

PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM A BENINESE
TRADITIONAL BEER'S FERMENT

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ABSTRACT: Lactic acid bacteria are widely used as probiotics in food industry for conservation and for the fight against some pathogenic strains. The aim of this work was to study probiotic properties of these bacteria isolated from "kpètè-kpètè" a ferment of traditional beers produced in Benin. Therefore, five species of lactic acid bacteria were tested for their probiotic properties such as colonization and survival in the gastrointestinal region, tolerance to acid and bile, ability to adhere to intestinal epithelial cells, the antimicrobial activity and resistance to antibiotics. For above mentioned tests and the technological skills, conventional methods were used. The results of our investigation showed that *Streptococcus thermophilus* was the most active amongst the tested pathogenic strains (8/10) with inhibition diameters ranging from 9.5 ± 0.7 mm to 22 ± 4.76 mm. All the lactic acid bacteria were resistant to different gastric conditions (pH 2, pH 2.5) and with variable survival rates (99-105%) according to the species. In addition, all species adhered to the epithelial cells of the colon and ileum of broilers. *Streptococcus thermophilus* and *Lactobacillus casei* showed high ability to colonize the cell surface (hydrophobicity), while with *Lactobacillus fermentum* this hydrophobicity was low. Concerning the technological skills, the tested strains produce exo-polysaccharides that improve the viscosity and texture of food. Moreover, they had a considerable proteolytic power ($24 \pm 5.30 \leq$ lysis diameter $\leq 27 \pm 8.48$ cm) and produce acetoin responsible for the food aromatization. These results indicate that these species isolated from "kpètè-kpètè" are probiotics and can be used to focus food ingredients with probiotic property.

Key words: *kpètè-kpètè*; Lactic acid bacteria; Antimicrobial activity; Probiotic properties; Technological skills

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INTRODUCTION

The fermentation of *tchoukoutou*, a traditional beer produced in Benin, is performed with a ferment called "*kpètè-kpètè*" in local language (Kayodé et al., 2006). So, the "*kpètè-kpètè*" is a traditional starter used not only for the beer fermentation but also in the treatment of diarrheal infections, dysentery and even malaria. This starter is also used for animal nutrition and also to cure some diarrheal diseases in domestic animals. It is also used as a condiment in the preparation of sauces in some parts of northern Benin (N'Tcha et al., 2014).

Previous studies have shown that this residue (*kpètè-kpètè*) from *tchoukoutou* has great potential in the functional microflora including lactic acid bacteria and yeasts (Kayode et al., 2012; Houndonougbo et al., 2011). The "*kpètè-kpètè*" is microbiologically similar to Turkish Kefir (fizzy of Caucasus, made with milk) (Greppi et al., 2013; Can et al., 2012).

The lactic acid bacteria displays the ability to ferment carbohydrates and degrade proteins and lipids results in the synthesis of a wide range of compounds such as organic acids, peptides, antimicrobial compounds (Mozzi et al., 2010). Thus, this group of bacteria live in various ecological niches with probably probiotic properties as they can be used in the formulation of new probiotic products (Merrified et al., 2014; Sonsa-Ard et al., 2015).

According to WHO (2001), probiotics are living microorganisms which, when ingested in adequate amounts, provide a beneficial effect on the health of their host. Currently, considerable interest has been developed around the use of lactic cultures for food and pharmaceutical applications (Kabir et al., 2004; Houndonougbo and al, 2011). Probiotics are used to stimulate the immune system in stock farming and to increase the productivity (Bahoua, 2008). Other studies have also shown that probiotics exert antibacterial activities against several pathogenic bacteria including those responsible both for infections in humans and animals such as broilers (Can et al., 2012.; Sharifuzzaman et al., 2009; Van Immerse et al., 2005; Panigrahi et al., 2005; Irianto et al., 2002). Antibiotics, anti-parasitic and vaccines are the means commonly used to fight against these diseases which affect the health and productivity of local stock farming. But the misuse of these antibiotics during medical treatment and stock farming induced the increase of bacteria resistant. It is therefore essential to find alternative therapeutic and/or protective way to control pathogenic microorganisms. The selected lactic acid bacteria, with probiotic properties, are reported to be used by specialized companies for the production of lactic ferments and bacteriocins (Beal et al., 2008).

Among the Beninese local commodities, several can shelter many lactic acid bacteria with probiotic properties. Indeed, before considering a lactic acid bacteria as probiotic, it must displays properties such as: i) colonization and survival in gastrointestinal region, ii) high tolerance to acid and bile, iii) ability to adhere to intestinal surfaces, iv) antibiotic resistance profile and antimicrobial activity and v) some technological (acidification, proteolytic, lipolytic, texturizing, coagulation, thickening and aromatization power) skills (Pitino et al., 2010). Due to its traditional use to cure infectious diseases, the ferment of tchoukoutou may be a tank of bacteria with probiotic properties as it incorporation to broilers food improved animals growth and decrease their death rate (Hondonougbo et al., 2011). However, before the promotion of the lactic acid bacteria with probiotic properties, it important to have a database of those bacteria's. Thus, the present study aims to investigate the probiotic properties of five lactic acid bacteria isolated from "kpètè-kpètè" samples collected in the northern Benin.

MATERIALS AND METHODS

Microorganisms Strains

The microorganisms used in this study include ten references strains and five species of lactic acid bacteria isolated from "kpete-kpete" samples. The ten references strains used were: *Escherichia coli* ATCC 25922, *Escherichia coli* O157, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* A24974, *Micrococcus luteus*, *Enterococcus faecalis* ATCC 29212, *Candida albicans* MHMR, *Salmonella typhi*, *Listeria monocytogenes*. The five species of lactic acid bacteria (*Enterococcus faecium*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus*) were those isolated in a previous study from "kpètè-kpètè" samples collected in Benin (N'Tcha et al., 2016).

PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA

Antimicrobial Activity of Lactic acid Bacteria against Reference Strains

The inhibitory activity of the native lactic acid bacterial supernatant (pH 6.5) was evaluated by disks diffusion method as previously described by Tadesse et al. (2004). First, the appropriate reference strains ($0.08 \leq OD_{660} \leq 0.1$) were used to flood the surface of Mueller-Hinton agar medium. Thus, after keeping dishes at room temperature (~25°C) for 15-30 min, sterile 5 mm diameter disks (aseptically placed on the surface of the previously flooded agar) on which a supernatant of young lactic culture (30 µl) was lodge. The supernatant was obtained after centrifugation (9400 rpm for 10 min) of young lactic acid bacteria cultures (~18 h) and it filtration with 0.22 µm Millipore. The Petri dishes were dried at room temperature and then incubated at 4°C for 4 h, then at 37°C for 24 h. Each sample was used in duplicate and after the incubation period, the inhibition zones of the reference strains around the disks were examined.

Resistance to some Conventional Antibiotics

The susceptibility to ten antibiotics (ampicillin, rifampicin, spiramycin, vanomycin, gentamicin, penicillin, tetracycline, sulfamethoxazole/trimethoprim, erythromycin and oxacillin) of the lactic acid bacteria was investigated by the disc diffusion methods of Kirby-Bauer on agar Mueller Hinton as recommended by Antibiogram Committee of the French Microbiology Society (SFM, 2015). After incubation at 37°C for 24 hours, the diameters of inhibition zones were measured.

