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Processing steps and lactic acid bacteria involved in traditional cultured milk (Kwerionik) production in Uganda

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Abstract

The production process of traditionally fermented milk (*Kwerionik)* from Eastern Uganda and the lactic acid bacteria (LAB) involved were investigated. Viable plate count method was employed for enumeration and isolation of LAB, coliforms and mesophilic aerobes from both the field and laboratory fermentation samples. Representative LAB isolates from the various stages of fermentation were grouped by PCR fingerprinting of the 16S-23S rRNA internal transcribed spacer region; and identified by carbohydrate fermentation tests using API 50 CH kit; and by sequencing of the 16S RNA gene. Fermentation was spontaneous; carried out in gourds and involves periodic removal of whey and addition of fresh milk. The field and the laboratory *Kwerionik* samples had comparable parameters. The pH ranged between 3.5 and 4.5; LAB levels were 10^9 cfu g^{-1}after one day but later decreased to 10^5–10^6 cfu g^{-1}. Mesophilic aerobes ranged from 10^6 to 10^{10} cfu g^{-1} while coliform levels up to 10^7 cfu g^{-1} were detected, but not later than 11 days. The pH and microbial counts at the different fermentation durations were significantly different ($p<0.005$) except for the coliforms. *Lactobacillus plantarum*, *Enterococcus faecalis*, *Lactobacillus paracasei* subsp *paracasei*, *Lactobacillus casei* subsp *casei*, *Lactococcus lactis* subsp *lactis*, *Enterococcus faecium* and *Leuconostoc mesenteroides* subsp *mesenteroides* were involved.

Keywords: Lactic acid bacteria, traditional milk fermentation, molecular and phenotypic identification

Introduction

The presence of organisms in traditional fermented foods is well known and has been the subject of scientific inquiry for over a century. The organisms in many cases determine the characteristics of the fermented food like acidity, flavor, and texture, as well as health benefits that go beyond simple nutrition. Fermentation by lactic acid bacteria has been reported to create better and preferable products with respect to taste, texture, nutrient bioavailability, flavour, palatability and shelf-life **(1, 2, 3, 4, 5, 6, 7)**. The microorganisms can be isolated at random from various natural sources and tested individually for their biosynthetic abilities. Identification and characterization of these microorganisms and successful processing of fermented products relies completely on choosing the right organisms for the specific type of fermentation **(8)**. Fermented milk production is a result of presence of bacteria, molds, yeasts or a combination of these and their enzymes **(4).**

Several traditional fermented milk products have been studied **(9, 10, 11, 12, 13, 14, 15, 16, 17)**. Many of these studies aimed at identifying the organisms involved, which is the prerequisite for development of starter cultures for the industrial processing of the products. Limited studies have been carried out on *Kwerionik* where the yeasts involved were identified and characterised **(18)**. However, the physicochemical and major bacteria associated with its fermentation were not yet known. Therefore, this study was carried out to document the production process and to establish the dominant lactic acid bacteria involved in *Kwerionik* fermentation, with the aim of selecting strains for use in starter cultures formulation if the traditional product has to be produced on an industrial scale.

Materials and methods

The *Kwerionik* production process was established through focus group discussion using semi-structured interviews held with the Sabiny people, who traditionally ferment *Kwerionik*, at the slopes of Mt. Elgon in eastern Uganda. Inquiries were made into the types and use of the milk products; the type and capacity of the containers employed and how they are treated; the source of milk (animal host and breed); persons who carry out the processing; the storage methods and conditions; and a step-by-step narration of the production process.

Laboratory fermentations for *Kwerionik* production were carried out in duplicate, simulating the traditional method using charcoal-treated gourds purchased from the local processors. The gourds (2 litres capacity) were filled with fresh cow's milk and fermentation allowed to take place at ambient temperature (25 – 30°C). Whey was withdrawn and more fresh milk was added every two days up to the 303rdday. Samples (25 g) were withdrawn every six hours until day 3, then daily until day 7, then every four days until one month and thereafter, fortnightly to the end. In addition, field samples of *Kwerionik*, made from cow's milk at days 7, 14, 30, 90 and 180 of fermentation, were obtained from the production area.

