophilus* and *L. fermentum*. *Enterococcus* spp. were the more prevalent species isolated. The gut of broiler chickens could be a potential source of probiotic LAB that could serve as alternative for antibiotics in poultry production.

## 1. INTRODUCTION

The use of antibiotics in poultry and livestock industry, as documented by several authors, (O'Sullivan, 2001; Acurcio *et al*., 2014) have resulted in the emergence of persistent residual antibiotics in organs, eggs, meat, poultry, milk and dairy products (Peters *et al* 2009, Done and Halden, 2016). Some of these residual products have been implicated as potential carcinogens, causes of neurologic and gastro-intestinal disorders, allergens in hypersensitive individuals and the development of antibiotic-resistant bacterial strains (FAO/WHO, 2004; Adewuyi *et al*., 2011). The use of probiotics has been found to be clinically important in the prevention and treatment of diseases in animal and man (Gauthier, 2002; CAST, 2007; McFarland *et al* 2014; Manuel *et al* 2016). Probiotics have also been reported to suppress acute and antibiotic-associated diarrhoea, alleviate lactose intolerance and post-operative complications, exhibit antimicrobial and

anti-colorectal cancer activities, reduce irritable bowel symptoms and prevent inflammatory bowel disease (CAST, 2007; D'Silva, 2011; Fontana *et al*., 2013). These positive effects are generally attributed to the ability of probiotics to regulate intestinal permeability, normalise host intestinal microbiota, improve gut immune barrier function and equilibrate the balance between pro-inflammatory and anti-inflammatory cytokines (Gomes *et al*., 2014).

Lactic acid bacteria of the species *Lactobacillus* and *Bifidobacterium* are most commonly used as probiotics, but the yeasts *Saccharomyces cerevisiae* and *Saccharomyces boulardii*, and some non-pathogenic strains of *Escherichia coli* and *Bacillus* species are also used as probiotics (WGO, 2011. Gareth *et al.*, 2016).

In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to be of host origin (to allow for easy adaptation and colonisation in the gastrointestinal tract), non-pathogenic, resistant to gastric acid and bile, adhere to gut epithelium or mucosa, possess antimicrobial activity by inhibiting pathogenic bacteria, modulate immune response safe for use *in vivo* and influence microbial activities such as vitamin production, cholesterol assimilation, lactose activity (FAO/WHO, 2001; Patterson and Burkholder, 2003; ICMR-DBT, 2011; Pundir *et al*., 2013; Morjgani, *et al* 2015). These characteristics, amongst others, are usually employed as suitable indices for screening probiotics.

Some probiotics that have been supplemented in animal feed include bacterial species, such as *Lactobacillus* spp., *Enterococcus faecium*, *Bifidobacterium thermophilum*, *Streptomyces* spp., *Micrococcus* spp., *Pseudomonas fluorescens*, as well as yeast, such as *Saccharomyces cerevisiae* (Tuan *et al*., 2013).

In Nigeria, there are no efficient regulatory and withdrawal guidelines for the use of antibiotics and antibiotic growth promoters (AGPs) in animal production yet. There is an unregulated access to veterinary drugs and antibiotics which are obtainable in open markets without prescription and supervision of administration to animals bred for food. This has led to indiscriminate administration of drugs and antibiotics to food animals. Thus, correct dosage and withdrawal periods of antibiotics are not usually adhered to and food safety quality assurance cannot be guaranteed (Olatoye and Ogundipe, 2009; Olatoye *et al*., 2011). It therefore, becomes imperative that these AGPs are eliminated and antibiotic use restricted in food animal production to minimise risk to both food animals and health of consumers.

The objective of this study is to isolate and identify lactic acid bacteria present in the gut of broilers for their probiotic properties, with a view to selecting suitable strains for use as potential probiotics for broilers.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection

Samples of intestinal contents were collected from three healthy broilers purchased from the Department of Animal Production and Health, Federal University of Agriculture Abeokuta, Ogun State, Nigeria. The birds were sacrificed and their digestive tracts were immediately collected. One gram of the intestinal contents of each bird was collected into 30ml sterile universal containers. The intestinal tissues were washed with sterile normal saline to remove intestinal contents and surface mucus. To obtain adhering bacteria, the intestinal epithelial tissues were scraped with a sterile surgical blade. One gram each of small (S) and large (L) tissues were then collected into 30ml sterile universal containers (Shokryazdan *et al*., 2014).

### 2.2. Isolation and Preliminary Identification of Lactic Acid Bacteria (LAB) strains

One gram of each sample was serially diluted up to $10^6$ using peptone broth as diluent. Inoculation of 1ml aliquot diluent was done on deMan, Rogosa and Sharpe (MRS) agar (SRL, India) using the pour plate technique (Olutiola *et.al* 1991). Inoculated plates were incubated in a candle extinction jar for 48 hours. After incubation, distinct colonies were randomly picked and purified by repeated streaking on MRS agar. The cultures were stored and maintained at 4 °C on MRS agar slates and all cultures were resuscitated in MRS broth for 16-24 hours prior to their use in each experimental step. Standard cultural characterisation, microscopic and some biochemical identification procedures were employed. Young active cultures of the isolates (24 hours cultures) were examined according to their colony morphology, Gram reaction and catalase reaction (Olutiola *et al*., 1991).

### 2.3. Tolerance to acidic pH conditions

The criteria for the *in vitro* selection of organisms to be used for probiotic are related to acid and bile tolerance, production of antimicrobial substances that inhibit the growth of other microorganisms as

well as the safety for food and clinical use (Hoque *et al*., 2010; Sieladie *et al.*, 2011). Ability of LAB to survive low pH ranging from 1-3 for up to 3hours is essential for their activity as probiotics (Shokryazdan *et al*., 2014).

