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FERMENTATION OF *KIVUGUTO*, A RWANDESE TRADITIONAL MILK: SELECTION OF MICROBES FOR A STARTER CULTURE

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Résumé

390 souches bactériennes ont été isolées à partir de quatre échantillons du lait *kivuguto*. Les isolats ont été sélectionnés par des méthodes phénotypiques et des techniques moléculaires par la PCR et le séquençage de l'ADNr 16S et / ou de la région intergénique 16S-23S ADNr (ITS). L'acidification du lait et la croissance dans des conditions environnementales extrêmes ont aussi été examinées pour les souches pré-sélectionnées. Des essais de fermentation avec des souches mixtes ont été effectués pour identifier les formulations microbiennes qui produisent du lait similaire au *kivuguto*. Afin de détecter des défauts organoleptiques et plus particulièrement l'apparition d'amertume, chaque lait formulé a été conservé sous réfrigération pendant 24 jours pour évaluer la durée de vie. Au cours de ces analyses, les bactéries identifiées sont les suivantes: *Lactococcus lactis* (souches CWBI-B1466 et CWBI-B1469) et *Leuconostoc mesenteroides subsp. mesenteroides* (souches CWBI-B1467 et CWBI- B1465) et *Leuconostoc pseudomesenteroides* (souches CWBI-B1468, des CWBI-B1470 et CWBI-B1471). Un *Leuconostoc* sp. a montré sa tolérance à un faible pH 2.5, à une concentration élevée en chlorure de sodium (jusqu'à 6,5%) et était résistante à une température élevée (55°C). Ces expériences ont montré que le *kivuguto* coagule en 8 heures à 30°C. Trois formulations de lait produit en utilisant des souches mixtes ont donné de bons résultats. La meilleure formulation suivant le critère goût et la stabilité sous réfrigération (35 jours) a été réalisée par un mélange de deux *Leuconostoc* spp. et *Lactococcus lactis*. Ces souches donnent au *kivuguto* ses propriétés caractéristiques.

***Mots clés**: kivuguto, séquençage, Lactococcus, Leuconostoc*

Abstract

A total of 390 bacterial strains were isolated from four *kivuguto* samples. Isolates were screened by phenotypic methods and molecular techniques using the PCR and sequencing of 16S rDNA and/or 16S-23S rDNA intergenic transcribed spacer (ITS) region. The rate of milk acidification and ability to grow under extreme environmental conditions were also examined for pre-selected isolates. Fermentation assays with mixed strains were performed to identify microbial formulations that produced milk similar to *kivuguto.* Such formulated milk was stored at 4°C on 24 days for shelf-life analysis to detect any organoleptic defect. Bacteria identified in *kivuguto* included *Lactococcus lactis* (strains CWB-B1466 and CWBI-B1469) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (strains CWBI-B1467 and CWBI-B1465) and *Leuconostoc pseudomesenteroides* (T) (NRIC177) (strains CWBI-B1468, CWBI-B1470 and CWBI-B1471). One *Leuconostoc* sp. grew at low pH 2.5, in high sodium chloride concentration (up to 6.5%) and was resistant to heat (55°C). Fermentation experiments showed that *kivuguto* coagulates in 8 hours at 30°C. Three milk formulations produced using mixed strains gave good results. The best-tasting and longest shelf-life (35 days) *kivuguto* was produced by a mixture of two *Leuconostoc* spp. and *Lactococcus lactis.* These strains give *kivuguto* its characteristic properties.

