



Assessment of Probiotic Potential of Lactic Acid Bacteria Isolated from Bottle Gourds (Calabash) of Milk Fermentation of Mbéré, Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Author TMMN collected the samples and carried out experiments and author NSFS contributed to paper writing. Authors BAM, MMJA, KP, IK and RM reviewed and edited the paper and LNT. Supervise the work. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: Lactic acid bacteria (LAB) became a field of interest by scientists in recent years due to their technological and probiotic properties. The aim of this work was to study the technological and probiotic properties of LAB isolated from the bottle gourds (calabashes) of milk fermentation, in Mbéré, Cameroun.

Methods: Five different bottle gourds from milk fermentation were collected and used for LAB isolation. These LABs were characterized using conventional cultural method, the technological

(such as proteolytic, lipolytic activities) and probiotic properties (including acid and bile salt tolerance, cholesterol assimilation and antioxidant activities) were assessed.

Results: From these samples, 30 LABs were isolated and among them, 21 exhibited great lipolytic and proteolytic activities with the maximum values of 18 and 29 mm respectively. In addition, 10 LAB isolates showed interesting antimicrobial activity against pathogens germs tested and good tolerance ability under acid and bile salt stress after 24h of incubation. Cholesterol assimilation and antioxidant tests revealed that isolated BC4 and BC3 have the greatest activity (35 and 39 mm respectively) while, BC4 and BL4 have the greatest antioxidant activity ($IC_{50} = 0,15$ and $0,13$ respectively).

Conclusion: LAB isolated from the bottle gourds (calabashes) of milk fermentation, in Mbéré, Cameroon can be used to develop dairy industry and manage the cardiovascular diseases.

Keywords: Lactic acid bacteria; technological and probiotic properties; bottle gourds; Mbéré.

1. INTRODUCTION

Probiotics are defined as living microorganisms which, when ingested in sufficient quantities, have positive effects on health beyond traditional nutritional effects [1]. They improve the intestinal microflora, the immune system response, the digestibility of dietary proteins, the lactose digestion and lowering serum cholesterol level, the blood pressure and synthesis of vitamins [2–4]. Probiotics are also important for the prevention of diarrhoea, *Helicobacter pylori* and *Clostridium difficile* infections [5,6].

However, the criteria for being considered as probiotic bacteria are several and strict. These criteria include that the bacteria should be of human origin and at least one clinical phase study must have been conducted [7,8]. To be considered as a probiotic, a microorganism must fulfil numerous criteria as non-pathogenic or without transferable genes of antibiotic resistance [9], acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to intestinal surfaces, temporary colonization, antagonism against pathogens and good technological properties [2,10,11]. The importance of these criteria depends on the final use of the probiotic and the most criteria is the survival of the specific microorganism with health benefits in the gastrointestinal tract [1].

Nowadays, probiotics have been mainly selected from the genera *Lactobacillus* and *Bifidobacterium*, because of their long history of safe, their natural presence in the human intestinal tract and their beneficial effects on health [12,13]. These include *L. acidophilus* LA1, *L. rhamnosus* GG, *L. plantarum* Lp01, *L. fermentum* RC-14, *L. paracasei* CRL431, *L. johnsonii* La1, *L. casei* Shirota, *B. breve* strain Yakult and *B. lactis* Bb-12, which are widely

produced and used in industrial processes [14,15]. However, many potentially probiotic microorganisms present in different types of food products remain unknown. Fermented foods are associated with desired and edible microbes which are beneficial for health [6]. Research in the field of exploring an interesting strain with probiotic potential from traditional fermented food products as a source of new isolates are feasible and necessary[2]. Traditional fermenting process is spontaneous and uncontrolled and could be a valuable source of autochthonous LAB [16]. Many authors reported that traditional fermented dairy products are the main source of isolation of active strains with greater biological activities [5,7].

In Cameroon as in many developing countries, the rural people still produce unpasteurized fermented milk by traditional methods using traditional container such as a bottle gourd. These bottle gourds are mainly used by the Mbororo's tribes in the Adamawa highlands of Cameroon for milk fermentation. In addition, there is a lack of information on the technological and probiotic properties of endogenous microflora. Preliminary studies carried out in the laboratory revealed that the internal wall of the calabashes of milk fermentation in the Mbéré (Adamawa, Cameroon) was colonized by LAB. The study aimed at assessing the technological and probiotic potential of LAB isolated from bottle gourds of milk fermentation.

2. MATERIALS AND METHODS

2.1 Culture Media and Chemical Reagents

De Mann Rogosa and Sharpe (MRS), Tryptic Soy Agar and broth and Mueller Hinton (MH) culture media, were obtained from Biolife (Biolife Italiana, Milano, Italy) while, porcine bile salt was

purchased from Sigma-Aldrich (Sigma Chemical Co., St-Louis, United States).

2.2 Microbial Strains

The microbial strains *Bacillus cereus* ATCC 19615, *Staphylococcus aureus*, *Listeria monocytogenes* ATCC 19115, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Candida albicans* were obtained from Food Microbiology and Biotechnology Laboratory, University of Ngaoundere were used for antimicrobial tests. All strains were subculture twice prior to the experiments.