Tolerance to Gastric Acidity and Resistance to Bile salts

Each sample was used in triplicate to investigate the ability of the lactic acid bacteria to withstand gastric acidity was determined according to the technique described by Ammor and Mayo (2007). The bacterial pellet of young culture obtained after centrifugation (13000 rpm for 4 min) was suspended in 10 ml of MRS broth at three different pH (pH 2, pH 2.5 and pH 6.5). The OD obtained at 660 nm of each culture and the number of viable cells (micro-dilution technique and the use of Malassez cell) was recorded respectively at the start of the experimentation (T_{0h}) and 2 h after incubation at 37°C (T_{2h}). The survival rate was calculated using the following formula:

$$\text{Survival rate (\%)} = [\log\text{UFC} (T_{2h}) / \log\text{UFC} (T_{0h})] \times 100$$

The ability of the pure lactic bacteria to resist bile salt was determined by the method described by Hydrominus et al., (2000). This methodology is similar to the test of tolerance to acidity above describe except that MRS broth was supplemented with 0.3% bile salts.

Evaluation of the Response to Stomach-Duodenal Stimulus

The response of the tested lactic acid bacteria to stomach-duodenal stimulus was evaluated *in vitro* by the method described Vizoso Pinto et al., (2006). It was performed on overnight bacterial cultures decimal dilutions to determine the OD_{600} and cell number of each dilution. After that, 10 ml of MRS broth (pH 3) was inoculated with 1 ml of the diluted bacterial suspension followed immediately (T_{0h}) by the determination of the OD_{600} and the evaluation of the number of cells. One hour after incubation, 4 ml of bile salts (10%) and 17 ml of a synthetic duodenal secretion (6.4 g/l NaHCO_3 , 0.239 g/l KCl and 1.28 g/l NaCl) were added before incubation at 37°C. Thus, the bacterial survival was determined by measuring the OD_{600} and determining the number of viable cells respectively at 1 h, 2 h and 3 h.

Adhesion to Epithelial Cells

The adhesion of the lactic bacteria was perform on the epithelial cells following the method previously described (Lin et al., 2007). The ileum and part of colon were isolated from 12 broilers coming from 4 different lots. Those fragments were used to seek *in vitro* the adhesion to epithelial cells capability. A given lot is composed of three broilers receiving the same feeding treatment (Table 1).

Table 1: Distribution of the tested broilers according to their feeding treatments.

Lot	Number of broilers	Feeding treatments		
		Currents food	Probiotic	Alfaceryl* Antibiotics
1	3	+	+ 3.5%	-
2	3	+	+ 1.5%	-
3	3	+	-	+
4	3	+	-	-

*100 g of alfaceryl contain: Erythromycin thiocyanate (3 mg), Furaltadone HCl (6 mg), Oxytetracycline HCl (5 mg), Streptomycin sulfate (3.5 mg), Neomycin sulfate (1 mg), (+) with and (-) without.

The performing of the test starts by the preparation of epithelial cells. The epithelial cells were prepared from the segments of the ileum and colon (intestine) collected from the appropriate lot broilers. Then, the tissues were opened and properly washed (~10 times) with sterile phosphate buffer saline (pH 7.2) and then kept at 4°C for 3 h. The epithelial cells used for the test were obtained by scraping the internal surface of the colon with a sterile blade. After that, four decimal dilutions (up to 10^{-4}) of the epithelial cells ($\sim 5 \times 10^4$ cells/ml) were performed and examined by microscope to make sure it was not contaminated.

In the other hand, 24 h bacterial pre-cultures were centrifuged (6000 rpm for 10 min) and the pellet of each strain was recovered in 2 ml of PBS ($\sim 10^8$ cells/ml) followed by microscopic observation (GX100). To end, 1 ml of each culture was mixed with 1 ml of the fourth epithelial cells suspension dilution (10^{-4}) and incubated at 37°C for 40 minutes. After incubation time, a preparation of smear and the staining with 0.5% crystal violet for 5 min was conducted to observe the adhesion to the optical microscope. The test was considered positive if the number of adhered bacteria was >15.

Hydrophobicity test

The hydrophobicity of the lactic bacteria was conducted according to the method described by Iyer et al. (2010). Briefly, the bacterial pellet of 18 h culture after centrifugation (12,000 rpm for 5 min) was recovered re-suspended in 1.2 ml of Magnesium Urea Phosphate Buffer (pH 6.5) and adjusted, if necessary, to DO₄₅₀ 1.0 (OD_{initial}). Then, xylene (0.6 ml) was gently added to 3 ml of the bacterial suspension and incubated at 37°C for 10 min. This mixture was correctly mixed and let to settle (~15 min). The aqueous phase was collected to measure once again the DO₄₅₀ (OD_{final}). The hydrophobicity cell surface (H%) was calculated using the following equation:

$$H\% = [(OD_{initial} - OD_{final}) / OD_{initial}] \times 100$$

Technological Abilities of Lactic acid Bacteria

Acidification power

To examine the acidification power was evaluated by determining not only the evolution of the lactic acid bacteria cultures pH but also the total titratable acidity (with N/9 NaOH). The bacterial culture used for this experimentation was performed on 250 ml of skimmed milk (10%). So, the culture composed of 1/100 (v/v) of bacteria was incubated at 37°C and the total titratable acidity was determined, using 10 ml of the culture, respectively at 2 h, 6 h and 24 h (Larparent, 1997). The acidity was calculated by multiplying per 10 the added volume of NaOH used for the titration. The same samples collected at 2 h, 6 h and 24 h were used to directly determine the pH with a pH meter.

Titratable acidity was measured as a function of time at Dornic degrees (°D) under these conditions 1 D corresponds to 0.1 ml of 0.1N sodium hydroxide. 1 D equals to 0.1 g of lactic acid / L of milk.

Proteolytic Power

To determine the proteolytic activity of the lactic acid bacteria, the MRS agar +10% skimmed milk was poured, solidified and dried and then sterile Whatman paper disks were placed on the surface of the agar. Each disk received 20 µl of the appropriate young culture and the proteolytic effect was determined after 24 h at 37°C (Veuillemard, 1986).

Lipolytic Power

Lipolysis was highlighted on a specific medium [MRS (pH 7) + Tween 80 (3% and 1%) + GINO® vegetable oil (3% and 1%)]. Sterile Wattman paper disks containing 10 µl of appropriate young culture were deposited on the surface of the agar medium. After incubation at 37°C for 24 h, lipolysis was revealed by a clear zone or a clear precipitate around the disks (Guiraud, 2003).

TEXTURIZING POWER

Production of exo-polysaccharides: The production of exo-polysaccharides was investigated a hyper-sucrose agar medium and an incubation at 37°C for 24 h. the observation of large and slimy colonies indicated the production of exo-polysaccharides (Leveau et al., 1991).

