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RESEARCH ARTICLE

PROBIOTIC PROPERTIES OF LACTOBACILLI STRAINS ISOLATED FROM RAW COW MILK IN THE WESTERN HIGHLANDS OF CAMEROON

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Abstract

The objective of this study was to characterize probiotic *Lactobacillus* sp isolated from raw cow milk focusing on their safety, antimicrobial, and cholesterol-lowering properties. A total of one hundred and seven colonies of lactobacilli isolated from thirty-two samples of raw cow milk were screened for their probiotic use. 15 isolates of lactobacilli were selected for acid and bile tolerance. Almost all the acid and bile tolerant isolates of lactobacilli were sensitive to eight of the nine antibiotics tested. None of the assayed strains showed hemolytic and gelatinase activity. In addition, isolate 29V showed strong antimicrobial activities against the used indicator pathogens. All isolates expressed bile salt hydrolase activity and had ability to assimilate cholesterol *in vitro*. The 15 selected isolates were identify to species level as *Lactobacillus plantarum* using API 50CH Kits. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was carried out to discriminate between three new best probiotic strains of *Lactobacillus plantarum.* According to these results, the *Lactobacillus* strains associated with dominant microflora that people from Mbororo's tribe in the western highlands of Cameroon use to manufacture fermented milk contain new potentially probiotic strains with antimicrobial and cholesterol-lowering properties.

Keywords: *Lactobacillus* cow milk, antimicrobial activity, cholesterol-lowering property.

Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microflora. *Lactobacillus* is one of the most important genera of LAB (Coeuret *et al.,* 2003).

During the last fifteen years, the *Lactobacillus* genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks (Coeuret *et al.,* 2003). Lactobacilli comprise

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a large and diverse group of gram positive, non-spore forming, catalase negative rod bacteria, able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Pelinescu *et al*., 2009). They are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications (Fuller, 1989). Probiotics, as defined in a FAO/WHO (2002) report, are "live microorganisms which when administered in adequate amounts confer a health benefit on the host".

Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Helland *et al.,* 2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance (Parvez *et al.,* 2006).

In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and prove safety for consumer (De-Vries *et al.,* 2006). To survive in the gut, the organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. Besides, quality assurance programmes associated with research, development, production and validation of the health benefits of these bacteria require their relevant characterization and identification.

Over the world, the research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present on the market.

In Cameroon as in many developing countries, the rural people still produce unpasteurized fermented milk by traditional methods. Such milk products are still consumed affectionately by rural population. There is a lack of information on the probiotic characteristics of traditional fermentative microflora that Mbororo's tribes used to produce fermented milk in the western highlands of Cameroon.

The main goal of our study was the probiotic characterization of *Lactobacillus* sp isolated from raw cow milk in the western highlands of Cameroon focusing on their safety, antimicrobial, and cholesterol-lowering properties.

Material and Methods

Isolation of bacteria

Thirty-two raw cow milk samples were collected from fifteen farms in the western highlands of Cameroon. Samples were incubated at 37°C until coagulation. Coagulated samples were then activated in MRS broth (Biolife, Italy) at 37°C for 24h in order to obtain enriched cultures. These cultures were streaked on MRS agar medium and incubated under anaerobic condition using a candle extinction jar with a moistened filter paper to provide a CO_2-enriched, water-vapor saturated atmosphere at 37°C for 48h. Single colonies picked off the plates were sub cultured in MRS broth at 37°C for 24h before microscopic examination. The cultures of rod-shaped bacteria were streaked on MRS agar medium for purification. Purified strains were stores at -20°C in sterile MRS broth supplemented with 20% glycerol. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus* (Hartemink *et al.,* 1997).

Preliminary identification of the isolates

Identification of the isolates at genus level was carried out following the criteria of Sharpe (1979) using morphological, phenotypic and biochemical methods. The cultures were examined microscopically for gram staining and catalase production (Harrigan and MacCance, 1976). In addition, all isolates were tested for growth at 10°C for 10 days, 45°C for 48h and CO_2 production from glucose.

Acid tolerance

Preliminary selection of acid tolerant lactobacilli using rapid method was determined according to

slightly modified methods as described by Pelinescu *et al*. (2009) to simulate gastric conditions. Tested lactobacilli isolate cultures were grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6h old culture was inoculated into 100ml MRS broth whose pH had been adjusted to 2, 3 or 7 using 1N HCL or NaOH. Bacterial growth was monitored by determination of optical density at 620nm after 6 and 24h incubation period at 37°C.

The percent difference between the variation of optical density (DO) at pH7.0 (ΔDO_{pH7}) and the variation of optical density (DO) at pH2 or 3 ($\Delta DO_{pH2\ or\ 3}$) would give an index of isolates surviving that can be expressed as follows:

$$Surviving\ (\%) = \frac{\Delta DO_{pH7} - \Delta DO_{pH2or3}}{\Delta DO_{pH7}} \cdot 100 \quad (1)$$

Classification criteria included four arbitrary level of acid condition tolerance: excellent if the isolate survived at pH 2 after 24h; very good if the isolate survived at pH 2 after 6h but not after 24h; good if the isolate survived at pH 3 after 24h but not at pH2; poor if the isolate did not survive in any experimental condition.

An isolate survived if it demonstrated a surviving percentage equal or greater than 50%.

In the present study, pH 3 was used as a representative gastric pH value. Isolates were cultured on MRS agar medium for 24h at 37°C. Colonies of isolates were collected and suspended in 0.1M citrate buffer pH3, and the turbidity of cells suspensions were compared to 4 Mc Farland (12X 10^8cfu/ml) followed by serial dilution and plate counting (Verdenelli *et al.,* 2009). At the time of 0 and 5h incubation at 37°C, each strain was cultured in MRS agar and was incubated anaerobically at 37°C for 48h. Results were expressed as the percent (log10cfu) of resistant cell.