MRS broth media were adjusted to different pH values (2.0, 2.5 and 3.0) using 1M HCl or 1M NaOH. The broth was inoculated with 100 µl of actively growing bacterial cultures and incubated anaerobically at 37 °C for 3 hours. Viable LAB count on MRS agar at each time interval (0, 1, 2 and 3 hours) was determined using the pour plate technique. Tolerance to acidic condition was estimated by comparing viable cell counts after exposure to acidic condition and the initial cell count (at 0 hour). The assay was performed twice, each in duplicate. Survival percentage of each strain to different pH conditions was then calculated as:

$$\text{pH survival (\%)} = \frac{\text{Viable LAB count (CFU/ml)after acid exposure}}{\text{Initial viable LAB count (CFU/ml)}} \times 100$$

(Kumar *et al.,* 2009)

An isolate was considered to have survived if it demonstrated- a survival percentage equal or greater than 70 % at pH 3.0 after 3 hours; a survival percentage equal or greater than 50 % at pH 2.5 after 3 hours and a survival percentage equal or greater than 50 % at pH 2.0 after 2 hours.

**2.4. Bile salt tolerance**

The modified method of Oluwajobi et al., (2013) was used to assay bile salt tolerance. This was done by supplementing MRS broth with 0.3 or 0.5 % (w/v) sodium deoxycholate (bile salt) (BDH, England). Inoculation was done using 1 % bacterial culture and incubation was done under anaerobic condition at 37 °C for 3 hours. Inoculum samples (1 ml) were taken at 1 hour interval for viable LAB count on MRS agar. Percentage resistance to bile salt was determined according to the equation:

$$\text{Bile salt resistance (\%)} = \frac{\text{Viable LAB count (CFU/ml)after bile salt exposure}}{\text{Initial viable LAB count (CFU/ml)}} \times 100$$

(Kumar *et al.,* 2009)

An isolate was considered to have survived if it demonstrated a surviving percentage equal or greater than 50 % (Sieladie *et al*., 2011).

**2.5. Antimicrobial metabolite inhibition**

The pathogenic microorganisms used in this study were isolated from poultry. *Escherichia coli* (isolated from chicken with colibacillosis), *Salmonella typhimurium*, *Salmonella arizonae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were laboratory strains obtained from Animal Care Services Konsult Diagnostic Laboratory, Ogere-Remo, Ogun State, Nigeria. *E. coli* O157:H7 was obtained from the Department of Microbiology, Federal College of Animal Health and Production Technology, Moor Plantation, Ibadan, Oyo State, Nigeria.

The inhibitory effects of the antimicrobial metabolite produced by the LAB isolates were determined using the method as described by Pundir *et al*. (2013). One ml of each test microorganism adjusted to 0.5 McFarland standards, was inoculated on Mueller Hinton agar using the pour plate technique. The agar plates were allowed to set and wells were bored using a sterile borer of 6mm diameter. A volume of 100µl of cell free supernatants was put in the wells cut into the Mueller Hinton agar. The diameter of the inhibition zone was measured with a ruler after 24 hours of incubation.

**2.6. Antibiotic sensitivity patterns**

The antimicrobial susceptibility testing was carried out on the isolated LAB on MRS agar using the disc diffusion method as described by Lavanya *et al*., (2011). Susceptibility patterns were assessed for erythromycin, gentamycin, streptomycin, amoxicillin, chloramphenicol, cotrimoxazole, ciprofloxacine, ofloxacin, ceftriaxone, ceftazidime, cefuroxime, cefixine, augmentin and nitrofurantoin. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition in millimeters. Results were expressed as: susceptibility, S (diameter ≥ 21 mm); intermediate susceptibility, I (diameter 16–20 mm); and resistance, R (diameter ≤ 15 mm) according to Vlková *et al*. (2006) and Sieladie *et al*. (2011).

**2.7. Haemolytic activity**

Haemolytic activity of isolates was carried out using 5 % human blood agar. Each blood agar plate was streaked with the LAB isolates and incubated at 37 $^{o}$C for 24 hours after which the plates were examined for haemolysis. *Escherichia coli* and *Staphylococcus aureus* were used as control for α- and β-haemolysis respectively.

**2.8. Further Physiological and Biochemical Characterisation**

The selected potential probiotic strains were further assayed to determine their temperature and sodium chloride sensitivity/tolerance, acid and gas

production from glucose and their sugar fermentation patterns.

### 2.9. Molecular Characterisation

Laboratory Molecular procedures were carried out at Stab Vida, Lisbon, Portugal. Bacterial cultures were inoculated and transported on FTA® cards. DNA was extracted using 150 µl each of FTA® wash reagent and RNAse/DNAse free water with incubation condition of 56 $^{o}$C for 15 minutes. Each wash was repeated twice. The cards were then dried at 56 $^{o}$C for 10 minutes.

For amplification of the 16S rRNA gene, two universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'- TACGGYTACCTTGTTACGACTT -3') were used as forward and backward primers respectively (Acurcio *et al*., 2014; Shorkryazdan *et al*., 2014) with the expected PCR product of 1.5 kbp. PCR amplification was carried out in a thermal cycler (SureCycler 8800, Agilent Technologies, USA) using Stab Vida's Surf Hot Taq DNA polymerase and reagents. For PCR, reactions were performed in the presence of 1 µl of dNTP (5mM), 1 µl each of the appropriate primers (10mM), 1.5 µl of $MgCl_2$ (25mM), 17.8 µl of $H_2O$ nuclease free, 2.5 µl of 10x PCR buffer and 0.2 µl of Surf Hot Taq (10U/µl ) in a final volume of 25 µl. Following initial denaturation at 96 $^{o}$C for 15 minutes, reactions were subjected to 37 thermal cycles with the following parameters: denaturation at 95 $^{o}$C for 30 seconds, hybridisation at 52 $^{o}$C for 30 seconds, elongation at 72 $^{o}$C for 2 minutes and a final extension step at 72 $^{o}$C for 7 minutes. Following PCR, products were verified to be of expected molecular weight by visualising 2µl of PCR product and 5µl of DNA ladder on a 1 % agarose TAE gel. Sequencing was performed using BigDye Terminator (BDT) v3.1 cycle sequencing kit technology (Applied Biosystems, USA), and running was made in an ABI 3730XL automated sequencer (Applied Biosystems, USA).