Quantification exo-polysaccharides: The exo-polysaccharides (EPS) were quantified on 2 volumes of ethanol 4° to 1 volume of supernatant obtained after centrifugation (6000 rpm for 20 min) of the 1% bacterial culture on MRS (100 ml) (Mozzi et al., 2001; Wu et al., 2010). The mixture (ethanol+supernatant) was incubated overnight at 4°C and centrifuged (6000 rpm for 5 min) to get the precipitates. The precipitates were suspended in of distilled water (2 ml) and filtrated (Ø=0.22 µm). Then to each filtrate (800 µl) a solution (40 µl of 80% phenol and 2 ml of concentrated sulfuric acid) was added and correctly mixed. The negative control was prepared by replacing the sample with distilled water. The recorded OD₄₉₀ was then the expressed in grams of EPS per liter (g/l).

AROMATIZATION POWER

The ability of lactic acid bacteria to produce flavor compounds during fermentation process was highlighted on skimmed milk. Therefore, each tube containing sterile skimmed milk was inoculated with one of lactic acid bacteria. After incubation for 24 h and milk coagulation, the Vogues-Proskauer (VPI and VPII) solutions were added. The presence of aroma was revealed by the appearance of the red color.

RESULTS

ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA ON SOME PATHOGENS

The Table 2 presents the results of the antimicrobial activity of native supernatant of lactic acid bacteria. Among the tested lactic acid bacteria, *Streptococcus thermophilus* showed a significant antagonistic effect by inhibiting the growth of 80% (8/10) of the pathogenic strains. The highest inhibition diameter (22 ± 4.76 mm) was recorded with *Streptococcus thermophilus* against *S. aureus* strain and the lowest inhibition diameter (9.5 ± 0.707 mm) was obtained with the same *Streptococcus thermophilus* against *Listeria Monocytogenes*, *Micrococcus luteus* strain was resistant to the effect of the 90% (9/10) of the tested bacteria. Only *St. Thermophilus* had an inhibitory effect against *M. luteus* with 17.5 ± 3.53 mm diameter of inhibition. Moreover, *Lb. casei* strain had an inhibitory effect on the *Ps. aerurogenosa* strain (12 ± 0 mm). There was no significant difference between the inhibition diameters recorded at 24 and 48 hours ($p>0.05$).

Table 2: Inhibitory activity of lactic acid bacteria's native supernatants against some reference strains

Strains	Diameter of inhibition (mm)									
	<i>En. faecium</i>		<i>Lb. fermentum</i>		<i>Lb. casei</i>		<i>Ln. mesenteroides</i>		<i>St. thermophilus</i>	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
<i>S. aureus</i>	17.5 ± 8.66	17.5 ± 8.66	13.5 ± 3.32	13.5 ± 3.32	14.5 ± 6.36	14.5 ± 6.36	11 ± 3.92	11 ± 3.92	22 ± 4.76	22 ± 4.76
<i>E. coli</i>	11 ± 2.86	11 ± 2.86	10 ± 0	10 ± 0	12 ± 0	12 ± 0	15.5 ± 1.77	15.5 ± 1.77	13.5 ± 4.04	13.5 ± 4.04
<i>M. luteus</i>	-	-	-	-	-	-	-	-	17.5 ± 3.53	17.5 ± 3.53
<i>En. faecalis</i>	-	-	-	-	-	-	-	-	18 ± 0	18 ± 0
<i>L. monocytogenes</i>	-	-	-	-	-	-	-	-	9.5 ± 0.707	9.5 ± 0.707
<i>C. albicans</i>	15.625 ± 0.75	15.625 ± 0.75	13 ± 2	13 ± 2	15 ± 0	15 ± 0	13.5 ± 0.01	13.5 ± 0.01	-	-
<i>Pr. mirabilis</i>	13 ± 0	13 ± 0	14 ± 0	14 ± 0	-	-	15 ± 7.1	15 ± 7.1	14 ± 0	14 ± 0
<i>Sp. aeruginosa</i>	-	-	12 ± 0	12 ± 0	-	-	-	-	-	-
<i>St. typhi</i>	13.62 ± 3.1	13.62 ± 3.1	15.75 ± 1.77	15.75 ± 1.77	-	-	13 ± 0	13 ± 0	16.75 ± 2.47	16.75 ± 2.47
<i>E. coli O 157</i>	10 ± 4.24	10.5 ± 4.95	14.75 ± 3.77	14.75 ± 3.77	14.75 ± 3.78	14.75 ± 3.79	14.75 ± 3.80	14.75 ± 3.81	16.25 ± 2.40	16.25 ± 2.40

En faecium: *Enterococcus faecium*; *Lb ferment*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Leu mesen*: *Leuconostoc mesenteroides*; *Strep thermo*: *Streptococcus thermophiles*.

Resistance to Antibiotic of Lactic Acid bacteria

Lactic acid bacteria showed variable sensitivity to the tested antibiotics (Table 3). Thus, among the 5 tested strains, 80% (*En faecium*, *Lb fermentum*, *Lb casei*, *Leu mesenteroides*) were resistant to penicillin and sulfamethoxazole trimethoprim.

Table 3: Susceptibility of lactic acid bacteria to the tested antibiotics.

LAB	SPN	AMP	PEN	SXT	RAF	VAN	ERY	OXA	GEN	TET	%S	%R
<i>En faecium</i>	S	S	R	R	R	S	S	R	R	S	50%	50%
<i>Lb ferment</i>	S	S	R	R	S	S	S	R	R	S	60%	40%
<i>Lb casei</i>	S	S	R	R	S	S	S	R	R	S	60%	40%
<i>Le mesen</i>	S	S	R	R	S	S	S	R	R	S	60%	40%
<i>St thermo</i>	S	S	S	S	S	S	S	R	R	S	80%	20%
%S	100%	100%	20%	20%	80%	100%	100%	0%	0%	100%		
%R	0%	0%	80%	80%	20%	0%	0%	100%	100%	0%		

SPN: Spiramycin; AMP: Ampicillin 10 µg; PEN: Penicillin 10 µg; SXT: sulfamethoxazole Trimethoprim (1.25/23.75) µg; RAF: Rifampicin 30 µg; VAN: Vancomycin 30 µg; ERY: Erythromycin 15 µg; OXA: Oxacillin 1 µg; GM: Gentamicin 10 µg; TET: Tetracycline 30 µg.

Adhesion test to Epithelial Cells

The adhesion of bacteria to the epithelial cells of the colon (Figure 1) show that all the tested lactic acid bacteria (100%) adhere to intestinal epithelial cells independently to the lot of broilers used (Table 4). Thus, we observed that there was a high adhesion with the epithelial cells of the chickens fed with 3.5% probiotic (Lot 1) in comparison with those of the chickens fed without probiotics nor antibiotics (Lot 4).