Bile salt tolerance

The tolerance of lactobacilli to bile salts (BS) was evaluated in MRS supplemented with bile salts using a modified method described by Dora and Glenn (2002). Test lactobacilli isolates cultures were grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6h old culture was inoculated into 100ml MRS broth with 0.2 or 0.4% (w/v) bile salts (Sigma, USA).

Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24h incubation period at 37°C.

The percent difference between the variation of optical density (DO) of culture without bile salts ($\Delta DO_{0\%BS}$) and the variation of optical density of culture containing 0.2 or 0.4% bile salts ($\Delta DO_{0.2\ or\ 0.4\%BS}$) would give an index of isolates surviving that can be expressed as follows:

$$Surviving\ (\%) = \frac{\Delta DO_{0\%BS} - \Delta DO_{0.2or0.4\%BS}}{\Delta DO_{0\%BS}} \cdot 100 \quad (2)$$

Classification criteria included four arbitrary level of bile salt tolerance: excellent if the isolate survived at 0.4% bile salt after 24h; very good if the isolate survived at 0.4% bile salt after 6h but not after 24h; good if the isolate survived at 0.2% bile salt after 24h but not at 0.4% bile salt; poor if the isolate did not survive in any experimental condition.

An isolate survived if it demonstrated a surviving percentage equal or greater than 50%.

Resistance to antibiotics

The antibiotic susceptibility of selected acidotolerant and bile tolerant isolates was determined towards nine antibiotics, namely, penicillin G (10 units), ampicillin (10µg), amoxicillin (10µg), erythromycin (15µg), tetracycline (30µg), chloramphenicol (30µg), Doxycycline (25µg), cotrimoxazole (25µg) and ciprofloxacine (5µg). Strains selection was based on their performance toward acid and bile salts.

Antibiotic susceptibility was determined semi-quantitatively using a modification of the agar overlay diffusion methods of the National Committee for Clinical Laboratory Standards NCCLS (1993).

Diameters of inhibition zones were measured and results were expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S), according to cut off levels proposed by Prescott *et al.* (1999), NCCLS (2002), Vlkova´ *et al.* (2006).

Gelatinase activity

Gelatinase activity of the most antibiotics sensitive isolates was investigated as described by Harrigan and McCance (1990). 2µl of a 6h old culture was spot-inoculated into nutrient gelatin agar (Oxoid, Basingstoke, Hampshire, UK). The plates were incubated anaerobically for 48h at 37°C after which they were flooded with saturated ammonium sulfate solution and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis). A strain of *Staphylococcus aureus* ATCC 25923 was used as positive control.

Haemolysis activity

Haemolysis activity of gelatinase negative isolates was investigated as described by Gerhardt *et al.* (1981). 2µl of a 6h old culture broth was spot-inoculated into sterile blood agar. The blood agar was prepared by adding 7% sheep-blood, that had been preserved in ethylenediaminetetraacetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48h after which they were observed for clear zones surrounding colonies (positive reaction for beta haemolysis). A strain of *S. aureus* ATCC 25923 was used as positive control.

Antimicrobial activity

Antimicrobial activity of the selected probiotic isolates was checked by using the agar-spot test (Mami *et al.,* 2008). Isolates were screened for production of antimicrobial against *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 25922, *S. aureus* (*MDR,* clinical isolate), *Streptococcus mutans* DSM 20523, *Enterococcus faecalis* ATCC10541, *Escherichia coli* ATCC 13706, *E. coli* (*MDR,* clinical isolate), *Salmonella typhi* ATCC 6539, *Pseudomonas aeruginosa* ATCC 20027, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae* (clinical isolate) as the indicator microorganisms.

An aliquot of 2µl of a 6h old producer isolate culture was spotted on MRS agar and plates were incubated anaerobically at 37°C for 48h to allow exhibition of antimicrobial compounds.

Cell suspensions of the indicator microorganisms were prepared as follows: each 24h old culture of the indicator strain on Mueller Hinton Agar slant was suspended in sterile physiological saline solution (NaCl 0.9%) and the turbidity was compared to 0.5 Mc Farland (corresponding to 10^8cfu.ml^{-1}). 50µl of the cell suspension was inoculated in 5ml of Plate Count Soft Agar and overlaid on colonies of producer isolates. After incubation at 37°C for 24h, plates were checked for zones of inhibition surrounding the producer colonies. Inhibition was recorded as positive if the width of the clear zone around the colonies of the producer was 2mm or larger.

The agar well diffusion technique was also used to discriminate antimicrobial activity of the selected probiotic isolates due to organic acid production. The method of Mante *et al.* (2003) was adapted. Isolates were cultured overnight before assay. Bacterial cultures were prepared into cell supernatant pH 7.0. 50µl sterilized free-cell neutralized supernatant was filled into the well against target microorganisms. After 24h of incubation time, the diameter of the inhibition zone was measured and scored. The representation of inhibition zone were not included in 6mm diameter of well. The inhibition zone larger than 2mm was scored positive.

Screening for bile-salt hydrolytic (BSH) activity

The isolates were screened for BSH activity by spotting 10µl aliquots of overnight cultures on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma, USA) and 0.37g/l of $CaCl_2$ (Schillinger *et al.,* 2005).

Plates were incubated anaerobically at 37°C for 72h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of bacteria.

Isolates were grouped into one of the three arbitrary classes based on the diameter of the precipitation zones on BSH screening medium according to Mathara *et al.* (2008): low BSH activity if the isolate demonstrated precipitation zone up to 10mm; medium BSH activity if the isolate demonstrated precipitation zone of 11 to 15mm; high BSH activity if the isolate demonstrated precipitation zone greater than 16mm.