DNA sequences were compared with reference sequences in GenBank, National Centre for Biotechnological Information (NCBI) using the BLASTn program. Sequences with ≥ 95% similarity to the previously published sequences were used as the criterion to indicate species identity.

Phylogenetic tree for the bacterial isolates as well as sequences of other claimed probiotics retrieved from the NCBI database was constructed using the maximum likelihood method (1000 bootstrap replicates, Jukes-Cantor substitution model) with MEGA 6.0.

### 2.10. Statistical Analysis of Data

Statistical Package for Social Sciences (SPSS, version 17.0) software was used to subject data to an analysis of variance (ANOVA) at a significant level of $\alpha = 0.05$. Significant differences between means ($p<0.05$) were separated using Duncan multiple range test.

## 3. RESULTS AND DISCUSSION

A total of 36 bacterial isolates displayed the general morphological characteristics of LAB, producing spherical to spindle shaped, white to cream coloured, shiny, opaque to translucent, flat to raised, smooth colonies. All of the isolates were Gram positive and catalase negative.

Table 1 shows the results of percentage survival of the isolates after acid exposure. Out of the 36 isolates assayed, 11 isolates were able to satisfy the criteria set for the acid tolerance test. The viable LAB count was observed to decrease with the incubation time ($p < 0.05$). This decrease observed during the 3 hours of incubation implies that the survival of LAB is largely dependent on the pH of the environment. At low pH, they are susceptible due to the action of the acidic environment on the cells which in-turn reduces their survival for both *in vivo* and *in vitro* experiments (Kailasapathy and Chin, 2000). The decreasing trend of viable LAB count obtained in this study is as a result of the hydrochloric acid (HCl) added to the medium, which has a bactericidal effect. HCl tends to oxidise some important biomolecules in the cells thus, leading to a reduction in the cell viability. HCl oxidation of cells has been observed to be strain dependent (Zavaglia *et al*., 2002). This result is in consonance with the findings of other similar research works where it has been documented that the bacterial count in acidified medium decreased with the time of incubation as well as the demonstrated capacity of these bacterial strains to tolerate acidic conditions (Chim-anage *et al*. 2008; Oloyede and Afolabi, 2013; Oluwajoba *et al*., 2013).

Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. According to Lin *et al.* (2003), the total bile salt concentration in the duodenum and cecum of chicken intestine has been reported to be 0.008 and 0.175 % respectively. In addition, Mokarram *et al*. (2011) reported that the type of food consumed affects the concentration and rate of secretion of bile. Thus, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentrations ranging from 0.15-0.30 % (Šušković *et al*., 2000).

In this study, the LAB strains were able to survive and grow in 0.3 and 0.5 % bile salt (sodium deoxycholate) concentrations for 3 hours (Table 2). It was observed that time had significant effect ($p<0.05$) on the viable LAB count as well as their survival percentages. Bacterial viable counts and survival percentages remained fairly stable for most of the isolates assayed. The bacterial count of all the isolates decreased just before the first hour. The percentage survival of some of the isolates decreased between the first and second hour. This could be attributed to the inability of the isolates to adapt initially in the bile salt medium hence, a delay in their growth. Jafari *et al*. (2011) reported growth delay of some *Lactobacillus* and *Enterococcus* strains in 0.3 % bile salt. The authors reported that the growth delay of the strains studied ranged from 15 minutes to more than one hour.

This reduction of bacterial survival in the bile supplemented medium is largely due to the fact that bile salts are able to inhibit the growth of bacteria, especially Gram positive bacteria. Bile acids act by dissociating the cell membrane of the already disrupted cell wall of the bacterial cell after acid exposure. The bile dissolves the phospholipids, cholesterol and proteins which make up the cell membrane thus, allowing disruption of cellular homeostasis, and further cell wall dissociation leads to cell lyses and leakage of bacteria content resulting in its low survival rate (Begley *et al*., 2005). Regardless of the resistance patterns observed in the presence of bile salt, all the isolates in this study could survive and grow in 0.3 and 0.5 % bile salt conditions for 3 hours. The growth in the bile salt medium could be attributed to the ability of the isolates to produce bile salt hydrolases. This hydrolytic activity decreases the toxic effect of the bile salt on the LAB as reported by Jafari *et al*., (2011).

Antibiotic activity is one of the important selection criteria for probiotics. In the present study, the LAB isolates were assayed for their antibiotic susceptibility patterns (Table 3). The isolates showed varying patterns in their resistance to ofloxacin and ciprofloxacin. All the isolates were susceptible to chloramphenicol and nitrofurantoin while being resistant to erythromycin, gentamycin, streptomycin, amoxicillin, cotrimoxazole, ceftriaxone, ceftazidime, cefuroxime, cefixime and augmentin. Although, most of the antibiotics used in this study are not common antibiotics used in poultry production, this trend in the antibiotic resistance pattern of the LAB isolates could be attributed to the routine use of antibiotics in poultry production. Antibiotics are routinely added in sub-therapeutic doses to the diet (drinking water or feed) of birds as treatment measures, control of diseases as well as for their growth-promoting effects. This regular practice tends to consistently expose the natural gut microflora to traces of antibiotics which accumulates with time. Enteric bacteria tend to develop resistance to the antibiotics used due to constant exposures. Similar trend in antibiotic susceptibility pattern has also been reported by Oloyede and Afolabi (2013) and Acurcio *et al*. (2014).

Table 4 shows the observed growth inhibition on agar well diffusion plates which indicated that the assayed LAB produced antimicrobial products which could be organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and/or bacteriocins that were able to inhibit the growth of *E. coli* O157:H7, *S. typhimurium*, *S. arizonae*, *K. oxytoca*, *P. aeruginosa*, *E. coli* and *S. aureus*, all of which were pathogenic laboratory strains from poultry. In consonance with this study, Chang *et al*. (2013), Oluwajoba *et al*. (2013) and several other authors have also reported the antagonistic activity of LAB against pathogens. Spanggaard *et al*. (2001) reported that this antagonism was the most influential factor preventing the establishment of the exogenous bacteria and indicates that the antagonistic part of an indigenous flora may offer a significant contribution to the control of unwanted (pathogenic) bacteria.