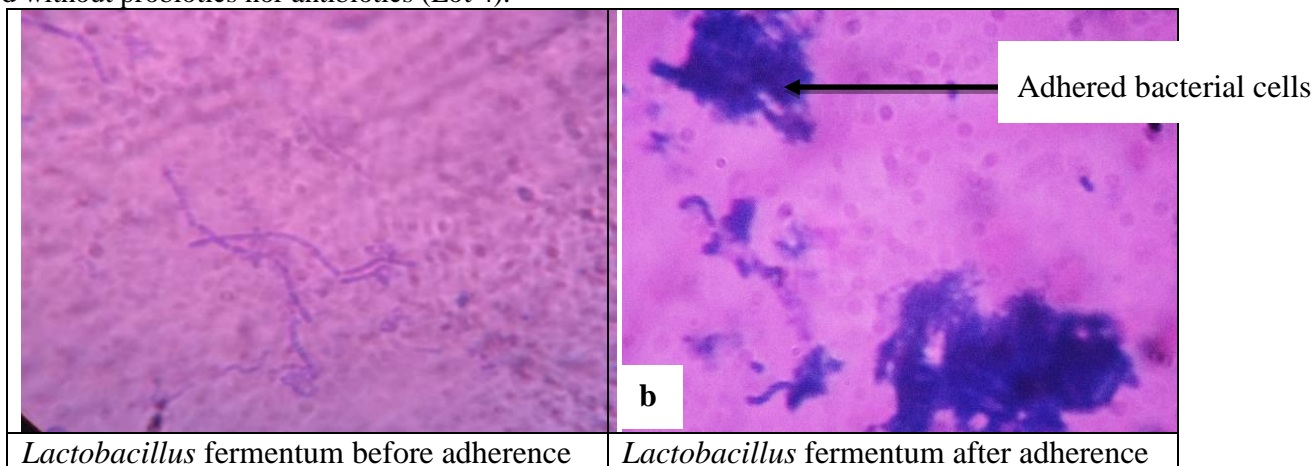


Figure 1: Illustration of the adhesion of *Lb. fermentum* to epithelial tissues of the colon.

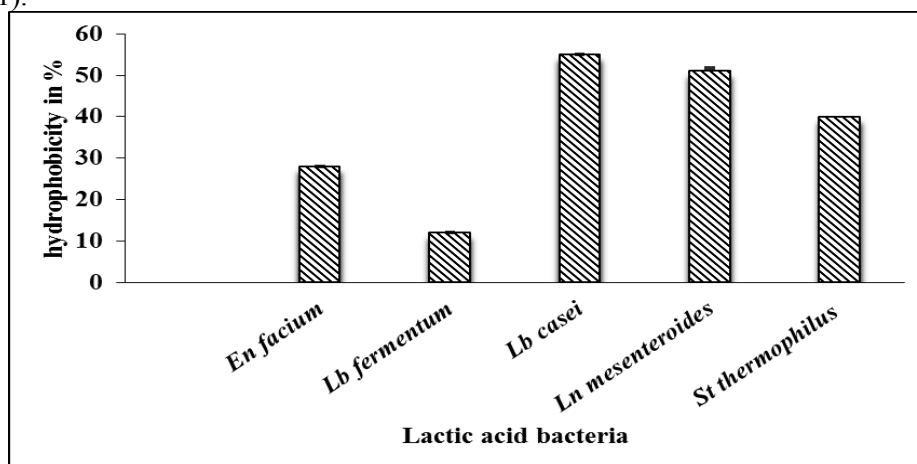
Table 4: Overview of the lactic acid bacteria adhesion to the epithelial cells according to the broilers' diet.

Strains	Lot 1		Lot 2		Lot 3		Lot 4	
	colon	ileum	colon	ileum	colon	ileum	colon	ileum
<i>En. faecium</i>	+++	++	+	++	+	+	+	++
<i>Lb. fermentum</i>	+++	++	+	+	+	+	+	+
<i>Lb. casei</i>	+++	++	+	++	+	+	+	++
<i>Le. mesenteroides</i>	+++	+	+	+	+	+	+	+
<i>St. thermophilus</i>	+++	++	++	++	+	+	++	++

+++ : Strong adhesion; ++ : Middle adhesion; + : Low adhesion.

Hydrophobicity of the Lactic acid Bacteria's Cell surface

The highest hydrophobicity percentages were obtained with *Lactobacillus casei* (55%) and *Leuconostoc mesenteroides* (51%) while the lowest percentage was recorded with the species *Lactobacillus fermentum* (12%) (Figure 2). The percentage of hydrophobicity recorded was significantly high for *Leu. Mesenteroides* ($p < 0.05$) and very significantly high with *Lb. casei* ($p < 0.01$).



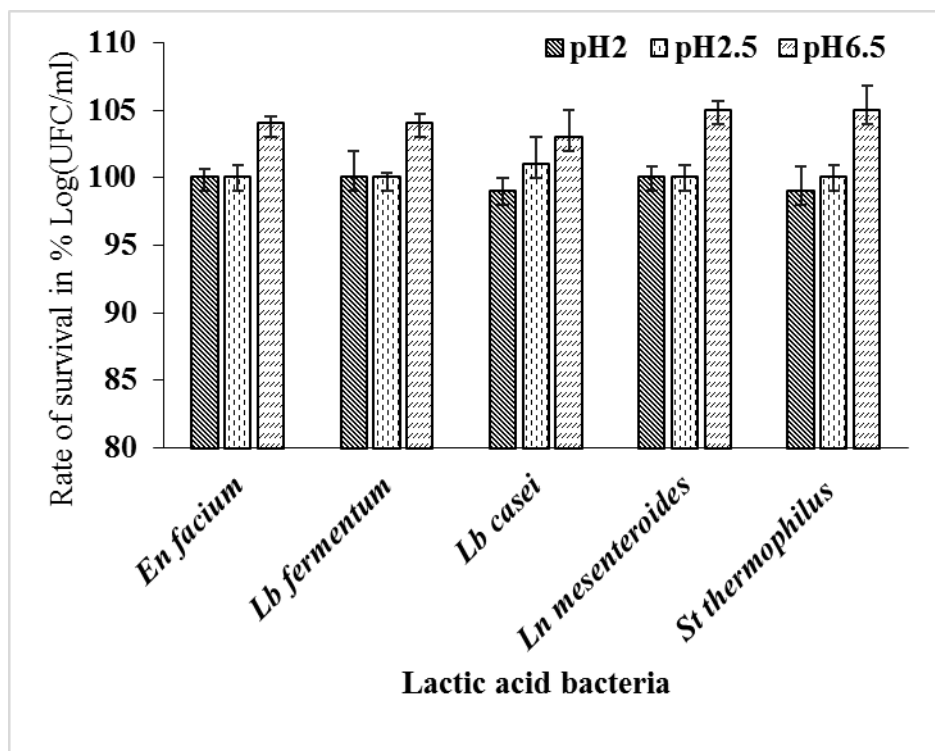
En faecium: *Enterococcus faecium*; *Lb ferment*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Leu mesen*: *Leuconostoc mesenteroides*; *Strep thermo*: *Streptococcus thermophilus*.

Figure 2: Hydrophobicity of lactic bacteria strains.

Resistance to the Digestive transit Conditions

Tolerance to gastric acidity

These results show the existence of a continuous viability of the strains tested in the acidic medium (Figure 3). A significant difference ($p < 0.05$) of survival rate was observed amongst the strains tested. All strains showed good growth on the control medium with a pH 6.5 ($> 100\%$) and hence the elevation of the initial number of cells with a maximum of survival recorded in *Leuconostoc mesenteroides* ($105 \pm 0.69\%$) and *Streptococcus thermophilus* ($105 \pm 1.8\%$). All the tested species showed good resistance ($99\% \pm 1\%$ and $101 \pm 2\%$) to low pH (2 and 2.5). But in general, resistance to acidic conditions decreases with decreasing pH of the medium up to $99 \pm 1\%$ (*Lactobacillus casei* at pH 2). There was no significant difference between the survival rate of lactic acid bacteria between pH 2 and pH 2.5. However, between pH 2 and pH 6.5, there was a significant difference ($p < 0.05$) survival rate achieved for *Leuconostoc mesenteroides* and a very significant difference ($p < 0.01$) for *Streptococcus thermophilus*.