In vitro cholesterol-lowering property

The ability of isolates to assimilate cholesterol was determined by a modified method described by Dora and Glenn (2002). A 9.9 ml aliquot of MRS broth containing 0.4% bile salt (w/v) and 0.01% (w/v) cholesterol (polyoxyethanyl-cholesteryl Sebacate; Sigma) was inoculated separately with 0.1ml overnight culture of each of the isolates. The inoculated bottles were incubated at 37°C under anaerobic conditions for 18h. The bacterial cells were removed from the culture broth by centrifugation at 4000rpm for 20min and the supernatant was used directly for measuring cholesterol. Total cholesterol was analyzed using an enzymatic procedure, which is a modification of the method of Allain *et al*. (1974). The amount of cholesterol removed from the growth medium was expressed as a percentage by the treatment compared with the control (MRS broth supplemented 0.4% bile salt) as follows: [1-(residual cholesterol in cell-free broth)/ (cholesterol of control broth)] x 100. Cell pellet was dry at 80°C until constant weight. Cholesterol assimilation of the isolates was expressed as the amount of cholesterol consumed in milligram per gram of cells.

Sugar fermentation profile

The carbohydrate fermentation profiles of purified isolates were determined using API 50 CH system (Biomérieux, Marcy l'étoile, France). Interpretation of these fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer-aid database Apiweb™ API 50 CH V5.1 software.

Genotypic characterization

DNA extraction

The genomic DNA of each bacteria strain was extracted and purified using the NucleoSpin Tissue Kit or UltraClean MEGA SOIL DNA Isolation Kit. Purified DNA samples were then stored at -20°C. Reference strains were obtained from the University of Camerino (*L. plantarum* strain N° 319, *L. paracasei* L.PRC502 and *L. rhamnosus* L.RHM501).

RAPD-PCR analysis

The primers M13 (5'-GAG GGT GGC GGT TCT-3'), RP (5' -CAGCACCCAC-3') and R5 (5'-AACGCGCAAC-3') were used for RAPD-PCR (Huey and Hall, 1989, Torriani et al., 1999). Conditions of PCR reactions and amplification were performed as described by Schillinger *et al.* (2003). Reactions were carried out in 25µl amplification mixtures with 12.5µl of 2x Master Mix (Fermentas, Burlington, Canada), 0.5µl of primer, 1µl of total DNA and 11µl of water.

The reaction mixtures with M13 primer, after incubation at 94°C for 2min, were cycled through the following temperature profile: 40 cycles 94°C for 60s, 42°C for 20s and 72°C for 2min. Final extension was carried out at 72°C for 10min.

The primer RP was used under the following amplification conditions: one cycle 94°C for 3min, 45°C for 45s, 72°C for 1min; 30 cycles 94°C for 45s, 45°C for 45s, 72°C for 1min; one cycle 94°C for 45s, 45°C for 45s, 72°C for 5min.

The reaction mixtures with R5 primer, after incubation at 94°C for 5min, were cycled through the following temperature profile: 40 cycles 94°C for 60s, 29°C for 90s and 72°C for 2min. Final extension was carried out at 74°C for 5min. The PCR was conducted in a Tpersonal Thermal Cycler (Biometra, Gottingen, Germany).

Agarose gel electrophoresis

Agarose at a concentration of 2% (w/v, Molecular Biology Certified) was used to separate PCR products. Electrophoresis was conducted in TAE 50X Buffer (242g of Tris base, 57.1ml acetic acid glacial, 100ml EDTA 0.5M, pH 8, in 1 liter of distilled water). DNA size marker (Boehringer XII) was used as standards. DNA bands were stained with ethidium bromide (GIBCO BRL Gaithersburg, USA) and then visualized and photographed under UV light using a Gel Doc EQ System (Biorad, Hercules, CA).

Results and discussions

Generic identification

One hundred and seven rod-shape, gram-positive, and catalase-negative bacteria were isolated from 32 samples of raw cow milk.

These isolates grew at 10°C and 45°C. None of them produced CO_2 from glucose. These characteristics suggest their classification as facultative heterofermentative lactobacilli.

Acid tolerance

Screening and selection of the 107 lactobacilli isolates under the acidic conditions using rapid selective method resulted in four groups. Sixty-six isolates out of the 107 tested demonstrated poor tolerances to acidic condition, 34 isolates showed good tolerance, 1 isolate demonstrated very good tolerance and 6 isolates presented excellent tolerance.

Table 1. *Survival (log 10cfu ml^{-1}) of selected lactobacilli isolates under acidic conditions after 5h of incubation in citric acid*

Isolates	Initial count (log ufc /ml)	Count after 5h (log ufc /ml)	Surviving percentage (%)
1RM	9.04	6.82	75.44
8RM	9.08	6.84	75.38
11RM	8.95	7.47	83.46
13RM	9.17	5.60	61.06
73RM	8.90	4.90	55.05
66RM	9.30	0	0
20RM	9.11	5.77	63.33
48RM	9.04	5.30	56.38
53RM	9.15	4,77	52.13
102RM	9.15	5.60	61.20
103RM	9.04	4.30	47.56
105RM	9.11	6.14	67.39
82RM	9.04	0	0
89RM	9.08	6	60.07
98RM	9.00	5.30	58.88
3V	9.08	6	66,07
18V	9.11	5.78	63.44
29V	9.00	6.58	73.11

Among the 41 lactobacilli isolates demonstrating at least good tolerance under the acidic conditions using rapid selective method, 18 best isolates were screened for their ability to tolerate acidic condition in citric acid, pH 3 after 5h (Table 1). Fifteen of these isolates demonstrated high tolerance to acidic conditions of pH 3 after 5h of exposure in citric acid at 37°C by showing surviving percentage greater than 50%. The highest resistance to acidic conditions was observed in isolate 11RM with maintenance levels of 83.46 after exposure to pH 3. Surviving percentage of one isolate (103RM) was below 50% after 5h of exposure in pH3. Two isolates (66RM and 82RM) lost their viability under exposure in citric acid pH 3 after 5h at 37°C. Additionally, the two methods used to screen isolates based on their tolerance to acidic conditions showed almost similar results. The fifteen isolates demonstrating surviving percentage greater than 50% were selected for the further investigations.