Safety is one of the recommended attributes in the FAO/ WHO (2002) guidelines on evaluation for probiotics. Haemolytic activity would break down the defensive epithelial layer, interfering with its normal functioning, and would cause pathways for infections. Absence of haemolytic activity is a selection criterion for probiotic strains, indicating to some extent that these bacteria are non-virulent (DeVuyst *et al.,* 2003). The presence of haemolysins as an indicator of potential pathogenicity must be evaluated in these microorganisms, before they can be used as probiotics and/or food additives (Bourouni *et al*., 2012).

All bacterial strains assayed in this study demonstrated non-haemolytic (gamma, γ-haemolytic) activity hence, they do not produce the toxic substance, haemolysin. Similar results have been reported for various LAB strains by Shivram and Vishwanath (2012), Chang *et al*. (2013) as well as Oloyede and Afolabi (2013).

The 16S rRNA gene sequencing was done and sequences with ≥ 95% homology with previously published sequences (NCBI) were selected to

indicate species identity. The selected eleven potential probiotic strains were identified as *Enterococcus faecium* (L1, 97%; S7, 99%; S11, 97%), *Enterococcus durans* (S2, 99%; S13, 100%), *Enterococcus faecalis* (S4, 98%; C10, 97%; S14, 99%), *Lactobacillus fermentum* (C3, 96%; C7, 97%) and *Lactobacillus acidophilus* (C14, 99%). All of the PCR amplicons of the isolates were found to have varying molecular weight between 1000 bp and 1500 bp (Figure 1). A phylogenetic tree was constructed based on the 16S rRNA gene sequence analysis which involved 29 nucleotide sequences including the 11 sequences obtained in this study. The results showed that the isolated strains from this study share some homology with the nucleotide sequences of probiotic LAB strains obtained from the NCBI database (Fig. 2).

## 4. CONCLUSION

Lactic acid bacteria were isolated from the gut of healthy broilers in the search to find strains for use as poultry probiotics. A total of 11 strains showed resistance to acidic pH, tolerance to bile salt and antimicrobial activity. *Enterococcus* spp. was the predominant genus isolated. *L. fermentum* and *L. acidophilus* were also isolated alongside with *E. faecium*, *E. faecalis* and *E. durans*. The strains isolated showed some probiotic properties which suggest their possible use as poultry feed supplements. In this study, the initial steps of selection criteria for probiotic microorganisms were determined and the selected isolates identified. Although the selection criteria are not limited to those determined, the isolates obtained from this study could be potential probiotic strains even if some further tests were applied. Nevertheless, some future studies should be performed in order to further prove their full probiotic potential as well as their more reliable application and efficacy.

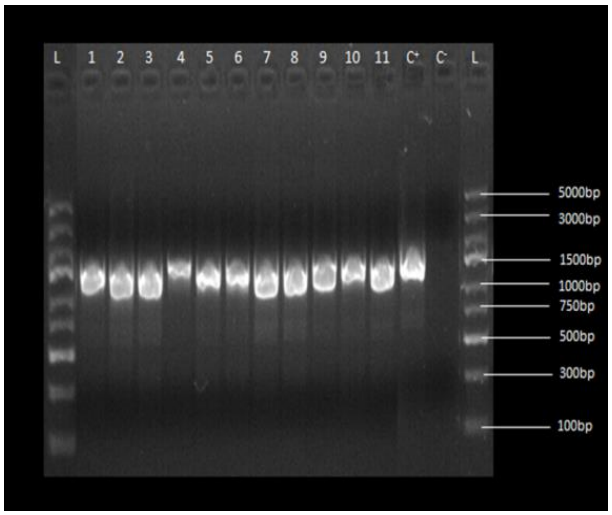


**Figure 1:** 1 % agarose gel electrophoresis of PCR amplicons of the 16S rRNA genes of bacterial isolates. Lane L: 100 bp DNA ladder, Lane 1 and 8: *Lactobacillus fermentum*, Lane 2, 3 and 7: *Enterococcus faecium*, Lane 4: *Lactobacillus acidophilus*, Lane 5, 6 and 10: *Enterococcus faecalis*, Lane 9 and 11: *Enterococcus durans*, $C^+$: positive control, $C^-$: negative control.