2.3 Sample Collection

Five samples of the inner walls of *bottle gourds* (calabashes) were randomly and aseptically collected from traditionally fermented milk producers in the city of Meiganga. Mbéré, Adamawa, Cameroon. They were collected in sterile sampling tubes, transported under in iced cool boxes at 4°C to Laboratory of Microbiology and Biotechnology, National School of Agro-Industrial Sciences, University of Ngaoundere for LAB isolation.

2.4 Isolation and Characterization of Lactic Acid Bacteria

The isolation of LAB was done following the method described by de Man et al. [17]. Serial dilutions (10^{-1} to 10^{-7}) of 25 g of each sample were done in 225 mL of saline water (NaCl, 0.9%, *m/v*). One hundred microliters of the appropriate dilution were surface-plated on MRS agar, incubated anaerobically at 37 °C for 48 – 72h. All whitish colonies, Gram-positive, catalase- and oxidase-negative bacteria were selected and characterized for growth at different temperatures (37, 45 °C), CO₂ production from glucose, fermentation of different sugars.

2.5 Proteolytic and Lipolytic Activity of LAB

Proteolytic activity of the LAB was performed using MRS agar supplemented with skimmed milk (25%, *m/v*). To do this, 10 µL of LAB suspension ($\sim 10^8$ CFU/mL) were inoculated as a spot on the surface of the supplemented agar medium and then incubated for 24 h at 37°C. The appearance of clear zone around the colonies indicates the production of the protease [18].

Concerning lipase activity, it was performed on MRS agar supplemented with Tween 80 (1%, *v/v*; Biolife Italiana S.r.l., Milano, Italie). As mentioned above, 10 µL of LAB suspension ($\sim 10^8$ CFU/mL) were inoculated as a spot on the surface of the supplemented agar medium and then incubated for 24 h at 37°C. After 72 hours of incubation at 37°C, the appearance of a clear halo around the colonies noted and the diameter was measured [19].

2.6 Probiotic Property of Isolated Lactic Acid Bacteria

2.6.1 Antimicrobial activity

The method of Fleming et al. [20] was used to evaluate the antimicrobial activity of the LAB. One hundred microliters of LAB suspension ($\sim 10^8$ CFU/mL) were inoculated in 10 mL MRS broth and incubated at 30 °C for 24 h. The aliquots (15µL) of this culture were spotted onto MRS agar, incubated for 18 h at 30 °C, overlaid with 7 mL of the Mueller Hinton soft agar (0.7% agar) inoculated with 100 µL of an overnight culture of the indicator bacteria with the total count range from 10^6 - 10^8 CFU/mL. The plates were incubated for 24 h at 30 °C. The diameter of the inhibition zone around each LAB colony was measured.

2.6.2 Acid tolerance

The resistance of the isolates was assessed as described by Minelli et al. [3]. Microbial suspension cells (100 µL, $\Sigma 10^8$ CFU/mL) of LAB prepared with overnight cultures (16 ± 2 hold) were inoculated in MRS broth adjusted to pH 1.0, 2.0, 3.0, and 6.5 using 1M HCl. Bacterial growth was evaluated by determining the optical density at 620 nm after 6 and 24 h of incubation at 37 °C. The difference of percentage between the change in optical density (OD) at pH3 (OD pH3) and pH2 (OD pH2) would give an index of survivors which can be expressed as follows:

$$\text{Survival (\%)} = 100 * \frac{DO_{pH\ 1,2\ or\ 3} - DO_{pH6.5}}{DO_{pH1,2\ or\ 3}}$$

The classification criteria included four arbitrary levels of tolerance to the acidic condition: excellent if the isolate survived at pH 2 after 24 h; Very good if the strain survives at pH 2 after 6 hours but not after 24 hours; Good if the strain survives at pH 3 after 24 h but not at pH 2; If the isolate does not survive in an experimental

condition. An isolate survived if it demonstrated a percent survival of 50% or greater.

2.6.3 Bile salt tolerance

The resistance of LAB to bile salts was tested according to the method of Minelli et al. [3]. Briefly, 1 mL of LAB culture of 18 h ($\Sigma 10^8$ CFU/mL) were inoculated into tubes containing 9 mL of bile salts solutions at 0.2 and 0.4% (w/v) and incubated at 37 °C for 24 h. The optical density was determined and the inhibition percentage was calculated using the following formula:

$$\text{Inhibition percentage} = \left(1 - \frac{A_x}{A_0}\right) * 100$$

Where A_x represents the absorbance at x (0.2 and 0.4) percent of bile salt; and A_0 the absorbance at 0% of bile salts at 620 nm.

2.7 Cholesterol Hydrolysis

Cholesterol hydrolysis was assessed using Saini method [21]. Briefly, overnight (18 h old) strains of LAB were cultured on MRS agar supplemented with the egg yolk (2%, v/v) and then incubated for 48 h at 37 °C. The presence of the lyse zones characterized the degradation of cholesterol and the diameters were noted.

2.8 Antioxidant Activity of LAB

Antioxidant activity was performed by determining the percentage of reduction of the 1,1 diphenyl-2-picrylhydrazyl (DPPH) as described by Chen et al. [22].

At this level, the retained strains were characterized and identified using API 50 CHL kit™. Results were recorded after 24 and 48 h at 37°C. Species were determined tentatively through the use of Apident 2.0 (BioMérieux, French) and standard taxonomic descriptions from Wood and Holzapfel (1995). The specificity of these galleries was at least 92%. Identity of isolates was confirmed using online API web services (<https://apiweb.biomerieux.com>).