***Keywords:** kivuguto, sequencing, Lactococcus, Leuconostoc*

ملخص

تم عزل 390 سلالة بكتيرية من أربع عينات من حليب kivuguto . تم إنتقاء هذه العزلات بواسطة طرق الدراسة المظهرية و التقنيات الجزيئية عن طريق الPCR وتسلسل rDNA-S16 أو بين الجينات منطقة S23-S16 rDNA (ITS). وبحث أيضا تحمض الحليب والنمو في الظروف البيئية القاسية للسلالات المختارة. أجريت تجارب تخمر مع سلالات مختلطة لتحديد الهيغ الميكروبية التي تنتج حليب مماثل لkivuguto. للكشف عن العيوب الحسية وخصوصا ظهور المرارة، تم تخزين كل صيغ الحليب تحت التبريد لمدة 24 يوما لتقييم مدة الحياة. في هذه التحليلات ، البكتيريا التي تم تحديدها هي : *Lactococcus lactis* (سلالات B1466-CWBI و B1469-CWBI), *Leuconostoc mesenteroides* subsp. *mesenteroides* (سلالة B1467-CWBI و B1465-CWBI) و *Leuconostoc pseudomesenteroides* (سلالة B1468 - CWBI، B1470-CWBI، و CWBI - B1471). واحد من *Leuconostoc* sp. أظهر سماحية لانخفاض الرقم الهيدروجيني (2.5) و نسبة عالية من كلوريد الصوديوم (بزيادة تصل إلى 6.5٪) وكانت مقاومة لهرجات الحرارة المرتفعة (55 درجة مئوية). أظهرت هذه التجارب أن تخثر kivuguto تم في 8 ساعات عند 30 درجة مئوية. ثلاث صيغ من الحليب المنتج باستخدام سلالات مختلطة كانت ناجحة واستنتج أن أفضل صيغة من حيث معيار الذوق والاستقرار تحت التبريد (35 يوما) بواسطة مزيج بين إثنين من *Leuconostoc* spp. و*Lactococcus lactis*. هذه السلالات تعطي لحليب kivuguto خصائصه المميزة.

***الكلمات المفتاحية:** kivuguto، الطرق المظهرية، PCR، تحديد التسلسل، rDNA-S16، S23-S16، rDNA (ITS)، التخمر، Lactococcus، Leuconostoc*

Fermentation has been used for many years to preserve milk in a number of countries, including Rwanda. *Kivuguto* is the term for traditional rwandese fermented cow's milk. It is a very consistent and rich dairy product with high nutritional value. Fermentation is carried out spontaneously by natural milk microflora and environmental contaminants without the addition of any starter culture [1]. During fermentation, milk microorganisms produce a large number of metabolites such as amino acids, vitamins and minerals. The reported health benefits of such microorganisms include, among others, boosting the immune system and inhibiting the growth of pathogens and preventing diarrhoea from various causes [2].

Rwandese local markets are dominated by traditional milk. However, Rwanda has five milk-processing plants (Inyange Industries, Nyanza Dairy, Eastern Savannah, Rubirizi Dairy and Masaka Farms). Although modern dairies put other types of fermented milk on the Rwandese market, artisanal *kivuguto* is popular, because consumers prefer locally-made natural fermented milk to fermented milk made using an imported mesophilic starter culture [3]. This preference for naturally fermented milk led researchers to isolate and characterize wild strains able to produce milk with similar sensorial properties to those of traditional products [4]. Therefore, an investigation was begun to identify those characteristics in traditional *kivuguto* in order to meet the needs of the Rwandese and regional marketplaces. This will allow standardization of quality without changing the fundamental properties of *kivuguto.* Artisanal *kivuguto* is made at the household level using raw milk. This makes the final product very variable and, as reported Tornadijo *et al.* [5], the lack of uniformity limits its acceptance and distribution on the market.

The purpose of this work definitely was to develop a starter culture from microbial strains selected from traditional *kivuguto*, with the ultimate objective of reproducing its major properties in dairy. In the present paper, we report the results of screening tests of isolates involved in *kivuguto* fermentation and the results of fermentation assays designed to identify a formulation with characteristics similar to *kivuguto*. To date, there are no reports of dairy starter formulations developed using this approach and no other report of microbial and technological study of *kivuguto* milk.