**Table 1.Tolerance to acidic pH 3.0, 2.5 and 2.0**

| Isolate code | Percentage survival (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 3.0 | | | pH 2.5 | | | pH 2.0 | | |
| | 1 hour | 2 hours | 3 hours | 1 hour | 2 hours | 3 hours | 1 hour | 2 hours | 3 hours |
| **C1** | 95.92 ± 0.019$^{hijk}$ | 91.03 ± 0.047$^{gh}$ | 86.18 ± 0.057$^{ijk}$ | 84.76 ± 0.015$^{defgh}$ | 73.05 ± 0.025$^{defghij}$ | 64.98 ± 0.042$^{fghi}$ | 12.03 ± 0.011$^{b}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |
| **C2** | 83.29 ± 0.024$^{abc}$ | 65.57 ± 0.010$^{a}$ | 50.38 ± 0.021$^{a}$ | - | - | - | - | - | - |
| **C3** | 95.41 ± 0.022$^{ghijk}$ | 90.04 ± 0.040$^{gh}$ | 83.54 ± 0.016$^{hijk}$ | 77.16 ± 0.090$^{abcd}$ | 68.08 ± 0.079$^{bcde}$ | 58.10 ± 0.079$^{defg}$ | 67.89 ± 0.009$^{fg}$ | 50.96 ± 0.009$^{d}$ | 27.28 ± 0.012$^{bc}$ |
| **C4** | 92.41 ± 0.025$^{fghijk}$ | 80.82 ± 0.010$^{cdefgh}$ | 72.47 ± 0.008$^{defgh}$ | 87.16 ± 0.013$^{fghi}$ | 74.58 ± 0.032$^{defghij}$ | 66.75 ± 0.026$^{fghi}$ | 58.82 ± 0.073$^{f}$ | 25.52 ± 0.030$^{c}$ | 0.00 ± 0$^{a}$ |
| **C5** | 79.25 ± 0.036$^{a}$ | 67.18 ± 0.059$^{abc}$ | 59.52 ± 0.085$^{abc}$ | - | - | - | - | - | - |
| **C6** | 87.75 ± 0.040$^{cdef}$ | 81.76 ± 0.031$^{defgh}$ | 77.52 ± 0.084$^{ghijk}$ | 80.44 ± 0.006$^{bcdef}$ | 71.59 ± 0.011$^{cdefghi}$ | 61.83 ± 0.038$^{efg}$ | 27.69 ± 0.032$^{c}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |
| **C7** | 97.43 ± 0.009$^{jk}$ | 92.49 ± 0.005$^{gh}$ | 88.31 ± 0.006$^{jk}$ | 94.20 ± 0.033$^{i}$ | 85.35 ± 0.061$^{jk}$ | 69.41 ± 0.022$^{ghij}$ | 74.66 ± 0.042$^{gh}$ | 61.23 ± 0.061$^{ef}$ | 36.97 ± 0.025$^{de}$ |
| **C8** | 91.82 ± 0.020$^{fghij}$ | 85.76 ± 0.015$^{efgh}$ | 81.29 ± 0.014$^{ghijk}$ | 87.72 ± 0.004$^{fghi}$ | 65.62 ± 0.067$^{abcde}$ | 44.85 ± 0.067$^{abc}$ | - | - | - |
| **C9** | 90.61 ± 0.013$^{defghi}$ | 86.77 ± 0.005$^{fgh}$ | 76.37 ± 0.062$^{ghij}$ | 86.34 ± 0.031$^{efghi}$ | 76.14 ± 0.013$^{efghij}$ | 65.14 ± 0.021$^{fghi}$ | 48.13 ± 0.013$^{e}$ | 29.48 ± 0.026$^{c}$ | 0.00 ± 0$^{a}$ |
| **C10** | 94.39 ± 0.013$^{ghijk}$ | 89.34 ± 0.014$^{gh}$ | 84.20 ± 0.014$^{hijk}$ | 81.83 ± 0.026$^{cdefg}$ | 70.54 ± 0.056$^{cdefg}$ | 59.80 ± 0.048$^{defg}$ | 72.46 ± 0.013$^{gh}$ | 56.95 ± 0.002$^{de}$ | 40.96 ± 0.052$^{e}$ |
| **C11** | 95.44 ± 0.019$^{ghijk}$ | 87.08 ± 0.010$^{fgh}$ | 80.65 ± 0.021$^{ghijk}$ | 81.17 ± 0.031$^{cdef}$ | 62.20 ± 0.104$^{abcd}$ | 43.41 ± 0.082$^{abc}$ | - | - | - |
| **C12** | 95.24 ± 0.010$^{ghijk}$ | 82.27 ± 0.087$^{defgh}$ | 74.99 ± 0.115$^{fghi}$ | 86.39 ± 0.024$^{efghi}$ | 68.46 ± 0.051$^{bcdef}$ | 51.57 ± 0.004$^{bcde}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |
| **C13** | 91.44 ± 0.028$^{efghij}$ | 72.17 ± 0.007$^{abcde}$ | 63.68 ± 0.043$^{bcdef}$ | - | - | - | - | - | - |
| **C14** | 89.45 ± 0.017$^{defg}$ | 83.50 ± 0.036$^{efgh}$ | 72.31 ± 0.004$^{defgh}$ | 83.92 ± 0.040$^{defgh}$ | 76.37 ± 0.040$^{efghij}$ | 66.99 ± 0.035$^{fghi}$ | 68.06 ± 0.027$^{fg}$ | 54.09 ± 0.003$^{d}$ | 39.14 ± 0.002$^{e}$ |
| **C15** | 93.07 ± 0.011$^{fghijk}$ | 84.71 ± 0.029$^{efgh}$ | 80.44 ± 0.006$^{ghijk}$ | 72.32 ± 0.031$^{a}$ | 59.42 ± 0.020$^{abc}$ | 50.04 ± 0.025$^{abcd}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |
| **L1** | 97.07 ± 0.024$^{jk}$ | 88.32 ± 0.023$^{gh}$ | 83.68 ± 0.006$^{hijk}$ | 91.94 ± 0.035$^{hi}$ | 81.82 ± 0.090$^{ghijk}$ | 67.61 ± 0.018$^{fghij}$ | 61.79 ± 0.033$^{f}$ | 54.90 ± 0.035$^{d}$ | 29.27 ± 0.024$^{bc}$ |