Both the field and the laboratory fermentation samples were analysed microbiologically as described below and their pH determined. Comparisons on the type and levels of microorganisms in the field and laboratory fermentation samples were made, and the microbial succession that occurs during *Kwerionik* production was explored.

For isolation and enumeration, samples were homogenized and ten-fold dilutions prepared using peptone-saline (1% Peptone (Difco, Detroit, Michigan), 0.9% NaCl, pH 7.0); and then various media inoculated. Unless stated otherwise, all the media were obtained from Difco, Detroit, Michigan. The media included Plate Count agar (PCA) for mesophilic aerobic counts, Violet Red Bile agar for the coliforms, M17 for the Lactococci; and de Man, Rogosa and Sharpe (MRS) for the Lactobacilli. The pour plating method was used in all cases except for PCA and the plates were incubated at 30°C aerobically except for MRS that were incubated anaerobically using BBL® Gas Pak® Anaerobic system envelopes (Becton Dickinson, Cockeysville, USA). Colony counts were established and five representative colonies from each M17 and MRS plates were selected for isolation and purification after initial screening as Gram positive, catalase negative cocci and bacilli respectively. Pure cultures were obtained using appropriate media. The isolates were phenotypically and genotypically characterized to establish their identity.

A total of 430 isolates were selected from the field samples and from the laboratory trials at 0h, 12h, 24h, 36h, 48h, three days, four days, seven days, 14 days, 30 days, 60 days, 180 days, 240 days and 300 days of the spontaneous laboratory fermentation. From the initial groups by PCR – ITS patterns, five representatives were subjected to carbohydrate fermentation and other phenotypic tests, but for the 16S gene sequencing, one representative was selected.

Extraction of DNA was achieved by heat-treatment of the LAB as described by Abdelgadir *et al* **(11)**. For DNA amplification and analyses, all the chemicals were obtained from Pharmacia Biotech, Uppsala, Sweden, unless stated otherwise. For amplification of the ITS region, a fluorescein labelled forward primer Cy5-16S-1500F and a reverse primer 23S-32R (DNA Technology A/S, Aarhus, Denmark) with sequences as described by Abdelgadir, *et al* **(11)** were used.

Amplification was performed as described by Abdelgadir *et al* **(11**) using a thermal cycler (Trio-Thermoblock, Biometra, Gottingen, Germany). The amplified products were electrophoresed on a 6% Polyacrylamide gel (5.5% Long Ranger [FMC, Vallensbæk strand, Denmark], 6 mol l^{-1} urea and 1.2 × TBE [1mol l^{-1} Tris-base, 0.83mol l^{-1} boric acid and 10mmol l^{-1} EDTA]) on the automatic DNA sequencer, ALFexpress (Pharmacia Biotech). A size marker, Cy5 sizer 50-500 was included as the sizing standard. The electrophoresis conditions were 700V, 60mA at 55°C for 200 min with 0.6 × TBE as the running buffer. Peak positions and intensity of the separated ITS fragments were analysed by use of Fragment Manager Software

(Pharmacia Biotech). Isolates with the same profiles of ITS fragment were grouped together manually and compared to those of the reference strains as shown in Table 1.

The isolates selected from the various PCR – ITS groups were tested for fermentation of various carbohydrates by use of API 50CH kit (BioMériux, Marcy-l'Itoile, France). The reactions were recorded visually and the organisms identified by APILAB Plus V3.2.2 software (BioMériux, France). The cocci were also tested for their ability to grow in 6.5% (w/v) NaCl-M17 broth and at 10°C and 45°C.

All the primers, both for gene amplification and sequencing were obtained from T-A-G Copenhagen ApS, Copenhagen, Denmark. Primers, 16S-27F and 23S-32R, whose nucleotide sequences were as given by Abdelgadir *et al* **(11)** were used to amplify nearly, full-length 16S rRNA gene of selected bacterial isolates for sequencing. Amplification was performed in 100 µL reaction mixtures whose composition was similar to that used for PCR-ITS as described by Abdelgadir *et al* **(11)**. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and used as the template during cycle sequencing.