MATERIALS AND METHODS

Sampling and origin of strains

Prior to choosing the samples for this work, a short survey was conducted at two sites in order to determine which households produce high quality *kivuguto*. Sites selection was based on geography, ecology and the absence of exotic fermented milk in the area. Samples of *kivuguto* were collected in february and september at two sites in Rwanda: the Huye District in the southern province (Sample S) and the Musanze District in the northern province (Sample N). Sample preparation followed IDF Standard 122C [6].

Isolation and enumeration

Media were prepared according to IDF Standard 149A [7]. Decimal dilution of samples followed IDF Standard 122C [6]. Eight selective and differential media were inoculated with 100 µl of diluted sample, prepared with 0.1% peptone water. The culture conditions are listed in **Table 1**.

Microorganisms were enumerated using the spread-plate method. Each experiment was carried out in triplicate. Single colonies (10 or 20) were picked randomly from each plate and subjected to Gram staining and assay for catalase activity. Gram staining followed the manufacturer's instructions (*BioMérieux, Marcy-l'Etoile, France*). Catalase activity was assayed by placing a drop of hydrogen peroxide solution on the colony. The presence of catalase was indicated by the formation of bubbles on the colony. Thereafter, Gram-positive and catalase-negative colonies were purified three times on MRS agar containing $CaCO_3$ for presumptive bacteria. Pure colonies were then stored at -80°C in Nalgene® cryogenic vials (Thermo Fisher Scientific Inc., Rochester, New York, USA) in 1 ml of 40% (v/v) glycerol.

Phenotypic and genotypic characterisation

The colonies and cells of dominant isolates (Gram-positive and catalase-negative) were initially analyzed through observations. Five colonies from each dominant isolate group were analyzed using an API 50®CH gallery kit (*BioMérieux Marcy-l'Etoile, France*), according to the manufacturer's instructions. Each dominant group was defined by the medium used for isolation. The API 50®CH galleries used API 50®CHL medium to identify isolates by characterizing their ability to ferment 49 carbohydrates.

The database associated with APILAB PLUS V3.2.2 (*BioMérieux*) software was used to aid in the identifications. Strains were therefore grouped based upon comparison of their API 50®CHL profile to that of organisms listed in the database. The 16S rDNA and/or 16S-23S rDNA intragenic spacer (ITS) regions of pre-selected strains were sequenced after a polymerase chain reaction (PCR) according to the method of Drancourt *et al.* [12]. The following primers were used for PCR of 16S rDNA and 16S-23S rDNA: 16SPO/16SP6 and ITS R/ITS F, respectively. Amplifica- tions were performed following the Fermentas Taq DNA Polymerase Protocol (Fermentas Life Sciences, Maryland, USA) in a My Cycler™ thermal cycler (Bio-Rad Laboratories, Inc., California, USA). The BigDye Terminator v1.1 Cycle Sequencing Kit Protocol [13] was used for the sequencing reactions with 8 primers for the 16S DNA and/or ITS R and ITS F for the 16S-23S rDNA ITS **(Table 2).** The data were analyzed using ABI

Genetic Analyzer Sequencing Analysis v5.2 software. The 16S rRNA and/or the 16S-23S rRNA ITS sequences obtained were submitted for BLAST searching (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) for comparison with sequences available in the GenBank databases.

Effect of temperature, pH and sodium chloride on growth

The ability to grow at 10, 15, 37, 44, and 55°C in MRS for *Leuconostoc* strains and in M17 broth for *Lactococcus* strains was evaluated. Growth was also analyzed at pH 3, 4, 7, and 9. Media were adjusted to the desired pH using NaOH (3 N) or HCl (3 N). Salt tolerance was determined by culturing each isolate in MRS broth containing 2%, 4% or 6.5% NaCl. At the end of the incubation time, changes in the color and turbidity of cultures were visually recorded as a simple indication of growth. Each treatment was repeated in 3 separate experiments.