2.9 Statistical Analysis

The analysis was done in triplicate, results were expressed as means \pm standard deviation. The data were analyzed by analysis of variance, differences between means were tested using the Duncan Multiple Range test and correlations

between variables were tested using correlation table of Pearson in Statgraphic® Centurion XVII software (Statpoint Technology, Inc. USA). The technological and probiotic characteristics (proteolytic and lipolytic activities, acid and bile salt tolerance, cholesterol assimilation) of the LAB were subjected to multivariate analysis to discriminate isolates using Statistical XLStat 2017 software.

3. RESULTS AND DISCUSSION

3.1 Lactic Acid Bacteria Isolates

A total of 30 Gram-positive, catalase- and oxidase-negative isolates were obtained and their characteristics are illustrated in Table 1. Based on these characteristics and when compared with the data reported in the literature, we noted the predominance of the presence of two main genera including *Lactobacillus* (80%) and *Leuconostoc* (20%). The presence in the inner walls of the bottle gourds could be associated to their isolation and identification in the fermented milks. The predominance of *Lactobacillus* genus in traditional fermented milk in Africa has been reported by several authors [23]. More specifically, the presence of *L. plantarum* in *kule naoto* (Kenya), in *omabere amaruranu* were reported by Mathara et al. [24] and Bititu et al. [25] respectively. Concerning *Leuconostoc* isolates, we note that they were not able to grow at 45°C and they fermented all sugar used except arabinose. Similarly, their presence in few fermented milks in different parts of Africa were reported the presence of *Leuconostoc* strain particularly *L. mesenteroides* species in the *suusac*, traditional fermented cow's milk, Ethiopia, [26].

In addition, *Leuconostoc* strain was also identified in *nunu*, Ghana [27]. *Leuconostoc* plays a key role during the milk fermentation because of their ability to convert citrate in acetoin and diacetyl, aromatic compounds responsible for the improvement of the sensory properties of the product [24]. In contrast, other authors that their presence in low levels compared to *Lactobacillus* could be due to the fact that *Leuconostoc* is commonly associated with fermented plant food products compared to milk products and especially cheese. This implies adaptation of strains introduced by the gourds or smoking material, involving their capability to ferment lactose, a carbohydrate not usually found in plant substrates [24].

3.2 Technological Properties of Lactic Acid Bacteria

Technological properties such as proteolytic and lipolytic activities of the 30 LABs are illustrated in Table 2. We noted that proteolytic ability was strains-dependent and all isolates exhibited proteolytic activity except BC18, BL15, BL16, BL22, BL30, BL34 and BL9. There is a significant difference between those activities ($P < 0.05$). The diameters of hydrolysis were ranged from 0 to 29 mm and isolates BL29 and BC10 showed the highest activity with the values of 29 and 22 mm respectively. We can classified the isolates in three groups: High proteolytic isolates ($\varnothing \geq 16$ mm, 23.33% of the isolates); average proteolytic isolates ($5 \leq \varnothing < 16$ mm, 50% of the isolates) and low proteolytic isolates ($\varnothing < 5$ mm, 26.66% of the isolates) The presence of proteolytic activity has been reported to genera *Lactobacillus* and *Leuconostoc* [28]. Proteolytic activity of the LAB is an important parameter associated with the development of flavour compounds characteristics of some fermented dairy products.

Concerning lipolytic activity, we also observed that it was strain-dependent and sixteen isolates exhibited clear hydrolysis zone excepted fourteen (BL9, BL5, BL34, BL32, BL31, BL30, BL28, BL25, BL24, BL20, BL19, BL16, BL14 and BL11). The highest activity was recorded by BL22 isolate with the inhibition zone of 22 mm. The presence of lipolytic activity has been reported to genera *Lactobacillus* [29].

3.3 Probiotics Properties of Isolated Lactic Acid Bacteria

3.3.1 Antimicrobial activity

The antimicrobial activity of LAB isolated from the bottle gourds of milk fermentation was carried and the results are illustrated in Table 3. It emerges from this table that 21 isolates have shown inhibitory activities against all the indicator strains used in this study excepted 4 isolates (BC18, BC5, BL15 and BL17). Statistical analysis (ANOVA) revealed a significant difference between isolates ($P < 0.05$). The inhibition zones ranged from 6 to more than 14 mm. The majority of these antibacterial activities shown by the 21 isolates could be classified as very good and strong according to their hydrolysis diameter.

The inhibitory effect can be attributed to antibacterial compounds produced by the strains