| **L2** | 90.01 ± 0.002$^{defgh}$ | 84.01 ± 0.049$^{efgh}$ | 77.17 ± 0.055$^{ghijk}$ | 72.94 ± 0.036$^{ab}$ | 61.88 ± 0.084$^{abcd}$ | 41.36 ± 0.036$^{ab}$ | - | - | - |
| **L3** | 93.32 ± 0.005$^{fghijk}$ | 88.51 ± 0.027$^{gh}$ | 80.22 ± 0.078$^{ghijk}$ | 76.98 ± 0.044$^{abcd}$ | 61.82 ± 0.060$^{abcd}$ | 43.35 ± 0.048$^{abc}$ | - | - | - |
| **L4** | 95.33 ± 0.029$^{ghijk}$ | 85.31 ± 0.031$^{efgh}$ | 77.90 ± 0.055$^{ghijk}$ | 77.72 ± 0.010$^{abcd}$ | 69.81 ± 0.033$^{bcdefg}$ | 64.44 ± 0.011$^{fghi}$ | 34.67 ± 0.050$^{cd}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **L5** | 91.24 ± 0.033$^{efghij}$ | 86.85 ± 0.048$^{fgh}$ | 81.80 ± 0.012$^{ghijk}$ | 86.59 ± 0.050$^{efghi}$ | 65.28 ± 0.006$^{abcde}$ | 56.91 ± 0.005$^{def}$ | 41.41 ± 0.034$^{de}$ | 0.00 ± 0$^{a}$ | 0.00 ± 0$^{a}$ |
| **L6** | 85.47 ± 0.018$^{bcde}$ | 79.98 ± 0.004$^{bcdefg}$ | 70.38 ± 0.011$^{cdefg}$ | 81.53 ± 0.043$^{cdef}$ | 66.26 ± 0.025$^{abcde}$ | 43.69 ± 0.051$^{abc}$ | - | - | - |
| **L7** | 83.01 ± 0.031$^{abc}$ | 66.54 ± 0.087$^{ab}$ | 55.82 ± 0.075$^{ab}$ | - | - | - | - | - | - |
| **S1** | 96.91 ± 0.013$^{jk}$ | 91.47 ± 0.016$^{gh}$ | 85.90 ± 0.026$^{ijk}$ | 89.86 ± 0.022$^{ghi}$ | 83.55 ± 0.015$^{hijk}$ | 73.80 ± 0.042$^{ijk}$ | 26.97 ± 0.037$^{c}$ | 14.10 ± 0.044$^{b}$ | 0.00 ± 0$^{a}$ |
| **S2** | 95.63 ± 0.015$^{ghijk}$ | 89.39 ± 0.055$^{gh}$ | 83.84 ± 0.034$^{hijk}$ | 87.92 ± 0.024$^{fghi}$ | 83.49 ± 0.031$^{hijk}$ | 78.29 ± 0.045$^{jk}$ | 79.82 ± 0.020$^{hi}$ | 63.72 ± 0.041$^{f}$ | 50.49 ± 0.015$^{f}$ |
| **S3** | 79.59 ± 0.032$^{ab}$ | 66.45 ± 0.026$^{ab}$ | 52.64 ± 0.023$^{ab}$ | - | - | - | - | - | - |
| **S4** | 96.79 ± 0.042$^{ijk}$ | 87.48 ± 0.244$^{fgh}$ | 75.03 ± 0.085$^{fghi}$ | 86.82 ± 0.040$^{efghi}$ | 80.88 ± 0.037$^{fghijk}$ | 52.92 ± 0.095$^{cde}$ | 68.14 ± 0.016$^{fg}$ | 53.58 ± 0.025$^{d}$ | 32.47 ± 0.027$^{cd}$ |
| **S5** | 84.67 ± 0.047$^{abcd}$ | 72.17 ± 0.021$^{abcde}$ | 61.02 ± 0.039$^{abcd}$ | - | - | - | - | - | - |
| **S6** | 93.63 ± 0.031$^{fghijk}$ | 88.46 ± 0.070$^{gh}$ | 78.25 ± 0.082$^{ghijk}$ | 78.93 ± 0.022$^{abcde}$ | 57.57 ± 0.067$^{ab}$ | 39.68 ± 0.044$^{a}$ | - | - | - |
| **S7** | 95.29 ± 0.040$^{ghijk}$ | 82.79 ± 0.013$^{defgh}$ | 72.93 ± 0.034$^{efgh}$ | 86.44 ± 0.042$^{efghi}$ | 71.10 ± 0.029$^{cdefgh}$ | 62.40 ± 0.035$^{efgh}$ | 67.50 ± 0.011$^{fg}$ | 62.41 ± 0.032$^{ef}$ | 37.62 ± 0.023$^{de}$ |
| **S8** | 82.41 ± 0.049$^{abc}$ | 67.51 ± 0.105$^{abc}$ | 54.02 ± 0.024$^{ab}$ | - | - | - | - | - | - |
| **S9** | 95.24 ± 0.003$^{ghijk}$ | 90.48 ± 0.019$^{gh}$ | 81.01 ± 0.018$^{ghijk}$ | 75.09 ± 0.022$^{abc}$ | 53.87 ± 0.106$^{a}$ | 44.46 ± 0.060$^{abc}$ | - | - | - |
| **S10** | 80.55 ± 0.036$^{ab}$ | 69.35 ± 0.033$^{abcd}$ | 62.99 ± 0.009$^{bcde}$ | - | - | - | - | - | - |
| **S11** | 96.33 ± 0.008$^{ijk}$ | 94.59 ± 0.008$^{h}$ | 85.51 ± 0.012$^{ijk}$ | 91.41 ± 0.008$^{hi}$ | 84.03 ± 0.023$^{ijk}$ | 73.62 ± 0.063$^{hijk}$ | 68.62 ± 0.012$^{fg}$ | 52.09 ± 0.038$^{d}$ | 25.94 ± 0.014$^{b}$ |
| **S12** | 89.51 ± 0.018$^{defg}$ | 73.44 ± 0.064$^{abcdef}$ | 56.12 ± 0.049$^{ab}$ | - | - | - | - | - | - |
| **S13** | 98.56 ± 0.013$^{k}$ | 94.78 ± 0.023$^{h}$ | 88.79 ± 0.028$^{k}$ | 93.71 ± 0.007$^{i}$ | 89.26 ± 0.006$^{k}$ | 82.61 ± 0.033$^{k}$ | 84.91 ± 0.040$^{i}$ | 72.29 ± 0.021$^{g}$ | 51.38 ± 0.060$^{f}$ |
| **S14** | 90.56 ± 0.003$^{defghi}$ | 84.69 ± 0.060$^{efgh}$ | 76.58 ± 0.111$^{ghijk}$ | 80.37 ± 0.031$^{bcdef}$ | 68.19 ± 0.024$^{bcdef}$ | 59.01 ± 0.051$^{defg}$ | 66.64 ± 0.127$^{fg}$ | 51.86 ± 0.007$^{d}$ | 27.12 ± 0.060$^{bc}$ |