Bile salt tolerance

After exposure to acidic conditions, 15 selected acidotolerant lactobacilli isolates were assayed for bile salt tolerance (Table 2). All isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C. These isolates were further investigated for their safety properties including sensitivity to antibiotic, haemolysis and gelatinase activity.

Table 2. *Surviving percentage of lactobacilli isolates in MRS broth supplemented with 0.2% or 0.4% bile salts after 6h and 24h at 37°C*

Isolates	0.2% bile salts		0.4% bile salts	
	6h	24h	6h	24h
1RM	50.73	57.88	51.67	49.61
8RM	82.56	81.34	71.38	62.18
11RM	66.06	54.01	59.38	40.44
13RM	62.21	81.05	49.66	48.49
20RM	102.98	105.46	54.62	79.03
48RM	92.93	103.11	55.12	93.23
53RM	96.19	98.96	77.29	89.26
73RM	115.57	119.92	51.94	85.53
89RM	72.43	95.98	11.15	62.94
98RM	90.94	81.52	39.43	70.84
102RM	63.13	67.68	54.63	55.60
105RM	77.77	83.09	51.71	73.71
3V	41.53	51.69	70.81	82.31
18V	89.60	93.55	59.37	93.73
29V	101.00	95.46	60.65	95.28

Table 3. *Susceptibility of potentially probiotic lactobacilli isolates to antibiotics using the disc diffusion method*

Isolates	Diameter of inhibition zone in mm								
	P_{10}	Ap_{10}	Am_{10}	T_{30}	D_{25}	Ch_{30}	E_{15}	C_5	B_{25}
1RM	$28_{(S)}$	$31_{(S)}$	$30_{(S)}$	$23_{(S)}$	$22_{(S)}$	$25_{(S)}$	$21_{(S)}$	$0_{(R)}$	$18_{(S)}$
8RM	$26_{(S)}$	$28_{(S)}$	$32_{(S)}$	$22_{(S)}$	$19_{(S)}$	$29_{(S)}$	$22_{(S)}$	$0_{(R)}$	$17_{(S)}$
11RM	$27_{(S)}$	$25_{(S)}$	$30_{(S)}$	$21_{(S)}$	$20_{(S)}$	$25_{(S)}$	$21_{(S)}$	$0_{(R)}$	$16_{(S)}$
13RM	$27_{(S)}$	$26_{(S)}$	$32_{(S)}$	$21_{(S)}$	$20_{(S)}$	$24_{(S)}$	$24_{(S)}$	$0_{(R)}$	$17_{(S)}$
20RM	$26_{(S)}$	$29_{(S)}$	$29_{(S)}$	$20_{(S)}$	$18_{(S)}$	$24_{(S)}$	$26_{(S)}$	$0_{(R)}$	$15_{(I)}$
48RM	$30_{(S)}$	$29_{(S)}$	$31_{(S)}$	$25_{(S)}$	$24_{(S)}$	$26_{(S)}$	$25_{(S)}$	$0_{(R)}$	$13_{(I)}$
53RM	$27_{(S)}$	$30_{(S)}$	$32_{(S)}$	$20_{(S)}$	$21_{(S)}$	$25_{(S)}$	$24_{(S)}$	$0_{(R)}$	$16_{(I)}$
73RM	$32_{(S)}$	$34_{(S)}$	$37_{(S)}$	$25_{(S)}$	$26_{(S)}$	$32_{(S)}$	$33_{(S)}$	$0_{(R)}$	$24_{(S)}$
89RM	$30_{(S)}$	$32_{(S)}$	$35_{(S)}$	$27_{(S)}$	$26_{(S)}$	$28_{(S)}$	$27_{(S)}$	$0_{(R)}$	$17_{(S)}$
98RM	$25_{(S)}$	$27_{(S)}$	$33_{(S)}$	$22_{(S)}$	$22_{(S)}$	$25_{(S)}$	$25_{(S)}$	$0_{(R)}$	$17_{(S)}$
102RM	$33_{(S)}$	$37_{(S)}$	$36_{(S)}$	$25_{(S)}$	$26_{(S)}$	$29_{(S)}$	$35_{(S)}$	$0_{(R)}$	$25_{(S)}$
105RM	$38_{(S)}$	$36_{(S)}$	$38_{(S)}$	$24_{(S)}$	$24_{(S)}$	$27_{(S)}$	$32_{(S)}$	$0_{(R)}$	$18_{(S)}$
3V	$31_{(S)}$	$32_{(S)}$	$30_{(S)}$	$27_{(S)}$	$25_{(S)}$	$30_{(S)}$	$26_{(S)}$	$0_{(R)}$	$19_{(S)}$
18V	$27_{(S)}$	$34_{(S)}$	$30_{(S)}$	$22_{(S)}$	$17_{(S)}$	$25_{(S)}$	$24_{(S)}$	$0_{(R)}$	$17_{(S)}$
29V	$30_{(S)}$	$32_{(S)}$	$27_{(S)}$	$20_{(S)}$	$28_{(S)}$	$30_{(S)}$	$30_{(S)}$	$0_{(R)}$	$21_{(S)}$

Antibiotics (Disk potency): P_{10} : Penicillin G (10units) ; Ap_{10} : Ampicillin (10µg); Am_{10} : Amoxicilline (10µg); T_{30} : Tetracycline (30µg); Ch_{30} : Chloramphenicol (30µg); E_{15} : Erythromycin (15µg); D_{25} : Doxycycline (25µg); C_5 : Ciprofloxacine (5µg); B_{25} : Cotrimoxazole (25µg). (S): sensitive; (R): resistant; (I): intermediate

Resistance to antibiotics

Fifteen potentially probiotic lactobacilli isolates were subjected to antibiotic susceptibility testing using the agar diffusion method (Table 3). All of them were sensitive to penicillin, ampicillin, amoxicillin, erythromycin, tetracycline, chloramphenicol, and doxycycline. Three isolates (20RM, 48RM, 53RM) demonstrated intermediate resistance to cotrimoxazole. Notable observation is the resistance towards ciprofloxacin expressed by all isolates.