Cycle sequencing of the 16S rRNA gene was achieved by use of eight internal primers designed from the conserved regions of the gene at temperature profiles as described by Abdelgadir, *et al* **(11)**, using the Thermo Sequanase fluorescent-labelled primer cycle sequencing kit (Amersham Life Science, Amersham, England). The primers included Cy5-16S-338F; Cy5-16S-357R; Cy5-16S-776F; Cy5-16S-810R; Cy5-16S-1050F; Cy5-16S-1193R; Cy5-16S-1391F; Cy5-16S-1500F and Cy5-16S-1540R.

Table 1: Reference strains of lactic acid bacteria, the source and the size of PCR-ITS products

Type Organism	Source	Fragment size (bp) of PCR-ITS
Enterococcus faecalis DSM 20478	DSM *, Germany	307; 313†; 331; 415; 431; & 518
Enterococcus faecium DSM 6177	DSM, Germany	419; 428; 447;& 524
Lactococcus lactis ssp *cremoris* AC1 (DSM 4645)	DSM, Germany	390; 475; & 496
Lactococcus lactis NCDO 2118	NCDO‡, England	392 & 493
Lactococcus. lactis ssp *lactis* C10 (MI 1025)	MLI-KVL§Denmark	389; 434; & 495
Lactococcus lactis ssp *lactis* biovar *diacetylactis* DRC1 (MI 1087)	MLI- KVL Denmark	389; 473; & 495
Lactobacillus casei 187	MLI- KVL Denmark	93; 208; 255; 295; 305; & 513
Lactobacillus paracasei BGL 17	MLI- KVL Denmark	207; 289; 306; 513; & 563
Lactobacillus plantarum L30	MLI- KVL Denmark	109;115; 291; 521; & 533

*DSM, the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

†**313**, The size of the major bands is bold.

‡ NCDO, the National Culture of Dairy Organisms, Reading UK.

§ MLI-KVL, Department of Food Science, Copenhagen University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark.

Electrophoresis of the cycle sequencing products was carried out on 6% Polyacrylamide gel on ALFexpress automatic DNA sequencer (Pharmacia Biotech). The sequence data was analysed and evaluated by the ALFwin v1.0 software (Pharmacia Biotech). The sequences were aligned and compared to those in the Basic Local Alignment Search Tools, BLAST.

For field *Kwerionik*, five samples per category (duration of fermentation) were analysed. Laboratory fermentation trials were carried out in duplicate. Data for bacterial counts were log10 transformed before statistical analysis. Mean comparison of the pH and microbial counts was done using least significant difference (LSD) for variables whose F values were found to be significant (Statistical Package for Social Scientists (SPSS) version 16.0, Apache Software Foundation, copyright 2008). Significant differences were calculated at 5% significance level.

Results

Kwerionik, which is produced in Eastern Uganda by the Sabiny ethnic group found on the slopes of Mt Elgon was made from short horn Zebu cow's milk. The fermented product had a mild sour flavour, a thick consistency and was grayish or white in colour depending on whether charcoal was added. *Kwerionik* was consumed as a refreshing beverage given to special visitors, convalescent individuals and nursing mothers and also as a sauce with cooked or boiled cassava or 'posho', a maize meal. It was mainly processed by women, who in some cases formed organized groups that produced large amounts that were sold at the local markets and hospitals.

Fermentation was performed in gourds that were reused. However, back-slopping from a previous lot was not practiced. The gourd was prepared from a dried fruit of *Lagenaria leucantha* by removing the seeds and cleaning the inside of the mature dried fruit. Before use for fermentation, some raw milk was left overnight in the new gourd and discarded, a process that could last for about one week. This helped in washing out the bitter substances. In some areas, *Kwerionik* from older gourds was used to condition the new ones. The capacity of the gourds depended on whether short term or long term fermentation was to be carried out, which ranged from ½ – 2 litres or more, respectively. In some cases, saucepans and plastic jerrycans (20 litre capacity) were used. However, if fermentation was to last for over a week, the product had to be transferred to the gourds. It was claimed that *Kwerionik* made in containers rather than the gourds often had an undesirable smell and taste.