Values are presented as mean ± standard deviation

Values with the same superscript within a column are not significantly different at $p < 0.05$.

Table 2. Tolerance to 0.3 and 0.5 % bile salt concentration

| Isolate code | Percentage survival (%) | | | | | |
|---|---|---|---|---|---|---|
| | 0.3 % bile salt | | | 0.5 % bile salt | | |
| | 1 hour | 2 hours | 3 hours | 1 hour | 2 hours | 3 hours |
| C3 | 88.41 ± 0.021$^{g}$ | 86.13 ± 0.047$^{f}$ | 85.87 ± 0.005$^{d}$ | 78.20 ± 0.016$^{fg}$ | 79.47 ± 0.020$^{fg}$ | 80.03 ± 0.037$^{e}$ |
| C7 | 74.70 ± 0.067$^{bc}$ | 84.02 ± 0.032$^{e}$ | 85.01 ± 0.026$^{d}$ | 71.02 ± 0.015$^{cd}$ | 77.53 ± 0.019$^{e}$ | 80.35 ± 0.016$^{e}$ |
| C10 | 74.43 ± 0.046$^{bc}$ | 75.05 ± 0.007$^{b}$ | 83.63 ± 0.016$^{cd}$ | 66.73 ± 0.034$^{b}$ | 70.72 ± 0.055$^{b}$ | 77.50 ± 0.052$^{cd}$ |
| C14 | 84.16 ± 0.004$^{e}$ | 81.23 ± 0.018$^{d}$ | 83.79 ± 0.039$^{cd}$ | 79.57 ± 0.005$^{gh}$ | 80.14 ± 0.093$^{g}$ | 80.24 ± 0.006$^{e}$ |
| L1 | 77.83 ± 0.005$^{d}$ | 73.18 ± 0.021$^{ab}$ | 74.88 ± 0.010$^{a}$ | 72.72 ± 0.29$^{de}$ | 70.35 ± 0.076$^{b}$ | 74.42 ± 0.046$^{bc}$ |
| S2 | 75.53 ± 0.045$^{cd}$ | 77.94 ± 0.019$^{bc}$ | 81.88 ± 0.027$^{c}$ | 70.22 ± 0.049$^{c}$ | 72.47 ± 0.060$^{bc}$ | 75.82 ± 0.045$^{c}$ |
| S4 | 68.74 ± 0.030$^{a}$ | 72.90 ± 0.033$^{a}$ | 80.22 ± 0.066$^{bc}$ | 63.26 ± 0.044$^{a}$ | 66.87 ± 0.054$^{a}$ | 69.76 ± 0.054$^{a}$ |
| S7 | 78.92 ± 0.086$^{de}$ | 78.54 ± 0.053$^{c}$ | 81.02 ± 0.051$^{c}$ | 73.97 ± 0.038$^{e}$ | 76.55 ± 0.078$^{de}$ | 79.06 ± 0.063$^{de}$ |
| S11 | 73.28 ± 0.019$^{b}$ | 78.80 ± 0.072$^{c}$ | 79.25 ± 0.011$^{b}$ | 72.69 ± 0.024$^{de}$ | 73.36 ± 0.034$^{c}$ | 75.00 ± 0.024$^{c}$ |
| S13 | 87.24 ± 0.047$^{fg}$ | 87.47 ± 0.028$^{g}$ | 88.74 ± 0.003$^{e}$ | 81.72 ± 0.054$^{h}$ | 85.04 ± 0.002$^{i}$ | 86.40 ± 0.011$^{g}$ |
| S14 | 88.55 ± 0.017$^{g}$ | 86.18 ± 0.023$^{f}$ | 89.98 ± 0.036$^{f}$ | 86.95 ± 0.004$^{i}$ | 83.94 ± 0.009$^{h}$ | 85.99 ± 0.034$^{fg}$ |

Values are presented as mean ± standard deviation

Values with the same superscript within a column are not significantly different at $p < 0.05$.

**Table 3.** Antibiotic susceptibility profile of lactic acid bacteria isolated from chicken gut

| Isolate code | ERY 5µg | GEN 10µg | STR 10µg | CHL 30µg | AMX 25µg | COT 25µg | CPR 10µg | OFL 5µg | CEF 30µg | CAZ 30µg | CRX 30µg | CXM 5µg | AUG 30µg | NIT 30µg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **C3** | R | R | R | S | R | R | S | I | R | R | R | R | R | S |
| **C7** | R | R | R | S | R | R | I | R | R | R | R | R | R | I |
| **C10** | R | R | R | S | R | R | R | R | R | R | R | R | R | S |
| **C14** | R | R | R | I | R | R | I | R | R | R | R | R | R | S |
| **L1** | R | R | R | I | R | R | I | R | R | R | R | R | R | S |
| **S2** | R | R | R | S | R | R | R | I | R | R | R | R | R | S |
| **S4** | R | R | R | S | R | R | I | S | R | R | R | R | R | S |
| **S7** | R | R | R | S | R | R | I | R | R | R | R | R | R | S |
| **S11** | R | R | R | S | R | R | S | S | R | R | R | R | R | S |
| **S13** | R | R | R | S | R | R | S | I | R | R | R | R | R | S |
| **S14** | R | R | R | I | R | R | R | R | R | R | R | R | R | S |

S: susceptible (diameter ≥ 21 mm); I: intermediate susceptible (diameter 16-20 mm); R: resistant, (diameter ≤ 15 mm); ERY: Erythromycin, GEN: Gentamycin, STR: Streptomycin, CHL: Chloramphenicol, AMX: Amoxycillin, COT: Cotrimoxazole, CPR: Ciprofloxacin, OFL: Ofloxacin, CEF: Ceftriaxone, CAZ: Ceftazidime, CRX: Cefuroxime, CXM: Cefixime, AUG: Augmentin, NIT: Nitrofurantoin.