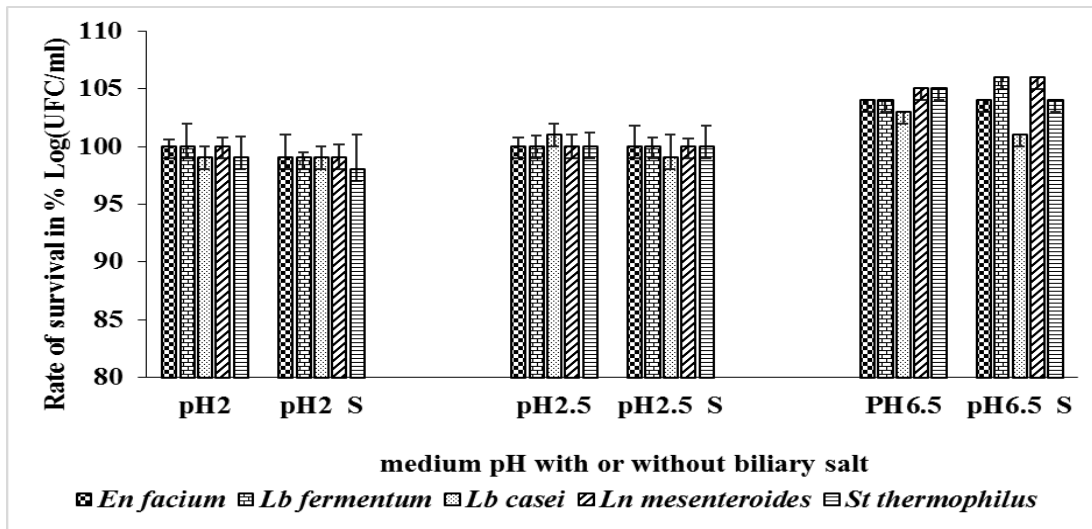
En faecium: *Enterococcus faecium*; *Lb ferment*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Leu mesen*: *Leuconostoc mesenteroides*; *Strep thermo*: *Streptococcus thermophilus*.

Figure 3: Resistance of lactic acid bacteria to acidity conditions.

Resistance to bile salts

It appears that all strains grew in acidic media supplemented with bile salts (Figure 4). There was a survival rate of 100% at pH 2.5 supplemented with bile salts. Nevertheless, there was a decrease in the survival rate in the media at low pH values ranging from 100% to $98 \pm 1.14\%$ at pH 2.5 and pH 2 and supplemented with bile salt. The resistance rates of strains obtained at pH 2 supplemented with bile salt were significantly different ($p < 0.01$) than that obtained with pH 6.5 supplemented with bile salt. In addition, *Leuconostoc mesenteroide* ($106 \pm 1\%$) and *Lactobacillus fermentum* ($106 \pm 0.79\%$) showed greater survival rates at pH 6.5 supplemented with bile salt.

The survival rate of lactic acid bacteria at pH 2 without bile salt varies (99% and 100%) whereas it varies between 98% and 99% at the same pH with bile salts.

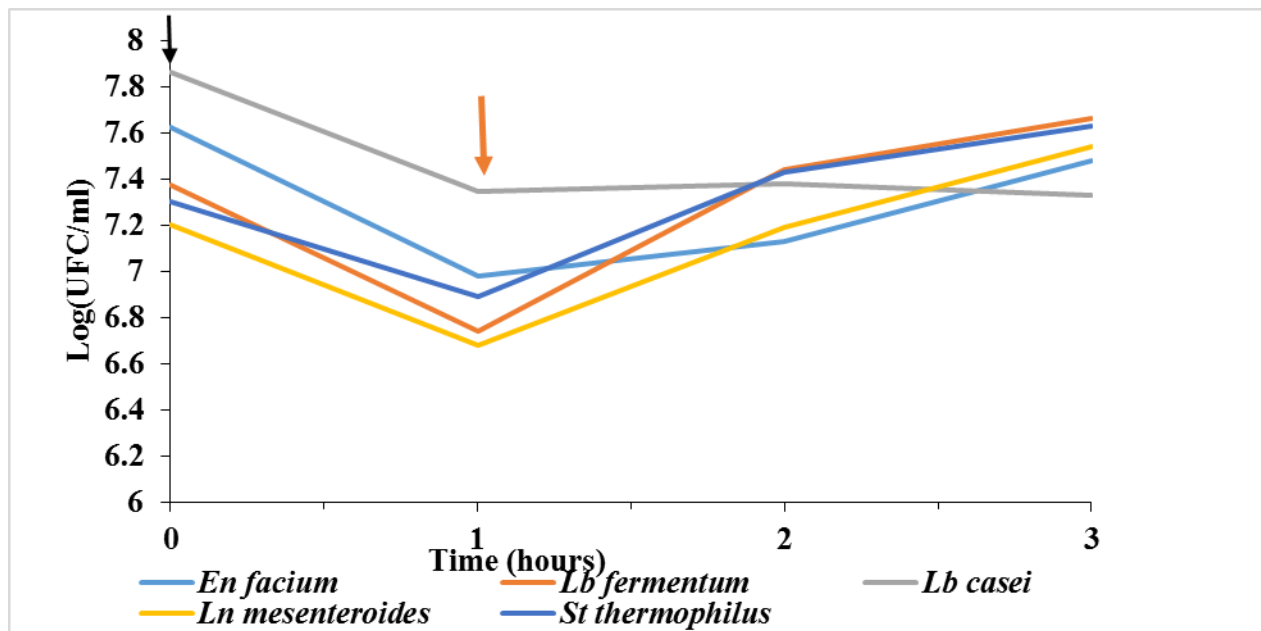


pH 2 S: pH 2 supplemented with 0.3% of bile salt, pH 2: pH 2 without bile salt.

Figure 4: Resistance of lactic acid bacteria to 0.3% bile salts.

RESPONSE TO STOMACH-DUODENAL STIMULUS

Figure 5 shows the response of lactic acid bacteria strains to stomach-duodenal stimulus. These results indicate that there was a resistance of most strains to adverse conditions imposed by the composition of this medium. All the tested strains resisted to pH 3 after 1 h of incubation. Nevertheless, there was a decrease in the number of germ with the species tested, such as *Lactobacillus casei* where the number of germ ranges from 7.85 log CFU / ml to 7.35 logCFU/ml between T0h and T1h.



En faecium: *Enterococcus faecium*; *Lb ferment*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Leu mesen*: *Leuconostoc mesenteroides*; *Strep thermo*: *Streptococcus thermophilus*.