The gourd was thoroughly washed with hot water, but not soap, and scrubbed with rough leaves using a palm stick locally referred to as 'Soschondet', then sun-dried. Prior to introduction of milk, the gourd was treated by smoking or adding charcoal obtained from selected shrub species, which often included *Combretum molle*, *Cassia didymobotyra*, *Vernonia auriculifera* and *Euclea divinorum*, locally known as 'Kombolyet', 'Senetwet', 'Tabongwet' and 'Wushet' respectively. A smoldering stem from a specific shrub was pushed into the clean gourd and rubbed against the walls. The charcoal there-from was crushed with the 'Soschondet' into finer particles and the gourd was then shaken to distribute them all over its walls. The excess and large particles were poured off and the gourd was ready for use. The charcoal and smoke were claimed to contribute to the flavour and to the prolonged shelf life of the product.

For production of *Kwerionik*, a charcoal treated or smoked gourd was filled with raw, rarely cool boiled milk; and covered with a lid. The gourd was then kept at ambient temperatures; ca. 16 – 22°C at the hills or ca.23 – 28°C in the valleys where coagulation takes approximately three days and one day respectively. After coagulation, the curd floats on top of the whey. Removal of whey and adding more milk was done at intervals of 2 - 4 days until the product was needed for consumption, which may go up to one year. Before consumption of the product or prior to adding more milk for continued fermentation, the surface layer was scooped off. Depending on the duration of fermentation, the product could be referred to as *Kwerionik*, *Katanik* and *Chekap-mwaka* after 3 – 7 days, 8 – 28 days and >29 days of fermentation, respectively.

Consumption of the product was done any time after 3 days of fermentation and all the contents were harvested at once and the gourd was washed and dried to make it ready for the next processing. After six months of fermentation, the product becomes very sour and had to be mixed with blood or porridge before consumption.

The pH, the levels of coliforms, mesophilic aerobes and the lactic acid bacteria on M17 and on MRS that were found in *Kwerionik* field samples at seven, 14, 30, 90 and 180 days of fermentation were as summarized in Table 2. The mean value and the standard deviation of the microbial counts for five field samples were given in each case. Comparable results (data not shown) for the spontaneous laboratory fermentation were obtained.

The pH was about 4 in the 7-day product and decreased further with the duration of fermentation to about 3.5. Significant differences in pH as the fermentation time increased ($F=27.6$, $p=0.000$) were noted. Multiple comparisons revealed that the difference was bigger with 7-days samples and all the other age groups ($p=0.000$); and between day 14 and day 30, day 90 and day 180 samples ($p=0.003$, $p=0.048$ & $p=0.001$ respectively). For laboratory trials, the pH of the milk decreased to a level of 4.5 within 12h and further to pH 4.0 after about 36h. After 30 days of fermentation, the pH reduced further to about 3.5.

Table 2: Microbial counts (log 10 cfu^{-g}) from field *Kwerionik* samples at different stages of fermentation

Fermentation Duration (days)	Mean values with Standard Deviation				
	pH	MAC*	Coliforms	LAB on M17†	LAB on MRS‡
7	3.99 ±0.06[a]	7.8±0.94[a]	1.5±1.85[a]	8.1±0.77[a]	8.4±0.63[a]
14	3.71±0.09[b]	8.6±0.68[ab]	1.2±1.17[a]	7.7±0.46[ab]	9.2±1.08[ab]
30	3.53±0.05[c]	7.9±0.26[a]	$0.7\pm1.05\times10^{-8\#a}$	7.1±0.44[b]	8.2±0.33[a]
90	3.60±0.15[c]	7.2±0.94[ac]	0.7±0.00[#a]	7.1±1.50[ab]	7.5±1.11[a]
180	3.49±0.02[c]	7.3±0.79[ac]	$0.7\pm1.05\times10^{-8\#a}$	5.3±0.34[c]	7.5±0.48[a]

*MAC Mesophilic aerobic count on Plate count agar
† LAB on M17 Lactic acid bacteria on M17 medium
‡LAB on MRS Lactic acid bacteria on de Man, Rogosa and Sharpe medium
Coliform counts below detectable levels
The mean bearing different superscript letters in the column differ significantly at $p<0.05$.