Acidification of milk

To prepare the pre-culture, 150 ml of sterile MRS broth was inoculated with each strain thawed from the stock culture, followed by streaking onto solid MRS. Fermented milk was prepared by inoculating 150 ml of Joyvalle UHT milk with the overnight (about 16 h) MRS broth pre-culture to a final concentration of 2% (v/v) in a 250 ml flask. The pH of the milk was determined every 4 h and the acidity was evaluated by titrating a 10 ml sample with NaOH (1/9N) using phenolphthalein as an indicator. This experiment was done in triplicate.

Fermentation with mixed strains and evaluation of storage stability

Flasks containing 150 ml of sterile Joyvalle milk were inoculated to 2% (v/v) with a mixture of overnight MRS broth cultures of strains that had been cultured separately and then mixed prior to inoculation. Milk was inoculated with two strains from the dominant Sample N groups and a single strain of flavoring bacteria. After coagulation, the fermented products were tasted to determine which microbial mixture produced a flavor similar to *kivuguto*. A total of four strains were used for the Sample S in the same manner. We also examined the effect of mixing two strains from Sample N milk with a single strain from Sample S milk. Milk formulations that tasted similar to *kivuguto* prepared using mixed strains were stored at 4°C for 24 days to examine their stability. Development of organoleptic defects, especially bitterness, was evaluated by tasting casually the milk on days 1, 8, 16 and 24. No trained taste panel or special taste analyses were employed for this experiment at this stage. Taste was evaluated by two individuals who were very familiar with the characteristics of *kivuguto*. A sensorial evaluation will follow the technological analyses in the next phase of this work. Two reference samples were used in the stability experiment: plain yogurt and Nyanza fermented milk made by the Nyanza Dairy in Rwanda.

Species interactions

To assess interactions between species during fermentation, milk samples were inoculated with three strains isolated from Sample N either individually or in co-culture. Each strain was pre-cultured overnight in MRS broth from colonies taken from cultures grown on MRS agar. In order to standardize the inocula, 50 ml of each pre-culture was centrifuged for 20 min at 7333 x *g*. The pellet was prepared to adjust cultures to the same concentration and resuspended in 50 mL of milk containing 10% glycerol and stored at -20°C for later use in initiating fermentation. Milk samples (150 ml of Joyvalle milk) were inoculated with the pre-cultures to a final concentration of 2% (v/v). The pH was recorded at the start (T_0) and the end (T_1) of fermentation. Lactic acid bacteria (LAB) populations were differentiated and enumerated at T_0 and T_1 by plating an aliquot of each milk sample onto tomato juice agar, Rogosa agar, and Mayeux agar with appropriate dilutions. Plates were incubated as described in **Table 1**.

RESULTS

Isolation and enumeration of microorganisms

The pH and coagulation time of various milk samples was evaluated during preliminary analyses. The average pH of raw milk samples we tested was 6.5-6.6, while the average pH of fermented milk was about 4.5. A total of 390 isolates were obtained from the milk samples we collected. The isolates were purified on MRS agar with $CaCO_3$ and then stored in glycerol at -80°C. Enumeration analyses showed that dominant strains grew on selective media such as tomato juice agar (3.57×10^7 cfu ml^{-1}), Rogosa (4.27×10^7 cfu ml^{-1}), Rogosa-raffinose (0.40×10^7 cfu ml^{-1}), and MRS (0.40×10^7 cfu ml^{-1}). Strains isolated on Mayeux agar (0.11×10^7 cfu ml^{-1}) were pre-selected for the flavor characteristics of *kivuguto* even if they were in low concentration.