studied, such as organic acid, hydrogen peroxide, biosurfactant, diacetyl, bacteriocin or the synergy between some of them [2,13,30,31]. The similar results have been noted by several studies on other LAB isolated in dairy products in Cameroon [11,32]. The Gram-positive bacteria are generally more sensitive to the bactericidal substances produce by LAB. This is based on the difference in cell envelope composition of the two groups of bacteria. The bacteriocins are mostly active on Gram+ pathogens *Listeria monocytogenes*, *Bacillus subtilis* and spores of *Clostridium perfringens* and act by forming pores in the cytoplasmic membrane which cause disruptions in cell function [13,16]. However, Song and Richard. [33] reported the presence of cells resistant in some strains of *L. monocytogenes* in Gram-positive bacteria, and explain that it could be linked to resistance of pore formation through a membrane changes in composition and properties, a decrease in surface hydrophobicity, and lower affinity of the bacterial surface to antimicrobial compounds. On the other hand, the resistance observed in Gram-negative bacteria is due to the outer membrane, which is a constituent of the cell envelope in all Gram-negative bacteria, and acts as an efficient permeability barrier against macromolecules and hydrophobic substances. This barrier function is largely attributed to the lipopolysaccharide (LPS) layer which contain anionic groups (phosphate, carboxyl), which contribute to the stability of the LPS layer through electrostatic interactions with divalent cations such as Mg^{2+} [34]. According to fungal species, Voulgari et al. [30] showed that LAB isolated from traditional fermented products produced protein-like compounds with very high activity on other pathogenic strains and alterations except on *S. cerevisiae* where no activity was detected. They explain that inhibitory activity was not only related to the production of lactic acid or acetic acid but other compounds with an antifungal activity, such as protein compounds such as phenyllactic acid, cyclic dipeptides, hydroxylated fatty acids, substances assimilated to bacteriocin and other low molecular compounds [31,35].

3.3.2 Acidity tolerance

Isolates with interesting antimicrobial properties were retained and the resistance under acidic and bile salts stress were tested. We observed in Table 4 that survival rate of the 17 isolates significantly increases during the first six hours with the maximum values of 38.8 % and 70.2% at pH1 and pH3 respectively. In addition, isolates

BL18, BL28, BL31, BL32, BL33, BC4 and BL5 showed a good survival rate (higher than 50%) at pH 1. At pH 3, we noted the reduction of survival rates for the isolates BL28, BL18 and BL5. At the same conditions, the high survival percentage was observed to the isolates BL18 (70.2%) and BL19 (70.1%).

Probiotic microorganisms should reach the site of action in a viable and the first barrier in the stomach is the gastric acid with inhibitory action related to low pH. Normal human gastric pH varies from 1 to 3 depending on fasting state of the individual [3]. Kalui et al. [36] obtained that all 18 strains of *L. plantarum* tested tolerated a pH of 2.5 after exposure for 3 h and 10% of these strains tolerated a pH of 2 and concluded that they were stable and could tolerate a low pH 2 but they were not able to grow. Various other studies have reported no growth in strains exposed to a low pH within the period of exposure. Sieladie et al. [11] had a survival percentage of less than 50% for all strains after 6 h exposure at pH 2. Similarly, Liong and Shah. [37] and Kalui et al. [36] working respectively with strains of *L. acidophilus*, *L. casei* and *L. plantarum*, *L. rhamnosus* observed that the viability of acid sensitive strains of *L. casei* and *L. plantarum* decreased gradually during the first three hours.

This variability of sensitivity can be explained by the genetic diversity of each strain and their ability to develop mechanisms of resistance to acid stress conditions. According to Cotter and Hill. [38], the mechanisms used by Gram-positive bacteria (especially probiotics) in acid resistance include the proton pumps F_1F_0 -ATPase encounter in some strains of *L. acidophilus* and *L. lactis*., the decarboxylation of amino acids (lysine, arginine, glutamate decarboxylases) encounter in *L. lactis subsp. lactis* and *L. brevis* and finally the progressive expression of regulators promoting changes in the parietal structure of the cell encounter in *B. subtilis*.

In addition, Guo et al. [14] observed that the incubation in PBS buffers at pH 2.5 resulted in significant decreases in the survival rate of the selected probiotics whereas *B. animalis* Bb12 was the most acid-sensitive strain. They explained that the acid tolerance of LAB has been linked to the induction of H^+ -ATPase activity and the variation in the acid tolerance of the selected probiotics might be related to the difference in H^+ -ATPase activity in the probiotics.

According to bile salt tolerance, tolerance was strains-dependent and were largely impacted by the bile salts concentration with a significant variation among them ($P < 0.05$). Indeed, all the 13 isolates retained and tested were able to tolerate presence of bile salt at the concentration of 0.2 and 0.4%. The less tolerant were the isolates BC3, BC4, BL11 and BL14 while, the more resistant isolates were BL18 (65.33%), BL33 (64.26%) and BL4 (54.25%). Resistance of strains belonging to *Lactobacillus* and *Leuconostoc* genera have been reported in the literature. For example, Begley et al. [39] demonstrated the tolerance of bile salt to *L. plantarum* and *Lactobacillus amylovorus*.

Bile salts are toxic for living cells, since they disorganize the structure of the cell membrane and bile salt tolerance is considered one of the essential properties required for LAB to survive in the small intestine. Before a probiotic can benefit human health, it must be able to tolerate bile salts as well as to grow in the lower intestinal tract [6,14,40]. The small intestinal transit tolerance of bile salts-resistant lactobacilli was found to be strain-dependent. The production of bile salt hydrolases is the main mechanism of resistance mentioned in literature. For example, authors established that probiotics such as *L. acidophilus* were found to excrete Bile Salt Hydrolase, the enzyme that catalyzes the hydrolysis of glycine- and taurine-conjugated bile salts into amino acid residues and free bile salts [37]. Therefore, interest has risen in the possibility of using bile salt by LAB to lower serum cholesterol levels in hypercholesterolemic patients and prevent hypercholesterolemia in normal people [11]. Singh et al. [41] obtained that all the selected strains could tolerate 0.5% and 1% bile concentrations and the most tolerant *Lactobacillus* were LR1, LR5, LR11, LR17, LR18, LR19, LR25, LR26 and LR34. However, when we look about the Bile Salt Hydrolase (BSH) activity only LR20 exhibited an intense level of BSH activity, while the rest of the 8 isolates showed moderate levels of BSH activity. This explains that there is not a significant relationship between bile salts tolerance and bile salt hydrolase production and activity. Nevertheless, the work of Schillinger et al. [42] demonstrated that not all bacteria had the ability to produce hydrolases such as 09 strains of *L. casei* and *L. rhamnosus* BFE 752. To improve this, the studies by Minelli et al. [3] observed that bacteria