**Table 4. Antimicrobial effect of lactic acid bacteria against pathogenic bacteria**

| Isolate | *S. typhimurium* | *S. arizonae* | *Staphylococcus aureus* | *Pseudomonas aeroginosa* | *E. coli* O157:H7 | *E. coli* | *K. oxytoca* |
|---|---|---|---|---|---|---|---|
| **C3** | 14.00 ± 0.000$^{ab}$ | 13.50 ± 1.500$^{ab}$ | 12.00 ± 3.000$^{a}$ | 12.50 ± 0.500$^{a}$ | 11.50 ± 1.500$^{ab}$ | 13.00 ± 0.000$^{abc}$ | 10.50 ± 0.500$^{a}$ |
| **C7** | 14.50 ± 0.500$^{ab}$ | 14.00 ± 1.000$^{ab}$ | 12.00 ± 2.000$^{a}$ | 12.33 ± 0.289$^{a}$ | 11.83 ± 1.756$^{bc}$ | 13.83 ± 1.258$^{abc}$ | 12.83 ± 0.289$^{a}$ |
| **C10** | 14.00 ± 1.000$^{ab}$ | 12.50 ± 1.500$^{ab}$ | 12.50 ± 3.500$^{a}$ | 13.00 ± 0.000$^{a}$ | 11.00 ± 1.000$^{ab}$ | 12.33 ± 0.764$^{a}$ | 13.00 ± 2.000$^{a}$ |
| **C14** | 13.50 ± 2.500$^{a}$ | 12.50 ± 1.500$^{ab}$ | 13.00 ± 0.000$^{a}$ | 13.50 ± 1.500$^{ab}$ | 12.00 ± 0.000$^{bc}$ | 13.00 ± 0.000$^{abc}$ | 12.50 ± 2.500$^{a}$ |
| **L1** | 15.00 ± 3.000$^{ab}$ | 11.00 ± 2.000$^{a}$ | 13.00 ± 2.000$^{a}$ | 13.50 ± 1.500$^{ab}$ | 12.00 ± 1.000$^{bc}$ | 13.50 ± 0.500$^{abc}$ | 12.50 ± 4.500$^{a}$ |
| **S2** | 14.50 ± 0.500$^{a}$ | 13.50 ± 1.500$^{ab}$ | 12.00 ± 3.000$^{a}$ | 12.33 ± 0.764$^{a}$ | 11.50 ± 1.500$^{ab}$ | 14.50 ± 0.500$^{c}$ | 12.50 ± 2.500$^{a}$ |
| **S4** | 14.50 ± 1.500$^{ab}$ | 14.00 ± 0.000$^{ab}$ | 12.50 ± 1.500$^{a}$ | 14.83 ± 0.764$^{b}$ | 9.50 ± 0.500$^{a}$ | 14.00 ± 1.000$^{bc}$ | 12.00 ± 2.000$^{a}$ |
| **S7** | 17.00 ± 2.000$^{b}$ | 12.00 ± 2.000$^{ab}$ | 13.50 ± 1.500$^{a}$ | 12.83 ± 1.258$^{a}$ | 13.00 ± 1.000$^{bc}$ | 14.00 ± 1.000$^{bc}$ | 11.50 ± 3.500$^{a}$ |
| **S11** | 14.33 ± 0.764$^{ab}$ | 11.83 ± 1.756$^{ab}$ | 11.50 ± 1.500$^{a}$ | 14.00 ± 1.000$^{ab}$ | 11.83 ± 0.764$^{bc}$ | 12.50 ± 1.500$^{ab}$ | 14.00 ± 1.000$^{a}$ |
| **S13** | 14.50 ± 1.500$^{ab}$ | 14.83 ± 0.764$^{b}$ | 13.00 ± 2.000$^{a}$ | 13.50 ± 0.500$^{ab}$ | 14.00 ± 2.000$^{c}$ | 12.50 ± 0.500$^{ab}$ | 13.83 ± 0.289$^{a}$ |
| **S14** | 12.50 ± 1.500$^{a}$ | 12.00 ± 3.000$^{ab}$ | 12.00 ± 2.000$^{a}$ | 12.50 ± 0.500$^{a}$ | 11.33 ± 0.289$^{ab}$ | 13.00 ± 0.000$^{abc}$ | 11.50 ± 2.500$^{a}$ |

Results are presented as mean ± standard deviation of triplicate values.
Values with the same superscript within a column are not significantly different at $p < 0.05$.

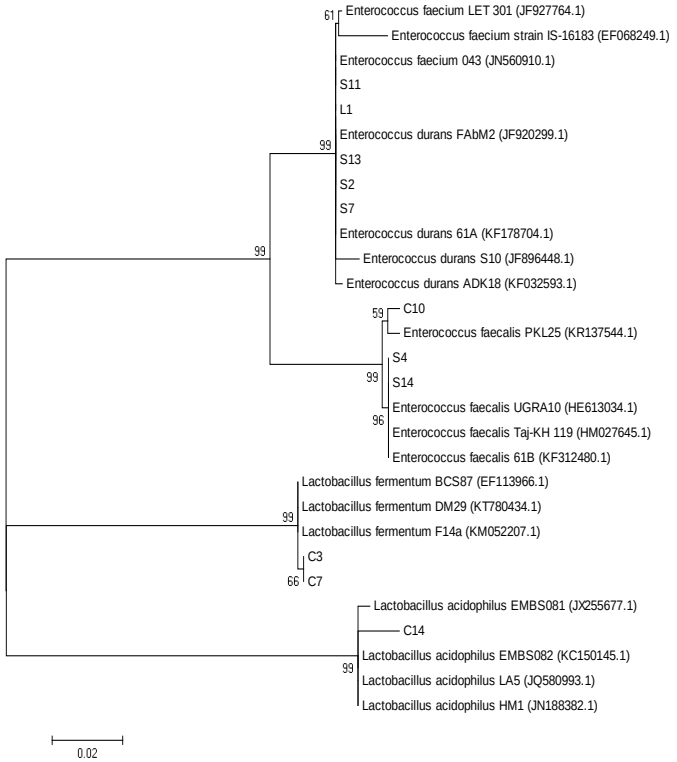


**Figure 2**: Phylogenetic tree based on 16S rRNA genes sequences of the isolates determined using the maximum likelihood method. Numbers above each node are bootstrap confidence levels (expressed as percentages) generated from 1000 bootstrap trees. The GenBank accession number is given in parentheses for each organism.
L1, S7 and S11: *Enterococcus faecium*, S2 and S13: *Enterococcus durans*, S4, C10 and S14: *Enterococcus faecalis*, C14: *Lactobacillus acidophilus*, C3 and C7: *Lactobacillus fermentum*.

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