Phenotypic and molecular characterization

In the first and the second samplings, all isolates were found to be *cocci*, Gram-positive, catalase-negative, and were therefore presumed to be LAB. Yeasts were present only at very low levels in all samples. About five similar dominant strain colonies were picked from each medium and subjected to API 50® CH testing. Colonies producing identical profiles were considered to be isolates of the same strain. However, a given strain could produce several profiles, differing only with respect to fermentation of 1 or 2 carbohydrates. API 50® CH testing of the microorganisms isolated during the first sampling (february) produced 7 profiles for Sample N and 5 profiles for Sample S. The

profiles produced by the Sample N isolates resulted in the identification of *Lactococcus lactis* ssp. *lactis* 1 (95.4%), *Lactobacillus helveticus* (68.1%) and *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 2 (65.6%). The profiles produced by the Sample S isolates resulted in the identification of *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 2 (99.7%), *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 1 (95.7%) and *Leuconostoc lactis* (52.9%). A total of 7 profiles for Sample N and 5 profiles for Sample S were also obtained upon API 50® CH testing of microorganisms isolated during the second sampling (september). Analysis of Sample N resulted in identification of *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 2 (55.4%), *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 1 (99.9%; 69.4%; 52.5%) and *Lactobacillus paracasei* subsp. *paracasei* 1 (92.4%). In Sample S, *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 2 (99.9%; 90.3%; 85.8%) and *Lactococcus lactis* subsp. *lactis* 1 (87.4%) were identified. To confirm our phenotypic identifications, the 16S rDNA gene and/or 16S-23S rDNA ITS of pre-selected strains were sequenced using PCR **(Table 3).** Sequencing indicated that the 16S rDNA of strain CWBI-B1466 had a high degree of identity (99%) with that of *Lactococcus lactis* subsp. *lactis* AB100805.1 (ATCC 13675). The strain was therefore identified as *Lactococcus lactis* and registered in GenBank under accession number JF313446. Strain CWBI-B1469 was assigned as *Lactococcus lactis* subsp. *lactis* AB008215.1 (97% identity) and the rDNA sequence was registered in GenBank under accession number JF313446. Strain CWBI-1467 was assigned as *Leuconostoc mesenteroides* subsp. *mesenteroides* CP000414.1 (97% identity) and was registered in GenBank under accession number JF313452. Strain CWBI-B1468 was 98% similar to strain *Leuconostoc pseudomesenteroides* AB290443.1. The accession number is JF313453. Strain CWBI-B1470 closely resembled to *Leuconostoc pseudomesenteroides* AB290443.1 (98% identity) and was registered in GenBank under accession number JF313454, while strain CWBI-B1471 was found to be comparable to *Leuconostoc pseudomesenteroides* AB290443.1 (99% identity) and was registered in GenBank under accession number JF313455. Finally, strain CWBI-B1465 may be *Leuconostoc mesenteroides* subsp. *mesenteroides* (GenBank accession number JF313445).

Effect of temperature, pH and sodium chloride on growth

The strains isolated from *kivuguto* were generally resistant to extreme environmental conditions (Table 4). All isolates grew in broth containing 4% NaCl. *Lactococcus* strains could not grow at 45°C. The viability of *Leuconostoc mesenteroides* strain CWBI-B1465 at 55°C was determined after analysis of its growth at 44°C and the residual viable count was determined at pH 2.5 and pH 3. The viable count was determined by dilution and plate counting on MRS agar before and after 4 h incubation in MRS broth under one of the specified conditions (55°C, pH 2.5, pH 3). The survival rate at 55°C and pH 2.5, 3, and 4 were 38.63% 25%, 61.76% and 97.36%, respectively.

Acidification, milk formulation and storage stability

Strains used for acidification of milk were taken from dominant groups belonging to different profiles. In the first pre-selection, we identified those strains that produced the most rapid acidification. Two *Lactococcus* strains isolated from Sample N were rapid acidifiers (8 hours), as was a *Lactococcus* strain isolated from Sample S (9 hours). Two *Leuconostoc* spp. isolated from sample N were not fermentative when cultured alone in milk. Two *Leuconostoc* spp. isolated from Sample S coagulated milk in 14 hours, while 2 others acidified milk in 16 and 20 hours.