→ MRS (pH3) → Addition of duodenal secretion + 4% bile salt

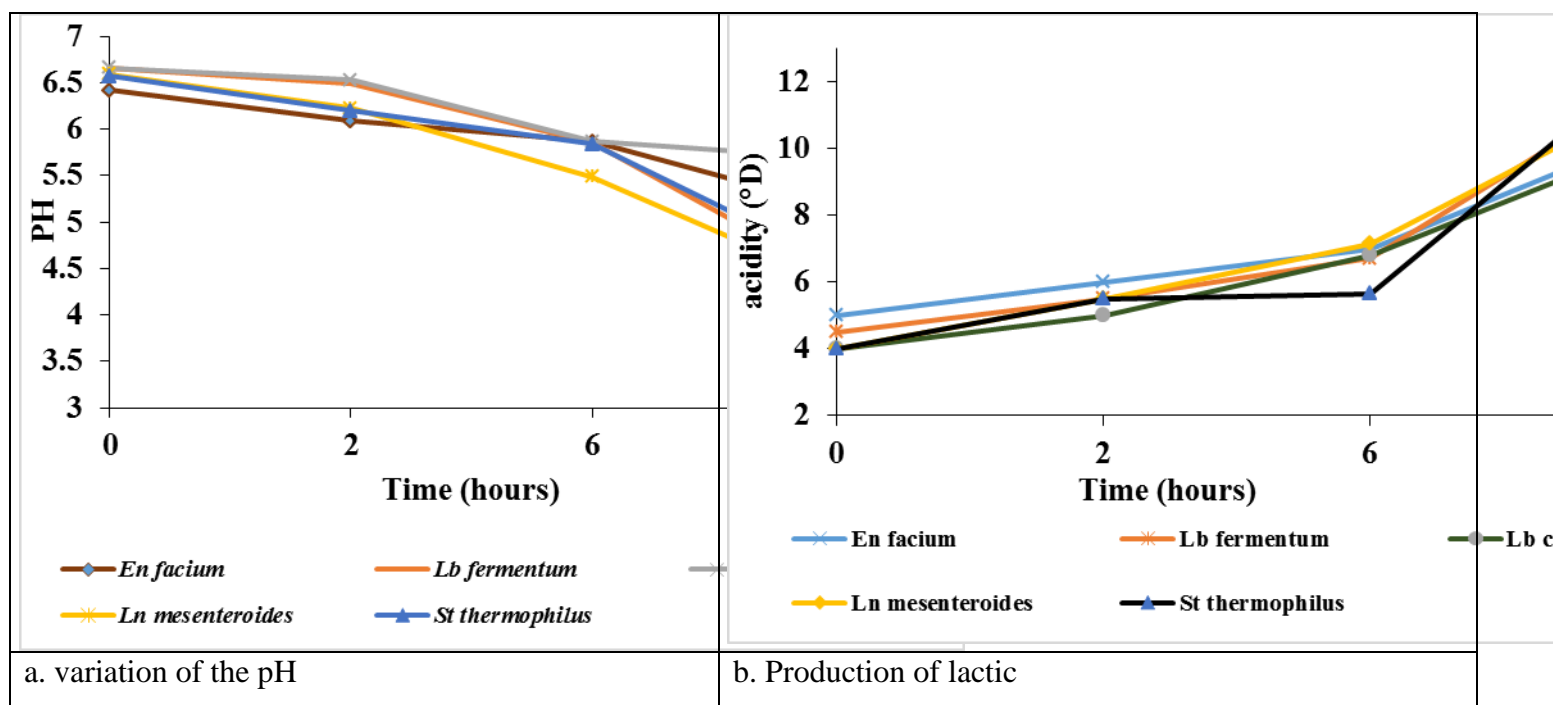
Figure 5: Response of strains to stomach- duodenal stimulus.

After adding synthetic duodenal secretions in the medium at T_{1h} , the behavior of species was variable. We noted an increase in the number of cells after 2 hours and 3 hours of incubation for most of the strains tested except in *Lactobacillus casei*. *Lactobacillus fermentum*, *Streptococcus thermophilus* were more resistant to stomacho-duodenal stimulus after 2 hours and 3 hours of incubation.

Technological Abilities of Lactic acid bacteria

Acidification power

The lactic acid bacteria exhibit a gradual production of lactic acid expressed by reduction of pH (Figure 6a) but increase the lactic acid concentration (Figure 6b). After 24 hours of incubation, the pH values decreased (\sim pH 4.35) whereas the amount of lactic acid produced increases (\sim 1.225 g/l). *St. Thermophilus* (1.225 g/l), *Lb. fermentum* (1.165 g/l) and *Ln Mesenteroides* (1.130 g/l) were the most acidifying species. The acidification kinetics showed that all species tested were acidifying strains. There was no significant difference between the amounts of acid produced by lactic acid bacteria tested ($p > 0.05$).



En faecium: *Enterococcus faecium*; *Lb fermentum*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Ln mesenteroides*: *Leuconostoc mesenteroides*; *St Thermophilus*: *Streptococcus thermophilus*.

Figure 6: Acidity power of lactic acid bacteria strains isolated from “kpètè-kpètè”.

Proteolytic power

Table 5 presents the results of the lactic bacteria's displays a clear halo around the discs ($24 \pm 5.30 \text{ mm} \leq \text{diameter} \leq 27.5 \pm 10.61 \text{ mm}$). The most proteolytic species was *Lactobacillus fermentum* ($27.5 \pm 10.61 \text{ mm}$) followed by *Enterococcus faecium* ($27 \pm 8.48 \text{ mm}$), *Lactobacillus casei* ($25 \pm 0 \text{ mm}$) and *Streptococcus thermophilus* ($25 \pm 0 \text{ mm}$). The less proteolytic strains were *Leuconostoc mesenteroides* ($24 \pm 5.30 \text{ mm}$).

Table 5: Proteolytic activity of lactic acid bacteria isolated from kpètè- kpètè samples.

Species	Observation	Diameter (mm)
<i>Enterococcus faecium</i>	Normal growth with proteolysis	27 ± 8.48
<i>Lactobacillus fermentum</i>	Normal growth with proteolysis	27.5 ± 10.61
<i>Lactobacillus casei</i>	Normal growth with proteolysis	25 ± 0
<i>Leuconostoc mesenteroides</i>	Normal growth with proteolysis	24 ± 5.30
<i>Streptococcus thermophilus</i>	Normal growth with proteolysis	25 ± 0

LIPOLYTIC POWER

The lipolytic activity was significant in MRS medium supplemented with natural lipid substrates (Gino vegetable oil) as compared with artificial lipid substrates. The Table 6 shows the majority of tested bacteria had a lipolytic activity. But *Lb fermentum* and *Lactobacillus casei* showed no deposit around the discs on the agar medium containing tween 80.

Table 6: Lipolytic activity of lactic acid bacteria isolated from kpètè- kpètè samples.