Haemolysis and gelatinase activity

The fifteen potentially probiotic lactobacilli isolates were tested for their haemolysis and gelatinase activity. All the isolates showed no positive haemolysis and gelatinase activity compared to the positive control strain of *S. aureus* ATCC 25923.

Table 4. *Inhibitory activity of potentially probiotic lactobacilli isolates*

Isolates	Indicator strains							
	LI	***SA1***	***SA2***	***SA3***	***EF***	***SM***	***EC1***	***EC2***
1RM	+	+	+	+	+	+	+	+
8RM	+	+	+	+	+	+	+	+
11RM	+	+	+	+	+	+	+	+
13RM	+	+	+	+	+	+	+	+
20RM	+	+	+	+	+	+	+	+
48RM	+	+	+	+	+	+	+	+
53RM	+	+	+	+	+	+	+	+
73RM	+	+	+	+	+	+	+	+
89RM	+	+	+	+	+	+	+	+
98RM	+	+	+	+	+	+	+	+
102RM	+	+	+	+	+	+	+	+
105RM	+	+	+	+	+	+	+	+
3V	+	+	+	+	+	+	+	+
18V	+	+	+	+	+	+	+	+
29V*	+	+	+	+	+	+	+	+

+: Diameter of inhibition zone ≥ 2mm; - : No inhibition; MDR: Multi Drug Resistant.

*Free-cell neutralized supernatant of the culture inhibited the growth of all indicator strains

LI: L. innocua ATCC 33090; *SA1: S. aureus* ATCC 25923; *SA2: S. aureus* ATCC 25922; *SA3:S. aureus (MDR)* Clinical isolate; *SM: S. mutans* DSM 20523; *EF: E. faecalis* ATCC10541; *EC1: E. coli* ATCC 13706; *EC2: E. coli (MDR)* Clinical isolate; *ST: S. typhi* ATCC 6539; *PA1: P. aeruginosa* ATCC 20027; *PA2: P. aeruginosa* ATCC 27853; *KP: K. pneumoniae* Clinical isolate.

DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ATCC: American Type Culture Collection, USA

Antimicrobial activity

Results for antimicrobial activity of fifteen safe probiotic lactobacilli isolates were as shown in Table 4. All isolates inhibited the growth of all pathogenic strains when agar spot method was used. The free-cell neutralized supernatant of 14 out of the 15 lactobacilli tested did not inhibit the growth of the tested pathogenic indicators. It was also noticed that, the neutralized free-cell supernatant from the culture of the isolate 29V inhibited the growth of all pathogenic indicators.

Bile salt hydrolase (BSH) activity

All the 15 isolates inhibiting the growth of pathogens, displayed BSH activity by providing the precipitation zone around colonies on plate assay. Four isolates exhibited medium BSH activity by demonstrating precipitation zone diameter of 12 to 15mm and eleven demonstrated high BSH activity by expressing precipitation zone diameter greater than 15mm.

Cholesterol assimilation

All the 15 isolates were tested for their ability to reduce cholesterol *in-vitro* in the presence of bile salts (Table 5). The amount of cholesterol assimilated ranged from 56.52 to 95.65%. Cholesterol assimilation of the isolates ranged from 16.20 to 37.23 mg of cholesterol per g of cells. The highest value of cholesterol assimilation was recorded in isolate 53RM.

Table 5. *Cholesterol assimilation of potentially probiotic lactobacilli isolates*

Isolates	% of cholesterol assimilated	mg of cholesterol assimilated per g of cells[a]
1RM	56.52	18.29 ±0.50
8RM	86.95	26.22 ±0,25
11RM	86.95	30,56 ±0.33
13RM	91.30	31.94 ±0.33
20RM	78.26	30.34 ±0.33
48RM	73.91	27.79 ±1.00
53RM	86.95	34.41 ±0.14
73RM	73.91	29.41 ±0.50
89RM	56.52	16.20 ±0.50
98RM	78.26	27.38 ±0.33
102RM	93.48	33.64 ±1.00
105RM	56.52	19.19 ±0.50
3V	95.65	32.63 ±0.50
18V	65.22	21.66 ±0.25
29V	82.61	29.66 ±0.33

[a]Results are presented as mean ± standard variation of triplicates

Table 6*: Origin and identification of lactobacilli isolates using API system*

Isolates	Origin of the isolates	API 5OCH Identification (% similarity)*
1RM	Raw cow milk from Santa Akum 1	*L. plantarum 99.9%*
8RM	Raw cow milk from Santa Akum3	*L. plantarum 99.9%*
11RM	Raw cow milk from Santa Akum4	*L. plantarum 99.9%*
13RM	Raw cow milk from Santa Akum 4	*L. plantarum 99.9%*
20RM	Raw cow milk from Bambui	*L. plantarum 99.9%*
48RM	Raw cow milk from Foto1	*L. plantarum 99.9%*
53RM	Raw cow milk from Foto1	*L. plantarum 99.9%*
73RM	Raw cow milk from Fotouni	*L. plantarum 72.5%*
89RM	Raw cow milk from Foto1	*L. plantarum 99.9%*
98RM	Raw cow milk from Fokoue2	*L. plantarum 99.9%*
102RM	Raw cow milk from Foto2	*L. plantarum 99.9%*
105RM	Raw cow milk from Foto2	*L. plantarum 72.5%*
3V	Raw cow milk from Fongo Tongo1	*L. plantarum 99.9%*
18V	Raw cow milk from Fongo Tongo2	*L. plantarum 99.9%*
29V	Raw cow milk from Fongo Tongo3	*L. plantarum 99.9%*

***:** The percentages following the scientific names of strains represent the similarities from the computer-aided database of the Apiweb™ API 50 CH V5.1 software.