Table 1. Phenotypical characteristics of LAB

Properties	Group 1	Group2	Group 3	Group 4
Cell morphology	Cocci	Rods	Rods	Rods
CO₂ production	+	-	-	+
Growth at 45°C	-	-	+	+
Substrate of fermentation				
Arabinose	-	+	+	-
Ribose	+	+	-	-
Xylose	+	-	+	-
Galactose	+	+	+	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Lactose	+	+	+	-
Sucrose	+	+	+	-
Maltose	+	+	+	+
Identity	<i>Leuconostoc mesenteroides</i> (6 isolates)	<i>Lactobacillus plantarum</i> (3 isolates)	<i>Lactobacillus casei</i> (12 isolates)	<i>Lactobacillus fermentum</i> and <i>L. cellulosus</i> (9 isolates)

Table 2. Proteolytic and lipolytic properties properties of LAB

Isolates	Proteolytic activity (mm)	Lipolytic activity (mm)
BC10	22.00 ± 0.00 ^o	14.33 ± 2.08 ^{gh}
BC18	0.00 ± 0.00 ^a	8.33 ± 1.15 ^{bc}
BC3	10.67 ± 1.53 ^{cde}	10.66 ± 1.15 ^{def}
BC4	18.00 ± 1.00 ^{lm}	3.00 ± 1.00 ^b
BC5	9.33 ± 1.15 ^b	1.67 ± 2.08 ^{ab}
BL1	17.00 ± 1.00 ^{kl}	10.00 ± 1.00 ^{cde}
BL11	12.33 ± 0.57 ^{efg}	0.00 ± 0.00 ^a
BL14	11.00 ± 0.00 ^{cde}	0.00 ± 0.00 ^{efg}
BL15	0.00 ± 0.00 ^a	12.33 ± 0.57 ^{ef}
BL16	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
BL17	2.00 ± 2.00 ^b	9.00 ± 2.00 ^{cd}
BL18	15.67 ± 1.15 ^{jk}	6.67 ± 1.15 ^b
BL19	14.67 ± 1.53 ^{hij}	0.00 ± 0.00 ^a
BL2	17.00 ± 0.00 ^{kl}	12.67 ± 1.15 ^{fg}
BL20	15.00 ± 0.00 ^{ij}	0.00 ± 0.00 ^a
BL21	10.33 ± 0.57 ^{cd}	14.67 ± 3.51 ^h
BL22	0.00 ± 0.00 ^a	22.00 ± 1.00 ⁱ
BL24	13.33 ± 2.30 ^{fghi}	0.00 ± 0.00 ^a
BL25	20.00 ± 1.00 ⁿ	0.00 ± 0.00 ^a
BL28	14.00 ± 0.00 ^{ghij}	0.00 ± 0.00 ^a
BL29	29.00 ± 1.00 ^p	18.67 ± 1.53 ⁱ
BL30	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
BL31	14.00 ± 2.00 ^{ghij}	0.00 ± 0.00 ^a
BL32	23.00 ± 1.00 ^o	0.00 ± 0.00 ^a
BL33	13.00 ± 1.75 ^{fgh}	8.67 ± 0.57 ^c
BL34	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
BL35	19.67 ± 1.15 ^m	10.00 ± 1.00 ^{cde}
BL4	12.00 ± 1.00 ^{def}	11.00 ± 0.00 ^a
BL5	12.00 ± 0.00 ^{def}	0.00 ± 0.00 ^a
BL9	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a

Values represented as mean ± SD of triplicate analyses; for each column, different subscripts lowercase letters indicate significantly different at $p < 0.05$

Table 3. Antimicrobial activity of LAB against pathogens

Isolates	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>S. typhi</i>	<i>L. monocytogenes</i>
BC18	-	-	-	-	-	-
BC3	-	-	+++	++	+++	+++
BC4	-	-	+++	-	+++	-
BC5	-	-	-	-	-	-
BL1	++	-	+++	-	+++	-
BL11	-	-	+++	-	-	-
BL14	++	+++	+++	++++	++++	++++
BL15	-	-	-	-	-	-
BL17	-	-	-	-	-	-
BL18	-	++	-	-	+++	-
BL19	-	-	++	-	-	-
BL2	-	-	-	-	-	-
BL20	-	+++	-	++	-	++++
BL21	-	+++	-	-	+++	-
BL24	-	+++	++++	++	+++	-
BL28	-	-	+++	++++	-	-
BL31	++	+++	++++	-	++++	++++
BL32	++	++++	+++	-	-	-
BL33	-	+++	+++	-	-	-
BL4	-	+	++++	-	-	-
BL5	+	-	+++	-	-	-

- no inhibition; + 1.0–3.0 mm (weak); ++ 3.1–6.0 mm (good); +++ 6.1–14.0 mm (very good); ++++ >14.0 mm (strong);

(04 strains of *L. casei*) could grow in MRS medium supplemented with 1% (w/v, Oxgall) but no bile salt hydrolase activity was observed by plate assay.