Milk was fermented with mixed strains and then tasted casually to determine the mixture of organisms that best reproduced the taste of authentic *kivuguto*. The fermented formulations were then stored at 4°C for up to 24 days to test stability. In that preliminary study, we obtained milk formulations that were fermented in only 8 hours by mixed strains from Sample N and one of the formulations presented a bitter taste after 15 days of storage. Based on these data, the strain of *Lactococcus* that was used was eliminated for further investigations. One association of organisms isolated from Sample S acidified milk in 9 hours. Another formulation failed the test of shelf-life, because one of the *Leuconostoc* sp. involved was not viable in acidic milk. When used alone, this species coagulated the milk in 14 hours. But such milk can't coagulate another raw milk. Another strain was also discarded because the milk formulation exhibited changes in flavor after only 1 week. Two strains from Sample N in association with the best acidifier identified from among the *Leuconostoc* spp. isolated from Sample S made a very tasty *kivuguto*, with no organoleptic defects detected up to 35 days. These results were reproducible in 3 replicated experiments.

Species interactions

Due to the presence of non-fermentative dominant species in Sample N, an examination of their interactions with the CWBI-B1466 *Lactococcus* strain was conducted to highlight their high biomass in the fermented milk. This examination revealed that the 2 non-fermentative *Leuconostoc mesenteroides* do not grow in milk when cultured singly. Instead, a high growth rate was observed for *Leuconostoc mesenteroides* strain CWBI-B1465 in co-culture. A similar result was obtained for *Leuconostoc mesenteroides* strain CWBI-B1467. A positive interaction thus occurs between *Lactococcus* and *Leuconostoc* species (detailed results not shown).

Table 1: Culture conditions, isolation and differentiation of microorganisms of *kivuguto.*

Enumeration media	Presumptive targeted microorganisms	Temperature of incubation (°C)	Duration of incubation (hours)	Incubation condition	pH
[1]**M17 agar**	total *lactococci*	30	48-72	Aerobiosis	7,15
Turner agar (arginine tetrazolium agar)	differentiation of *Lactococcus lactis ssp. lactis and L. lactis ssp. lactis bv. diacetylactis*	30	48-72	Anaerobiosis	6,0
Tomato juice	differentiation of *L. lactis ssp. lactis bv. diacetylactis*	25	72	Anaerobiosis	6,1
[2]**Mayeux agar**	total *Leuconostoc*	25	120	Aerobiosis	-
[3]**Rogosa agar**	total *Lactobacilli*	30	72	Anaerobiosis	5,4
Rogosa raffinose agar	differentiation of *Lb. plantarum*	30	72	Anaerobiosis	5,4
[4]**MRS agar**	differentiation of *Lb. acidophilus*	37	72	Aerobiosis	6,1-6,4
[5]**PDA**	Yeasts	30	48	Aerobiosis	5,6

[1]The modified M17 agar [8] was prepared for the isolation of cocci (*Lactoccus* and *Streptococcus*); [2]Mayeux agar [9] for *Leuconostoc* spp., is an elective medium for the detection and enumeration of *Leuconostoc* in milk, dairy products and sweet foods. It was supplemented with sodium azide and sucrose. Sodium azide was used for inhibition of *Lactococci* and Gram negative bacteria. *Leuconostoc mesenteroides subsp. mesenteroides* and *Leuconostoc mesenteroides subsp. dextranicum* utilize the sucrose in the medium to synthesize polysaccharides (dextrans) which impart a gelatinous appearance to the colonies; [3]Rogosa agar [10] targeted all *Lactobacilli*. However, glucose was replaced by raffinose for presumptive *Lactobacillus plantarum* in Rogosa raffinose agar; [4]MRS [11] was modified and incubation was made in the conditions of *Lactobacillus acidophilus* [7]. [5]Potato dextrose agar (PDA) was used for yeasts.