Santa Akum1, Santa Akum3, Santa Akum4, Santa Akum4, Bambui, Foto1, Fotouni, Fokoue2, Foto2, Fongo Tongo3, Fongo Tongo2 and Fongo Tongo1 are the localities where samples of raw cow milk were collected.

Phenotypic identification

Fifteen isolates selected according to their good probiotic potential were identified at phenotypic level as *L. plantarum* using API 50 CHL technique (Table 6). These isolates were from samples of various origins. Furthermore, a genotypic method was essential to discriminate between strains.

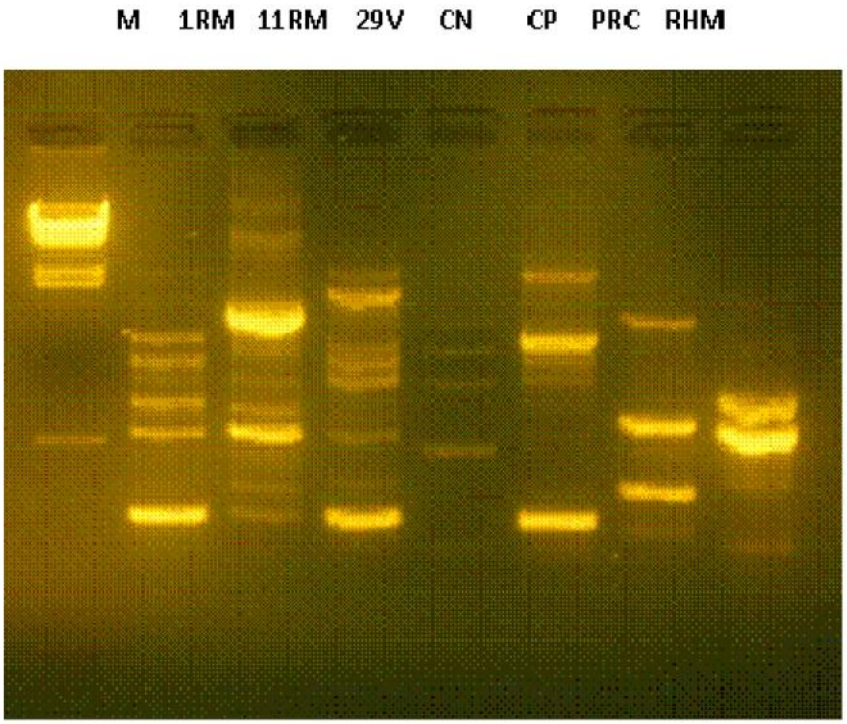

Figure 1: Agarose gel showing RAPD-PCR product profiles for primer M13 obtained from:

1RM: *L. plantarum* isolated in raw cow milk from Santa-Akum1 North West region- Cameroon

11RM: *L. plantarum* isolated in raw cow milk from Santa-Akum4 North West region- Cameroon

29V: *L. plantarum* isolated in raw cow milk from Fongo - Tongo3 West region- Cameroon

CN: negative control (ultra pure distilled water)

Genotypic identification

RAPD-PCR method was used to estimate the diversity between three potential probiotic strains of *L. plantarum* including 1RM, 11RM and 29V (Figure 1). *L. plantarum* 1RM and 11RM were selected for their high acidotolerance. Selection of *L. plantarum* 29V was based to its high acidotolerance and strong antimicrobial activity. Among the three primer used (M13, RP and R5), primer M13 was retained for its reproductivity. The profiles of RAPD-PCR products for primer M13 obtained from *L. plantarum* 1RM, 11RM and 29V differed from each other and from those of patented strains from Camerino University (*L. plantarum* N° 319, *L. rhamnosus* L.RHM501 and *L. paracasei* L.PRC502). These profiles are little similar to the one of *L. plantarum* N° 319. Therefore, strains of *L. plantarum* 1RM, 11RM and 29V constitute different new potentially probiotic lactobacilli.

Lactic acid bacteria have been consistently demonstrated to be responsible for the spontaneous lactic acid fermentation of homemade dairy products in Cameroon, where three genera have previously been identified: *Lactobacillus*, *Enterococcus* and *Lactococcus* (Zambou *et al.*, 2004). Zambou *et al.* (2008) demonstrated that strains of *Lactobacillus plantarum* constituted the dominant species of lactobacilli present in dairy products from western highland region of Cameroon. In our study, all the 107 isolates belonged to facultative hetero-fermentative group. Lactobacilli have the most claims to be selected among potential probiotics (FAO/WHO, 2002).

Probiotics are health-promoting microorganisms. The criteria used to select potential probiotics are related to acid and bile tolerance, production of antimicrobial substances, cholesterol metabolism, production of useful enzymes and safety for food and clinical use (Ouwehand *et al.*, 1999).