3.3.4 Cholesterol activity

All the 11 LAB strains were able to assimilate cholesterol in the medium materialize by a clear hydrolysis zone beside the colonies. The diameter of hydrolysis ranged between 10.69 and 39.44 mm and there were significant differences between obtained results. The greatest activity was collected to the isolates BC (39.4 mm), BC4(34.7 mm), BL4(33.3 mm) and BL14 (33.1 mm). Several studies established that the management of obesity is cholesterol-lowering through administration of probiotics strains. Literature mentioned that probiotic could use several mechanisms for cholesterol reduction such as cholesterol assimilation, incorporation of cholesterol to their cell membrane or attachment to the bacterial cell surfaces, deconjugation of bile acid inducing the coprecipitation of cholesterol [43].

3.4 Grouping of All Evaluated Parameters

Multivariate analysis including dendrogram and principal component analysis were purchased for

grouping the isolates and distribution is illustrated in Fig. 1 and Fig. 2. The principal component analysis (PCA) revealed that first two principal components (F1 and F2) explained 54.97% of the total variation and F1 and F2 accounted for 36.60% and 18.37% respectively. As shown in Fig. 1 representing the distribution plots of variables on the plane of the first two principal components, we observed that all selected and analyzed properties were correlated to F1 and F2, suggesting that these variables contribute to probiotic strain clustering. Projection of LAB isolates in two-dimensional space of the F1 and F2 loading factors could differentiate three main clusters. The same observations are obtaining using hierarchical ascending classification analyses. BL14 and BL31 (C1) were related to antimicrobial activity against pathogens strains tested. On the other hand, BL2, BL32 and BL28 (C2) were related with higher proteolytic activity, while, BL5, BL11, BL19, BL24, BL1, BL21, BC3, BC4 (C3) were related with the higher lipolytic activity, acid and bile salt tolerance and cholesterol assimilation. These previous isolates were retained for biochemical characterization and identification using API 50 CHL kit™. Data analysis and the comparison using different tools of identification revealed the presence of 4 main species such as *L. casei* (BL1, BL14, BL19,

Table 4. Survival (%) of LAB under acidic, bile stress conditions and cholesterol hydrolysis

Isolates	pH			Bile salts (% m/v)		Cholesterol removal
	1	2	3	0.2	0.4	
BC3	40.5 ± 0.2 ^c	44.2 ± 0.1 ^e	45.6 ± 0.4 ⁱ	48.6 ± 0.4 ^{gh}	45.2 ± 0.6 ^e	39.4 ± 0.7 ^k
BC4	42.4 ± 0.5 ^{de}	43.5 ± 0.0 ^c	50.4 ± 0.1 ^k	57.4 ± 0.1 ⁱ	40.5 ± 0.2 ^d	34.7 ± 2.0 ^{ij}
BL1	38.8 ± 0.1 ^a	41.6 ± 0.6 ^b	36.8 ± 0.4 ^{de}	30.8 ± 0.8 ^c	49.6 ± 0.7 ^h	25.8 ± 0.9 ^g
BL11	38.8 ± 0.4 ^{ab}	41.6 ± 0.3 ^b	36.3 ± 0.8 ^d	44.2 ± 1.1 ^f	40.5 ± 0.5 ^d	25.8 ± 0.1 ^{gh}
BL14	42.4 ± 0.4 ^{de}	4.2 ± 0.7 ^a	39.7 ± 0.6 ^{fg}	39.58 ± 0.3 ^d	11.3 ± 0.9 ^b	18.1 ± 1.1 ^{cd}
BL18	63.4 ± 0.9 ^j	65.3 ± 0.7 ^m	70.2 ± 1.2 ^{op}	67.2 ± 2.1 ^a	65.3 ± 0.4 ^{ik}	21.5 ± 0.7 ^e
BL19	41.8 ± 0.3 ^d	43.4 ± 0.2 ^{cd}	39.5 ± 0.7 ^f	52.3 ± 0.7 ⁱ	45.4 ± 0.1 ^{ef}	33.1 ± 0.4 ⁱ
BL2	42.3 ± 0.1 ^{de}	45.5 ± 0.1 ^g	40.7 ± 0.5 ^g	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
BL20	40.9 ± 1.5 ^{cd}	49.8 ± 0.8 ^h	57.8 ± 1.5 ^j	58.8 ± 0.2 ^k	47.9 ± 0.3 ^g	10.6 ± 0.5 ^b
BL21	43.8 ± 0.9 ^f	44.7 ± 0.3 ^{ef}	41.5 ± 0.2 ^h	40.8 ± 0.5 ^e	49.2 ± 0.8 ^{gh}	22.6 ± 1.2 ^{ef}
BL24	43.8 ± 0.6 ^f	44.7 ± 0.9 ^{ef}	41.5 ± 1.2 ^{hi}	49.3 ± 1.0 ^h	45.3 ± 0.3 ^{ef}	17.4 ± 0.3 ^c
BL28	72.1 ± 0.9 ^l	66.9 ± 0.1 ^{mn}	34.7 ± 0.7 ^c	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
BL31	50.5 ± 0.4 ^g	61.1 ± 0.6 ^{kl}	61.6 ± 1.5 ^{mn}	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
BL32	55.4 ± 0.3 ⁱ	51.6 ± 0.8 ^{hi}	28.2 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
BL33	57.3 ± 0.5 ^a	69.2 ± 0.4 ^o	70.1 ± 0.7 ^o	59.7 ± 0.5 ^{kl}	64.2 ± 0.6 ⁱ	0.0 ± 0.0 ^a
BL4	53.0 ± 0.0 ^h	56.9 ± 0.2 ^j	59.7 ± 0.5 ^m	66.3 ± 2.1 ^m	54.2 ± 0.4 ⁱ	0.0 ± 0.0 ^a
BL5	69.2 ± 0.3 ^k	60.1 ± 0.9 ^k	32.4 ± 0.1 ^b	29.4 ± 0.2 ^b	38.5 ± 0.1 ^c	0.0 ± 0.0 ^a