Coliforms were found at levels up to 10^4 cfu g^{-1} in the 7-day and 14-day samples, in only one of the field samples in each case, but were not detected (< 30 cfu g^{-1}) in products fermented beyond 30 days; which was not statistically significant ($F=0.774$, $p=0.555$). For the laboratory trials, the level of the coliforms was about 10^6 cfu g^{-1} at the beginning of fermentation, increased slightly but fell below the detection levels by the 11^{th} day.

The mesophilic aerobic counts, which comprised mainly of Gram positive cocci, ranged from 10^7 cfu g^{-1} to 10^9 cfu g^{-1} with the 14-day old group of field samples having the highest levels. For the laboratory trials, the mesophilic aerobic counts peaked to 10^{10} cfu g^{-1} at 30 hrs and ranged between $10^8 – 10^9$ cfu g^{-1} up to day 11, then stabilized at levels of $10^6 –10^7$ cfu g^{-1} thereafter.

For the LAB determined on M17, the counts decreased from 10^8 cfu g^{-1} for the one-week old field product to 10^5 cfu g^{-1} for the 180-day old. The counts on MRS increased from day 7 to day 14 reaching levels of 10^9 cfu g^{-1} and after 90 days of fermentation the levels finally declined to about 10^7 cfu g^{-1}. Significant differences between the LAB counts on MRS were noted ($F=3.865$, $p=0.017$). Differences were significant between samples of 14 and 30 days, 90 and 180 days of fermentation with p values of 0.047, 0.006 and 0.002 respectively. In addition, significant differences between the LAB counts on M17 existed ($F=7.706$, $p=0.001$). The laboratory fermentation trials gave comparable

findings, where the LAB counts were about 10^6 cfu g^{-1}; and after 72 days of fermentation, the levels on M17 and on MRS were between $10^5 – 10^6$ cfu g^{-1} and $10^7 – 10^8$ cfu g^{-1} respectively.

Table 3 summarizes the identification results obtained. Of the 430 isolates selected, 405 (94%) successfully gave PCR products and the PCR finger printing of the 16S-23S ITS region established six major groups that were designated A-F. The sequences of the 16S gene of the representative LAB strains that were established were deposited to the European Molecular Biology Laboratory (EMBL) and the accession numbers are indicated in Table 3.

Group A organisms had a major PCR-ITS product of size 413 - 416 base pairs (bp); and presence of additional products results in encountering five sub-groups. This profile resembled that of *Enterococcus faecalis* reference strain DSM 20478 (Table 1). Members were identified as *L. lactis* subsp *lactis* by API, but all grew at 45°C. Most of them grew in M17 broth with 6.5% (w/v) NaCl except those with the 355 bp product and were all identified as *Enterococcus faecalis* by sequencing and comparison with the BLAST. However, the group with the 363 bp product gave slightly higher identity to *Enterococcus hirae* (97%) than to *Ent. faecalis* (96%). The *Enterococcus faecalis* were isolated from all the field samples and from the samples obtained during the first week of the spontaneous laboratory fermentation, some did not grow in the presence of 6.5% NaCl. Those identified as *Enterococcus hirae* was isolated mainly from the 7- and 14- days- field samples and from one of the trials between four and seven days of fermentation.

Group B had major PCR-ITS products of 431 bp and 526 bp with some additional products of 516 bp. These profiles resembled that of *Enterococcus faecium* DSM 6177 (Table 1), but were also identified as *L. lactis* subsp *lactis* by API. However, the growth characteristics in 6.5% (w/v) NaCl and at 10°C and 45°C and 16S-gene sequence confirmed the genus *Enterococcus*, which concurred with the BLAST sequence search identification as *Enterococcus faecium*.

Group C isolates had a PCR product of 466 bp, a profile resembling that of some Leuconostocs isolated from cheese from other studies (results not shown) and were identified as *Leuconostoc mesenteroides* subsp *mesenteroides* by both API and BLAST sequence search for the 16S gene.