In vitro survival of bacterial strains in low pH is a more accurate indication of the ability of strains to survive passage through the stomach. The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999). The rapid and the classical screening techniques were the two methods used in this study to test the ability of *Lactobacillus* strains to tolerate gastric acidic conditions. Acid tolerance capacity of the selected isolates under these two conditions showed similar results indicating that the two techniques could be used individually in this purpose. Many scientific reviews demonstrated the capacity of *L. plantarum* strains to tolerate gastric acidic conditions. Kalui *et al.* (2009) demonstrated that all the 18 *L. plantarum* strains tested were tolerable to pH 2.5 after exposure for 3h and 10% of these strains could not at pH 2. Sirilun *et al.* (2010) reported that a viable rate of more than 90% of 43 out of 114 strains at pH 3 after 2h of incubation was found; at the pH 2 a surviving percentage that was higher than 50% could be observed in 27 strains. Many studies reported that tolerance to acid and other gastrointestinal stresses is strain specific (Morelli, 2000; Huang and Adams, 2004). For strains to survive and colonize the gastrointestinal tract, microorganisms should express tolerance to acid and bile salts (Gibson, 1998). It has been suggested that food intake could protect bacteria during gastric passage (Charalampopoulos *et al.,* 2002). The pH, physical and chemical characteristics of a food carrier in which potential probiotics are relayed into the gut may have a buffering effect and significantly influence survival of the microorganisms (Patel *et al.,* 2004).This may also explain why *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*, known to exhibit poor survival when challenged *in vitro* to gastric acidity, showed high survival rates in the terminal ileum of fistulated minipigs fed with yoghurt (Lick *et al.,* 2001). From these observations the question may arise whether resistance to gastric acidity in a ''non-food'' model is an obligatory prerequisite for a strain to survive the gastrointestinal transit, and what is the relevance of this functional property as undefined selection criterion for probiotic strains. It seems to make more sense to study the tolerance to gastric conditions with strains incorporated in the final product (e.g. yoghurt). There is therefore a need for consideration of inclusion of foods during *in vitro* characterizations for probiotic potential. In the present study, results suggest that these 41 selected isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. Additionally, the tolerance to gastric transit was also observed to be variable among strains tested.

Bile salt tolerance is the second selection criterion for probiotics. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to 700ml per day (Hoffman *et al.,* 1983). The relevant physiological concentrations of human bile range from 0.1 to 0.3% (Dunne *et al.,* 2001) and 0.5% (Mathara *et al.,* 2008). Thus, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v) (Šuškovi et al., 2000). Kalui *et al.* (2009) reported that 18 of the 19 *L. plantarum* tested were able to grow in broth supplemented with 0.3% bile salts following exposure to pH 2.5. Bile salt hydrolytic (BSH) activity may contribute to resistance of lactobacilli to the toxicity of conjugated bile salts in the duodenum and therefore is an important colonization factor (De Smet *et al.,* 1995). The deconjugation activity may play a role in maintaining the equilibrium of the gut microflora (Taranto *et al.,* 1996). It has been also suggested that bile BSH enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress (De Smet *et al.,* 1995). Schillinger *et al.* (2005) demonstrated that among lactobacilli isolated from probiotic yoghurts, BSH activity was only for the strains of the *L. acidophilus* group and not for *L. paracasei* and *L. rhamnosus*. In a recent study, 17 strains of *L. casei* (most probably *L. paracasei*) were found to grow in the presence of 0.5% bile without hydrolyzing the bile acids (Bertazzoni et al., 2004). Data from Moser and Savage (2001) indicate that bile salt hydrolase activity and resistance to bile

salts are unrelated in lactobacilli. In our investigations, high bile salt tolerance and BSH activity was observed for all *L. plantarum* strains tested. For a strain to be considered as probiotic, it should be able to survive at pH 3 and in the presence of 0.1% bile salt (Dunne *et al.,* 2001). According to this statement, 18 out of the 107 lactobacilli tested in this study could be considered as potentially probiotic strains.

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, the antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh, 2005). One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable resistance genes. A recent study reported that the lactobacilli isolated from commercial products in Europe comprised strains resistant to tetracycline (29.5%), chloramphenicol (8.5%), and erythromycin (12%) and overall, more than 68% of the isolates exhibited resistance to two or more antibiotics (Temmerman *et al.,* 2003). Rojo-Bezares *et al.* (2006) reported that the most resistant species to the tested antibiotics were *L. plantarum* and *P. pentosaceus*; these authors also demonstrated that, *Lactobacillus* strains showed high minimum inhibitory concentration (MIC) values (indicating high resistance) to ciprofloxacin [MIC of 50 to 64 µg/ml]. Similar results were previously reported by Elkins and Mullis (2004). Glycopeptide, aminoglycoside and sulfamethoxazole resistance has been formerly described in LAB species (Mathur and Singh, 2005), and in all cases it has been associated with their natural and intrinsic resistance, probably due to cell wall structure and membrane impermeability, complemented in some cases by potential efflux mechanisms (Elkins and Mullis, 2004). Liasi *et al.* (2009) demonstrated that, the 3 lactobacilli isolates tested (including one strain of *L. plantarum*) were susceptible to β-lactam group of antibiotic which include penicillin G, amoxicilline and ampicillin. The isolates were also susceptible to erythromycin, chloramphenicol and tetracycline. Furthermore, all isolates were also resistant to quinolones and sulfonamides. Herreros *et al.* (2005) demonstrated that in general, strains of lactobacilli showed significant resistance to ciprofloxacin and trimethoprim. In our investigations, almost all tested isolates were sensitive to 8 of the 9 antibiotics used. This could be due to limited use of antibiotics among the Mbororo community on livestock husbandry. Resistance of all *L. plantarum* strains to ciprofloxacine could be due to their natural and intrinsic resistance, probably due to the cell wall structure and membrane impermeability, complemented in some cases by potential efflux mechanisms (Ammor *et al.,* 2007).

Safety is one of the recommended attributes in the FAO/ WHO (2002) guidelines on evaluation for probiotics. The mucoid lining constitutes the target across which many physiological substances are exchanged. Haemolysis activity would break down the epithelial layer while gelatinase activity would derange the mucoid lining. These impairments interfere with the normal functioning of these very important linings and would cause pathways for infections. Absence of haemolytic and gelatinase activity is a selection criteria for probiotic strains, indicating that these bacteria are none virulent (De Vuyst *et al.,* 2003). Kalui *et al.* (2009) reported the situation where all the *L. plantarum* strains tested were haemolytic-negative and gelatinase-negative. Similar results were published by Mami *et al.* (2008) for 8 strains of *Lactobacillus*. This study showed that *L. plantarum* isolated from raw cow milk in the western highlands of Cameroon were haemolytic-negative and gelatinase-negative. They may therefore be considered as safe with regard to these activities.