Values represented as mean ± SD of triplicate analyses; for each column, different subscripts lowercase letters indicate significantly different at $p < 0.05$; Table 5: Antioxidant activity of different yeast strains

Table 5. Antioxidant activity of different yeast strains

Isolates	IC ₅₀ (mg/mL)
BC3	0.55 ± 0.07 ^f
BC4	0.15 ± 0.02 ^b
BL1	0.19 ± 0.03 ^{cd}
BL11	0.41 ± 0.01 ^a
BL14	0.18 ± 0.02 ^c
BL18	0.61 ± 0.03 ^{fg}
BL19	0.33 ± 0.05 ^e
BL20	0.63 ± 0.01 ^{gh}
BL21	0.17 ± 0.02 ^{bc}
BL24	0.64 ± 0.01 ^h
BL4	0.13 ± 0.05 ^{ab}
Vitamin C	0.01 ± 0.00 ^a

Values represented as mean ± SD of triplicate analyses; for each column, different subscripts lowercase letters indicate significantly different at $p < 0.05$;

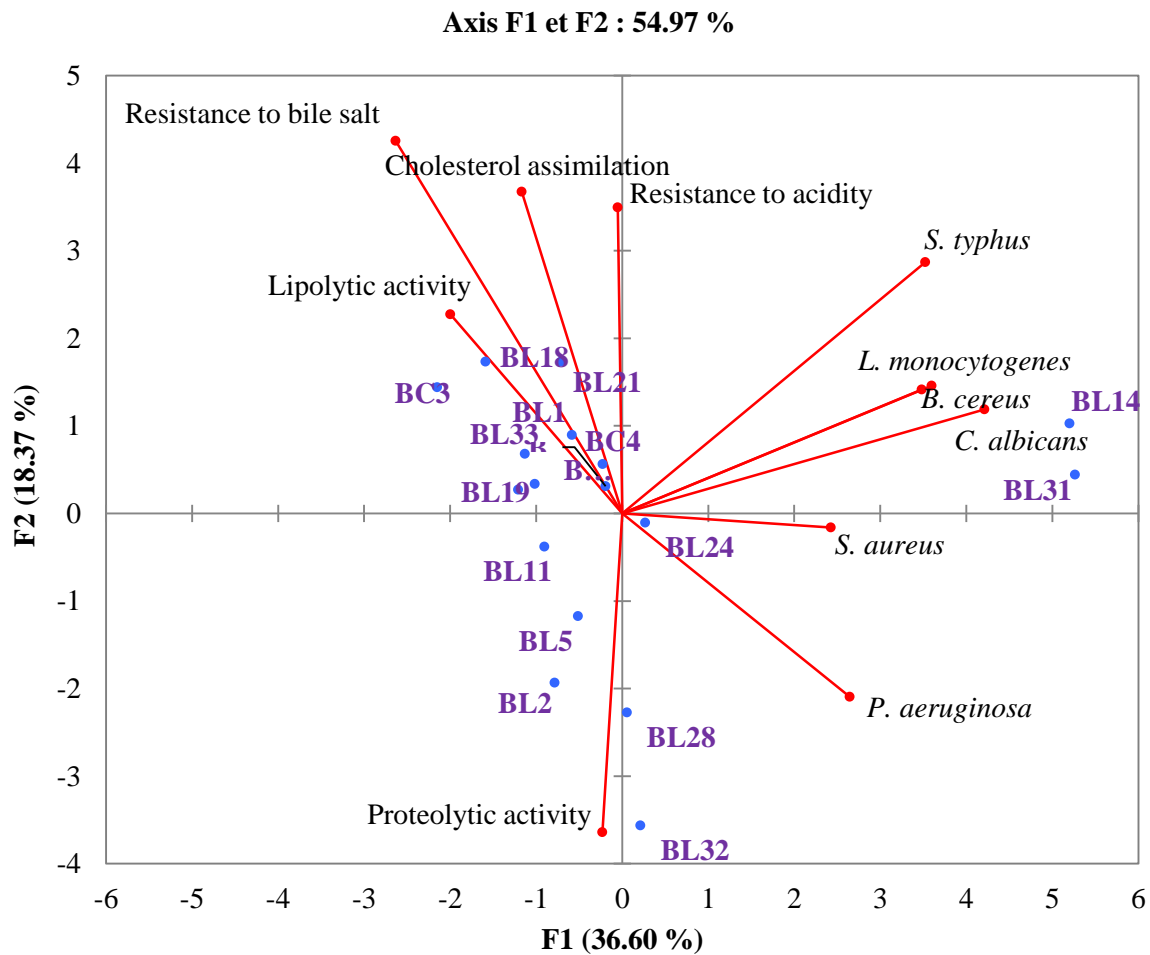


Fig. 1. Projection of the LAB, technological and probiotics parameter on F1 and F2 axes