Table 2: Oligonucleotides primers, length and function used for PCR and sequencing

Primers	Sequence	Technique	Sense	Source
16SPO	5'-AAGAGTTTGATCCTGGCTCAG-3'	PCR	Forward	[14]
6SP6	5'-CTACGGCTACCTTGTTACGA-3'	PCR	Reverse	[14]
ITS R	*5'-GTCCTTCATCGGCTBYTA-3'	sequencing	Reverse	-
ITS F	5'-TACACACCGCCCGTCAC-3'	sequencing	Forward	-
F1	*5'-CTGGCTCAGGAYGAACG-3'	sequencing	Forward	Sigma-Proligo
F2	*5'-GAGGCAGCAGTRGGGAAT-3'	sequencing	Forward	Sigma-Proligo
F3	*5'-ACACCARTGGCGAAGGC-3'	sequencing	Forward	Sigma-Proligo
F4	*5'-GCACAAGCGGYGGAGCAT-3'	sequencing	Forward	Sigma-Proligo
R1	5'-CTGCTGGCACGTAGTTAG-3'	sequencing	Reverse	Sigma-Proligo
R2	*5'-AATCCTGTTYGCTMCCCA-3'	sequencing	Reverse	Sigma-Proligo
R3	5'-CCAACATCTCACGACACG-3'	sequencing	Reverse	Sigma-Proligo
R4	*5'-TGTGTAGCCCWGGTCRTAAG-3'	sequencing	Reverse	Sigma-Proligo**

***** Degenerated sequences.
** Synthetic primers used for sequencing were deduced from alignment of 16S rDNA genes coding for bacterial RNA collected from EMBL (European Molecular Biology Laboratory) databases and have been supplied by Sigma-Proligo.

Table 3: API50 CHL Test vs molecular characterisation of bacterial species selected in *kivuguto* milk

Strains	API 50CHL identification			16S rDNA Sequencing			16S-23S rDNA ITS Sequencing		
	Similar Species	% of identity	Sequence length (bp)	Accession Number	Similar species*	% of identity	Similar species	Accession Number	% of identity
CWBI-B1465	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	65.6%	1436	AB362705.1	*Ln. mesenteroides subsp. mesenteroides*	98%	-	-	-
CWBI-B1466	*Lactococcus lactis subsp. lactis1*	95.4%	1512	AB100805.1	*Lactococcus lactis*	99%	-	-	-
CWBI-B1467	*Lactobacillus helveticus*	68.1%	578	CP000414.1	*Ln. mesenteroides subsp. mesenteroides*	98%	*Ln . mesenteroides subsp. mesenteroides*	CP000414.1	97%
CWBI-B1468	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	91.9%	1422	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	98%
CWBI-B1469	*Lactococcus lactis subsp. lactis1*	87.4 %	1550	AB008215.1	*Lactcoccus lactis*	97%	-	-	-
CWBI-B1470	*Ln.mesenteroides subsp. mesenteroides/dextranicum2*	99.9%	1489	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	98%
CWBI-B1471	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	91.9%	1483	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	99%