Different reports showed that most lactobacilli strains produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages. In general, the antimicrobial activity of lactobacilli may be due to organic acids, hydrogen peroxide, bacteriocins or other inhibitory substances from metabolites (Kuwaki *et al.,* 2002; Testa *et al.,* 2003; Todorov et al., 2010). In this study, observed growth inhibition on agar-spot plates indicates that the assayed lactobacilli produced antimicrobial

products such as organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins that were able to inhibit growth of *L. innocua*, *S. aureus*, *S. mutans*, *E. faecalis*, *E. coli*, *S. typhi*, *P. aeruginosa*, *K. pneumoniae*, all of which are food contaminants and pathogens. Furthermore, the absence of antimicrobial activity in neutralized free-cell supernatant of 14 out of the 15 strain indicates that the growth inhibition observed on agar-spot plates was due to organic acid production by lactobacilli strain. The inhibitory effects of *L. plantarum* 29V, whose free-cell supernatant pH7 showed activity could be due to other antimicrobial products except organic acid. Varadaraj and Manjrekar (1993) observed moderate inhibition on some food borne pathogens and other bacterial species by neutralized culture filtrates of LAB using a well diffusion assay. McLean and McGroarty (1996) also showed that about 60% of the antimicrobial activity of culture filtrates of LAB was lost when the filtrates were neutralized to pH 6.5 with NaOH. Herreros *et al.* (2005) demonstrated that none of the strains tested was active against *L. monocytogenes* CECT 4031, *S. aureus* CECT 240, *E. faecalis* CECT 481 or *C. tyrobutyricum* CECT 4011, in an agar well diffusion assay after prior treatment of the extracts with catalase. Todorov *et al.* (2010) reported that thirty colonies of *L. plantarum* inhibited the growth of *E. faecium* HKLHS imbedded in MRS agar. Sirilun *et al.* (2010) showed that 4 strains of *Lactobacillus* sp. had ability against all the seven microbial indicators; the antagonistic activities of these strains were effective in all fractions including normal supernatant, neutralized supernatant 7.0 and neutralized supernatant containing catalase. De Waard *et al.* (2002) reported that antimicrobial activities of the tested *Lactobacillus* spp. Had broad inhibitory spectrum, against yeast and bacteria both of gram-negative and gram-positive. Further studies should be done on the strain *L. plantarum* 29V in order to determine the nature of the antimicrobial compound other than organic acid produced by this strain.

Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease (Pereira *et al.*, 2003). Although therapeutic drugs are available to relieve this problem, they are often expensive and can have side effects. Several studies indicated that *Lactobacillus* species were able to reduce cholesterol via several mechanisms including bile salt deconjugation (Liong and Shah, 2005).

Another phenomenon related to the presence of the deconjugation activity is the reduction of serum cholesterol (Corzo and Gilliland, 1999). Dashkevicz and Feighner (1989) demonstrated that bile salt hydrolase activity was measured in 71% of the *Lactobacillus* cultures tested, representing both homo- and heterofermentative species. Ramasamy *et al.* (2009) reported that all the 12 *Lactobacillus* strains tested were able to deconjugate bile salt *in vitro* but the level of deconjugation differed significantly among the strains. Recently, Sirilun *et al.* (2010) demonstrated that among the 4 lactobacilli strains tested, BSH activity was observed in 2 strains which were identified as *L. plantarum*. In this study, all the lactobacilli strains tested expressed BSH activity.

Other hypocholesterolemic mechanism(s) of lactobacilli may be involved in the removal of cholesterol from growth media. The removal of cholesterol by lactobacilli *in vitro* could be due to an uptake or assimilation of cholesterol by bacterial strains. Liong and Shah (2005) demonstrated that a portion of the cholesterol assimilated by *Lactobacillus* strains was incorporated into the cellular membrane. Hyeong *et al.* (2004) reported that strains of lactobacilli tested removed 31.5 to 58.5% cholesterol in the growth medium. Ramasamy *et al.* (2009) reported that all the 12 *Lactobacillus* strains tested were capable of removing cholesterol from the growth medium after 20h of incubation, but the percentage of cholesterol removal varied considerably among the strains (26.74 to 85.41% cholesterol). Sirilun *et al.* (2010) demonstrated that the four *L. plantarum* isolated from food origins were considered as the effective probiotics with cholesterol-lowering property capable of reducing 25.41% to 81.46% from the growth medium after 24h of incubation. Dora and Glenn (2002) demonstrated that some strains of lactobacilli tested express cholesterol assimilation capacity of 0.09 to 29.73 mg cholesterol/g cell. Cholesterol assimilation is widely influenced by the growth rate in the

medium (Dora and Glenn, 2002). In our study, all the 15 tested strains of *L. plantarum* removed 56.52 to 95.65% cholesterol from the growth medium after 18h of incubation and demonstrated cholesterol assimilation capacity of 16.20 to 34.41mg of cholesterol per g of cells.

Quality assurance programmes associated with research, development, production and validation of the health benefits of probiotic *Lactobacillus* require their relevant identification using modern techniques. Several combinations of tests and ready-to-inoculate identification kits such as API 50 CH, enzymatic tests can be used for the rapid and theoretically reproducible phenotypic identification of pure cultures. They have been used for the characterization and identification of lactobacilli in milks (IDF, 1995; Zambou *et al.,* 2004). RAPD-PCR has been used to monitor population dynamics in food fermentation and to estimate the diversity of *Lactobacillus* strains. In this study, RAPD-PCR enables to distinguish between three potential probiotic strains of *L. plantarum* strains (1RM, 11RM and 29V) and patented lactobacilli strains (*L. plantarum* strain N° 319, *L. paracasei* L.PRC502 and *L. rhamnosus* L.RHM501).

Conclusion

Consequently to our investigations, it could be noticed that, the *Lactobacillus* strains associated with dominant microflora that people from Mbororo's tribe in the western highlands of Cameroon use to manufacture fermented milk contain new potentially safe probiotic strains with antimicrobial and cholesterol-lowering properties.

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