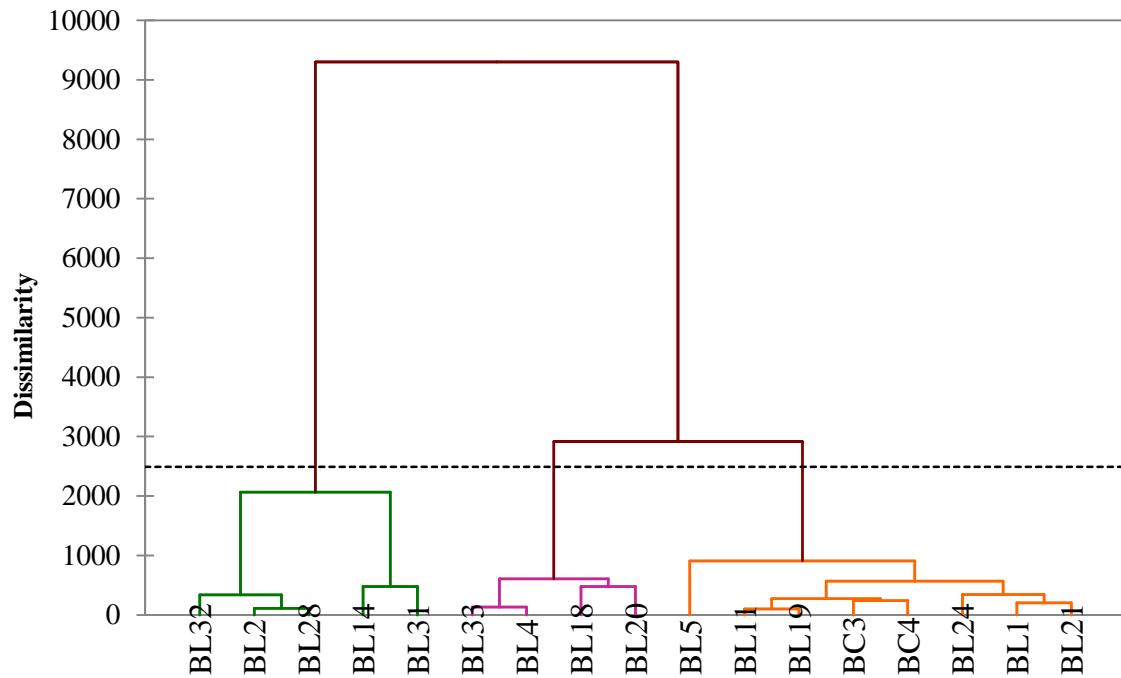


Fig. 2. Clustering of LAB based on their technological and probiotic properties

BL20), *L. fermentum* (BL21, BL24), *L. plantarum* (BL4, BC4) and *L. mesenteroides* (BL11, BL18 and BC3).

3.5 Antioxidant Activities

Antioxidant potential using the 1,1 diphenyl-2-picrylhydrazyl (DPPH) scavenging method of retained and identified isolates and vitamin C used as control are illustrated in Table 5. Five isolates BL4 (0.13 mg/mL), BL21 (0.17 mg/mL); BL1 (0.19 mg/mL), BL14 (0.18 ± 0.02 mg/mL) and BC4 (0.15 ± 0.02 mg/mL) recorded the highest antioxidant activities characterized by the lowest value of the IC₅₀. In contrast, the lowest activities were recorded with isolates BLC3 (0.55 mg/mL); BL18 (0.61 mg/mL); BL20 (0.63 mg/mL) and BL24 (0.64 mg/mL). Analysis of variances (ANOVA) showed a significant difference between isolates (P<0.05). The antioxidant activity of several LAB strains isolated from dairy products have been mentioned [44,45]. Globally, the antioxidant activity of LAB would be linked to their ability to produce some peptide compounds and relevant oxidative enzymes such as tanase during their growth. Concerning compounds, several authors reported bacteria produced secondary metabolites of antioxidant nature such as glycerol, techoic acid, peptidoglycans, valine, arginine, tyrosine and tryptophan, pathetoic and nicotinic acid, and manganese during

fermentation, thus contributing to the stabilization of the finished product. Kudoh et al. [46] reported a strong antiradical activity to DPPH with inhibition percentages ranging from 63.02 to 91.75% from *L. delbrueckii* subsp. *bulgaricus* isolated from fermented milks and explained that this was due to the presence of κ-casein derived peptides an antioxidant peptide.

4. CONCLUSION

The bottle gourds used for milk fermentation in Mbéré division (Cameroon) have microbial diversity characterized by LAB predominance including *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* with interesting technological and probiotic potential. In addition, they showed great antimicrobial activity, high ability to assimilated cholesterol and interesting antioxidant activity. This capacity has to be verified through *in vivo* tests before being used as functional foods to hypercholesterolemic patients.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding authors on request.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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