Media used	Species	Observations	Conclusion
MRS with 1% vegetable oil	<i>Enterococcus faecium</i>	Growth with deposit	++
	<i>Lactobacillus fermentum</i>	Growth with deposit	++
	<i>Lactobacillus casei</i>	Growth with deposit.	+++
	<i>Leuconostoc mesenteroides</i>	Growth with deposit	+++
	<i>Streptococcus thermophilus</i>	Growth with deposit	++
MRS with 3% tween 80	<i>Enterococcus faecium</i>	Growth with deposit	++
	<i>Lactobacillus fermentum</i>	Growth with deposit	+
	<i>Lactobacillus casei</i>	Growth with deposit	+++
	<i>Leuconostoc mesenteroides</i>	Growth with deposit	+++
	<i>Streptococcus thermophilus</i>	Growth with deposit	++
MRS with tween 80 (Peptone+)	<i>Enterococcus faecium</i>	Growth with deposit	++
	<i>Lactobacillus fermentum</i>	Growth with deposit	-
	<i>Lactobacillus casei</i>	Growth with deposit	-
	<i>Leuconostoc mesenteroides</i>	Growth with deposit	+
	<i>Streptococcus thermophilus</i>	Growth with deposit	++

+++ : high lipolytic activity; ++ : middle lipolytic activity; + : low lipolytic activity; - : no activity.

Texturizing power

Detection of colonies on hyper-sucrose agar

Table 7 shows that all the tested bacteria were capable to grow on hyper-sucrose agar indicating a production of exo-polysaccharides.

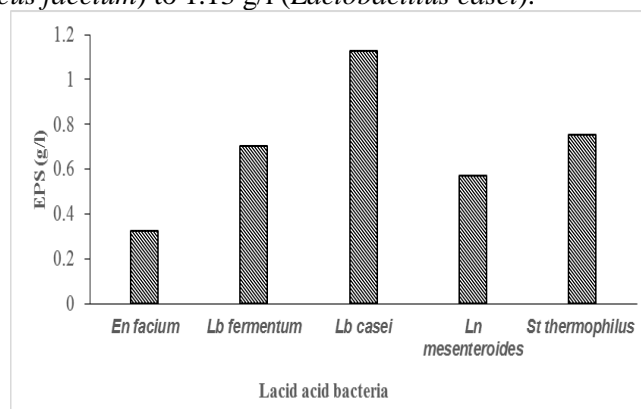
Table 7: Capability of the lactic acid bacteria isolated from kpètè-kpètè samples to produce exo-polysaccharides.

Species	Observation	Test conclusion
<i>Enterococcus faecium</i>	Large and slimy colonies	+
<i>Lactobacillus fermentum</i>	Large and slimy colonies	+
<i>Lactobacillus casei</i>	Large and slimy colonies	+
<i>Leuconostoc mesenteroides</i>	Large and slimy colonies	+
<i>Streptococcus thermophilus</i>	Large and slimy colonies	+

(+) test positive

Quantification of exo-polysaccharides

The Figure 7 shows that all the tested lactic acid bacteria strains produced exo-polysaccharides in different amounts ranging from 0.33 g/l (*Enterococcus faecium*) to 1.13 g/l (*Lactobacillus casei*).



En faecium: *Enterococcus faecium*; *Lb ferment*: *Lactobacillus fermentum*; *Lb casei*: *Lactobacillus casei*; *Ln mesenteroides*: *Leuconostoc mesenteroides*; *Strep thermo*: *Streptococcus thermophilus*.

Figure 7: Production of polysaccharides by lactic acid bacteria isolated from kpètè-kpètè samples.

Coagulant power

Table 8 shows that all the tested bacteria were capable to coagulate the milk. This property is needed in fresh cheese industry for which the dominant lactic coagulation gel is firm and a bit strong.

Table 8: Coagulant power of lactic acid bacteria isolated from kpètè-kpètè samples.

Species	coagulation test
<i>Enterococcus faecium</i>	+++
<i>Lactobacillus fermentum</i>	+++
<i>Lactobacillus casei</i>	++
<i>Leuconostoc mesenteroides</i>	++
<i>Streptococcus thermophilus</i>	++

+++ : High coagulant power; ++ : Average coagulant power

Aromatization power

Among the tested strains, *Enterococcus faecium* produced more acetoin than the others. So, the tested bacteria showed flavoring power that will contribute to the organoleptic characteristics of fermented products (Table 9).

Table 9: Flavoring power of lactic acid bacteria isolated from kpètè-kpètè samples.

Species	Production of Acetoin
<i>Enterococcus faecium</i>	+++
<i>Lactobacillus fermentum</i>	++
<i>Lactobacillus casei</i>	+
<i>Leuconostoc mesenteroides</i>	+
<i>Streptococcus thermophilus</i>	-

+: positive test; -: negative test.

DISCUSSION

The aim of this study was to investigate the probiotic properties of lactic acid bacteria isolated from “kpètè-kpètè”, ferment used in the production of traditional beer tchoukoutou in Benin. The results on the antimicrobial activity indicate that the lactic acid bacteria are able to inhibit the growth of both pathogenic bacteria and the yeast. All the strains do not have the same spectrum of action but most of lactic strains are active against Gram-positive pathogens. This result corroborates those of Onda et al. (2003) when they reported that those gram-positive bacteria are generally more susceptible to the bactericidal effect of lactic acid bacteria.

Moreover, lactic acid bacteria are known to produce a large variety of antimicrobial compounds such as organic acids, acetic acid, bacteriocins, diacetyl and hydrogen peroxide (Titiek et al., 1996; Bourgeois and Larpent, 1996; Aslam Qazi, 2010). The inhibitory ability of our lactic acid bacteria *in vitro* against the tested pathogenic germs confirms the production of antimicrobial agent and can presage to a good probiotic (Ammor et al., 2006).

Apart of the capacity to inhibit the growth of some pathogenic microorganism, the tested strains display a variable susceptibility to conventional antibiotics molecules. Indeed, our data indicate a high sensitivity of the tested lactic acid bacteria to *vancomycin*, *rifampicin*, *erythromycin* and *tetracycline*. These results are similar to those obtained during the studies on probiotic properties of *E. faecium* and *L. mesenteroides* isolated from Tunisian freshwater fish (Rim et al., 2015) and on properties of *Lactobacillus* strains isolated from infant feces (Tulini et al., 2013; Xanthopoulos et al., 2000). However, some authors reported a resistance of *Enterococcus* to rifampicin (Amariah, 2010) and of *Leuconostoc sp.* to Vanomycin, Gentamicin and Chloramphenicol (Ogier et al., 2008). We observed that all the *Lactobacillus* strains isolated from kpètè-kpètè samples were susceptible to vancomycin, and tetracycline. Thus, the profile of resistance to antibiotics is an important parameter of a probiotic strains (Ammor and Mayo, 2007) even its realization *in vivo* is difficult (Donohue, 2004).

The ability of adhesion to the intestinal mucosa is also one of the criteria for selection of the most important probiotic because it is considered a prerequisite for colonization and growth of bacteria (Schillinger et al., 2005; Guglielmotti et al., 2007). The colonization is needed to exercise their beneficial effects by inhibiting undesirable bacteria and the stimulation of the immune system. In our study, all tested lactic acid bacteria adhere to epithelial cells (colon and ileum) of broilers. Our results confirm those of Shukla et al (2014) who showed in their study on probiotic properties of *Leuconostoc mesenteroides* NRRL B-1149 that this strain adhered to cells.

However, the origin of probiotics may play a role in the adhesion ability (Gu et al., 2008). But, it was reported that *Lactococcus* (*Lc. lactis ssp. lactis* et *Lc. lactis ssp. cremoris*) strains isolated from various dairy products can adhere to human intestinal epithelial cells (Kimoto-Nira et al., 2009). The low adhesion observed in epithelial cells of chickens fed with antibiotic could be explained by the fact that most of the active molecules of this antibiotic cocktail (neomycin, streptomycin, erythromycin and tetracycline) are used to prevent and fight against bacterial infections.