DISCUSSION

Raw milk typically has a pH of 6.6. Mathara *et al.* [15] found that the average pH of the Kenyan fermented milk product *kule naoto* is 4.4 (range 4.17-5.19). Only four *kule naoto* samples examined had a pH exceeding 4.5. Abdelgadir *et al.* [16] tested the pH of two samples of *rob*, a Sudanese fermented milk, and found values of 3.87 and 4.02 when the pH was tested immediately upon sampling. The pH of *kivuguto* falls within the normal acidic pH range for fermented milk. People living in this region therefore typically consume acidic milk, and those who consume *rob* are consuming an even lower pH milk. For the purpose of this work, we put more emphasis on the dominant strains because the quality of a fermented milk product is judged by its flavor, appearance and consistency [17]. Nevertheless, the main technological property of strains fermenting milk is the acidifying activity, but also the growth in milk and survival in acidic milk [18]. And strains sustaining these properties are major strains at the end of the fermentation. Thus, the strains developed on tomato juice were acidifiers and represented by *Lactococcus lactis.* Mayeux agar and other media were used to isolate strains imparting both flavor and consistency, such as *Leuconostoc* spp., which produce aromatic compounds and dextran. The enumeration and the phenotypic characterization experiments demonstrated that the *kivuguto* microflora is composed primarily of *Lactococcus* and *Leuconostoc* species. The group of bacteria isolated from Rogosa agar was presumed to be composed of lactobacilli. Indeed, 3 strains were assigned as *Lactobacillus helveticus* by the API software, but at a low percentage (61.1%). It is known that leuconostocs grow on Rogosa agar, unlike *lactococci* [19]. Phase contrast microscopy indicated that these bacteria were not rods, and therefore could be *Leuconostoc* spp. It is typically quite difficult to differentiate heterofermentative lactobacilli and leuconostocs due to difficulty to distinguish bacilli to cocci [20]. Many authors have confirmed the difficulty of identifying *Leuconostoc* by phenotypic means due to heterogeneity in the biochemical and physiological characteristics of these organisms [21, 22]. Furthemore, some *kivuguto* strains exhibited unusual tolerance to high temperature and very low acidic media. McDonald *et al.* [23] showed that growth of *Leuconostoc mesenteroides* stops when the internal pH reaches a value of 5.4 to 5.7. This is a very useful characteristic in dairy technology, because the ability to grow at high temperature is a desirable trait as it could translate into an increase in the rates of growth and lactic acid production [24]. Such a strain could be preserved by freeze- or spray-drying.

After isolation, enumeration and identification by phenotypic (morphology and API tests) techniques, molecular methods confirmed the identification for six pre-selected strains. The study was followed by tests of acidification because the primary function of dairy starter cultures is rapid acidification [25]. Following the acidification tests, the shelf-life of the fermented milk formulations was examined in order to prevent proteolysis and lipolysis, since some LAB produce peptidases that produce bitter-tasting peptides that detrimentally impact the organoleptic quality of milk [26]. Due to issues regarding strain compatibility, the first starter formulations were composed of strains from the same sample. The choice of strains used was based on resistance to extreme environmental conditions and the functional role of the strain in the fermentation process. Using such an approach, complex and costly analyses were avoided. This is in agreement with a number of findings, suggesting that these species could be used as starters or starter adjuncts [27, 28]. Ultimately, selected strains showed that no yogurt strain enters in the *kivuguto* selected strains. The CWBI-B1466 *Lactococcus lactis* is found in *leben* of Morocco, in *zivda* of Israel, in Indian dahi, in Zimbabwean *amasi*, in sudan *rob* and in other types of fermented milk worldwide. With *Leuconostoc* sp., it composes the starter of filmjölk of nordic countries, which is the most similar both in consistence and taste. But the selected strains confer to *kivuguto* its sole biochemical identity.

CONCLUSION

In the present study, we isolated and identified the microorganisms that produce the fermented milk *kivuguto*. Phenotypic and molecular characterisation studies enabled a screening of seven strains used to formulate three *kivuguto* starters, and we found that based on fermentation time, organoleptic aspects, and shelf-life at 4°C, the best *kivuguto* was produced by a mixture of *Lactococcus lactis* CWBI-B1466, *Leuconostoc mesenteroides* CWBI-B1465, and *Leuconostoc pseudomesenteroides* CWBI-B1470. Using this formulation, milk fermented in 8 hours at 30°C, and no organoleptic defects were detected after 35 days of storage. Of particular interest to the dairy industry, most of the *Leuconostoc spp*. isolated ferment milk and produce dextran, and one of these isolates were particularly resistant to extreme environmental conditions, a characteristic that would be beneficial in terms of the cost of its production. Since the three strains in the present study are technologically interesting bacteria, development of a standardised processing method coupled to a sensorial analysis will ensure the production of a highest microbial and nutritional quality *kivuguto*.

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