The colonization of the cell surface of lactic acid bacteria strains is also a criterion for the selection of probiotic strains. This test demonstrates a selectivity of membrane surfaces and it shows the ability of these strains to colonize the intestinal mucus. The hydrophobicity seems to be a factor assisting in the adhesion, but it does not contribute to strong adhesion (Roos and Jonsson, 2002; Guglielmotti et al., 2007). The strains used in this study showed 51% of hydrophobicity with *Lactobacillus casei*. Similar results were obtained by González Arias et al (2013) in their study of the lactic acid bacteria such as *Lactobacillus paracasei* (87.34%) and *Enterococcus faecalis* (69.05%) and by Ly-Brown et al., (2010) on *Lactobacillus* (40%) with hexadecane. However, our data on *Streptococcus thermophilus*' hydrophobicity (40%) is two times higher than those of Iyer et al., (2010) on two streptococci was (~20%).

The test of the resistance to gastric acid showed a viability of strains despite the acidification of the medium (\downarrow pH). All species tested showed a better resistance to low pH with a survival rate from 99% to 100% at pH 2 and pH 2.5. Several studies have shown that lactic acid bacteria are able to survive under acid conditions (Mathara et al., 2008; Rim et al., 2015). In the same line, Even et al., (2003) founded a significant effect of acidification on the glycolytic activity of cells. Under acidic conditions, the specific glucose consumption rate increased, enabling a large energy input which allows lactic acid bacteria to be more resistant to pH acid. *Ln. mesenteroides* strain isolated in our work showed a good resistance to gastric acidity at pH 2 and pH 2.5 whereas some previous studies reported the stop of the bacteria (*Ln mesenteroides* and *Lb. plantarum*) grows at pH 3 (McDonald et al., 1990). Thus, we can say that the tested lactic acid bacteria isolated from "kpètè-kpètè" samples withstand gastric acidity.

The test of resistance to bile salts showed a high survival rate of lactic acid bacteria isolated from "kpètè-kpètè" samples ranging from 98 to 100%. The strains also had better resisted to stomacho-duodenal stimulus. These lactic acid bacteria can then grow in the presence of detergents (bile salts) released by the duodenum after ingestion of meals. This survival rate is higher than the 40% recorder with the lactobacilli isolated from milk and cheese (Zago et al., 2011). The difference observes between our results and those of the above cited authors may be not only due to the origin of the tested strain but also to the culture conditions.

The survival rate (100%) obtained in our study for *Lactobacillus fermentum* strain is greater than those obtained by Tulumoglu et al., (2013) in their study on probiotic properties of *Lactobacillus* isolated from child feces. In fact, these authors observed a survival rate of 51%-77% for *Lactobacillus fermentum* strains. The difference observed between the two results may be due to the percentage of bile salts used and the origin of the strains because the resistance of lactic acid bacteria with bile are associated with the composition of the growth medium (Kimoto-Nira et al., 2009). Thus, the resistance to the bile salts of some *Lactobacillus* strains has been changed when the sugar in the medium is changed from glucose to lactose. Then, the exclusion of bile by bacteria requires energy and the carbohydrates are easily metabolized to bring this energy increase resistance to bile salts. Also, it was reported that resistance to bile salts (0.15 to 0.3%) is recommended for human use probiotic selection (Gilliland et al., 1984).

The variation of acidity and pH during the growth of tested strains on skimmed milk show a difference among the lactic acid bacteria species. Similarly these bacteria are all acidifying with lactic acid production from 1 to 1.225 g/l. The result obtained in this study confirm those of Wang et al., (2010) who showed that lactobacilli isolated from feces are resistant to low pH 3.0 and that these bacteria are acidifying.

Concerning the ability to produce extracellular proteinases (proteolysis), the species of lactic acid bacteria tested in this study were found to be proteolytic with variable proteolysis diameters (24 ± 5.30 mm to 27.5 ± 10.61 mm). This result is consistent with that obtained by Idoui and Karam, (2008), who found that lactic acid bacteria isolated from the traditional cow butter from Jijel region (Algeria), had a proteolytic character. The proteolytic activity of the lactic acid bacteria is essential for their growth in fermented products and for the development of organoleptic properties of different food products (Savoy and Hebert, 2001; Hassaïne et al., 2007). Thus, proteinases hydrolyze protein, by providing essential amino acids for bacteria growth. It is known that the proteolytic system of lactic acid bacteria breaks down proteins and therefore changes the texture, taste and aroma of fermented products (El-Ghaish et al., 2011, El-Jenia et al., 2015).

As for the lipolytic activity of our kpètè-kpètè isolated most of them revealed deposits around the discs. However, *Lactobacilli* and *Leuconostoc* showed lipolytic activity more or less higher than streptococci and enterococci. These results agree with those of Karam et al., (2012) in Algeria, when they showed that the lipolytic activity was important in lactobacilli and *Leuconostocs*. The production of exopolysaccharides (EPS) by lactic acid bacteria is a favorable phenomenon for many food industrial processes (Walling et al., 2001).

In our study all strains tested produced EPS. *Lactobacillus casei* produced the highest amount of EPS (1.13 g/l) followed by *Streptococcus thermophilus* (0.75 g/l) and *Lactobacillus fermentum* (0.70 g/l) and the lowest amount were produced by *Enterococcus faecium* (0.33 g/l). The main advantage of the use of EPS producing lactic acid bacteria in the lactic ferments during the production of fermented products is to improve the texture and viscosity. It was also suggested that some of EPS produced by lactic acid bacteria may confer beneficial effects for consumer health (Wu et al., 2010). Thus, the EPS produced by *St. mutans* and *St. salivarius* are involved in bacterial colonization and dental plaque formation (Cerning, 1990) whereas *Lc. lactis ssp. cremoris* produced EPS that can prevents the reduction of cheese stickness (Hassan et al., 2005).

The lactic acid bacteria strains tested in this study have very good texturizing, flavoring and coagulant a power. These results confirm those of several authors who showed that lactic acid bacteria are able, from pyruvate, to synthesize various compounds (diacetyl, acetoin, 2,3-butanediol and α -acetolactate) responsible for aroma of fermented products (Raynaud et al., 2003; Leroy and De Vuyst, 2004). Indeed, α -acetolactate is an unstable compound that can be spontaneously transformed into diacetyl and/or acetoin (aromatic Béal molecules) during the lactic fermentation (Monnet et al., 2008). Lactic acid bacteria that metabolize citrate play an important role in many dairy processes because, in these bacteria, the metabolism of citrate and lactose results in the production of diacetyl, acetoin and CO₂, participating in aromatic and textural qualities of products (Raynaud et al., 2003).

CONCLUSION

This study allowed us to set up a collection of indigenous strains of lactic bacteria with probable food applications. In this study, resistance of lactic acid bacteria to the acidic media supplemented with bile salts was observed, and which makes possible their living passage in the digestive tract. After various tests conducted we confirm that the selected strains have probiotic properties and can be used for food applications.

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