Group D members had two PCR–ITS products of 390 and 409 bp, comparable to *Lactococcus lactis* NCDO 2118 (Table 1). All the identification procedures including the sequencing suggested that they were *Lactococcus lactis* subsp *lactis*. *Lactococcus lactis* was isolated from all the field samples and from the spontaneous laboratory fermentation, but were relatively in low numbers after one month of fermentation as indicated in Table 2.

Group E isolates, with three sub-groups, had major PCR-ITS products of 208, 308 and 515 - 520 bp. When compared to the reference organisms, they resembled *Lactobacillus casei* 187 (Table 1). Two of the three subgroups had double bands at 208 and 308 bp, but one was identified as *Lactobacillus paracasei* subsp *paracasei* by both API and on sequencing; and the other as *Lactobacillus curvatus* by API and as *Lact. casei* subsp *casei* on sequencing. The third subgroup was also identified as *Lact. paracasei* subsp *paracasei* by both API and sequencing. *Lact. paracasei* subsp *paracase*i were isolated on all occasions but *Lactobacillus casei* subsp *casei* was isolated from one of the fermentation trials only.

Group F with its two sub-groups had major and common PCR products of about 290 and 518 - 526 bp. Some had an additional PCR product of 110 bp and their profile was similar to that for *Lact. plantarum* L 30 (Table 1) and were identified as such by the API as well as on sequencing. *Lact. plantarum* were isolated on all occasions, and were the predominant Lactobacilli after 14 days of fermentation.

As shown in Table 3, of all the 405 isolates that were successfully amplified by PCR, their identifications were as follows: *Lact. plantarum* (44.2%), *Ent. faecalis* (29.1%), *Lact. paracasei* subsp *paracasei* (9.4%), *Lact. casei* subsp *casei* (9.4%), *L. lactis* subsp *lactis* (4.9%), *Ent. faecium* (1.7%) and *Leuc. mesenteroides* subsp *mesenteroides* (1.3%).

Table 3: Grouping of Lactic acid bacteria isolates by ITS fragment size and identification by various methods

ITS group & (# of isolates)		Fragment size (bp)*	Genus/species†	16S rRNA gene sequence‡	EMBL Accession #§
	(41)	**355**‖ & **416**	*Streptococcus*	*Ent. faecalis* (96%)	AJ271855
	(22)	293, **355**‖, **416** & 510	*Streptococcus*	*Ent. faecalis* (97%)	AJ271848
A	(18)	363 & **416**	*Enterococcus*	*Ent. hirae* (97%)/*faecalis* (96%)	AJ272200
	(30)	303‖, **413**‖ & 511	*Enterococcus*	*Ent. faecalis* (98%)	AJ271847
	(7)	311 & **413**‖	*Enterococcus*	*Ent. faecalis* (96%)	AJ271856
B	(7)	**431**‖, 516 & **526**	*Enterococcus*	*Ent. faecium* (98%)	AJ271849
C	(5)	**466**	*Leuc. mesenteroides*	*Leuc. mesenteroides* subsp *mesenteroides* (99%)	AJ271850
D	(20)	**390** & **490**	*L. lactis* subsp *lactis*	*L. lactis* subsp *lactis* (99%)	AJ271851
	(35)	192, **208**‖, **308**‖& 516	*Lact. paracasei* subsp p*aracasei*	*Lact. paracasei* subsp *paracasei* (96 & 99%)	AJ271854 &AJ271853
E	(38)	**208**‖, **308**‖ & 520	*Lact. curvatus*	*Lact. casei* subsp *casei* (99%)	AJ272201
	(3)	157, 264, **299** & 515	*Lact. paracasei* subsp *paracasei*	*Lact. paracasei* subsp *paracasei* (98%)	AJ272010
F	(127)	110, **290** & **518**	*Lact. plantarum*	*Lact. plantarum* (97%)	AJ271852
	(52)	**290** & **526**	*Lact. plantarum*	*Lact. plantarum* (96%)	nd

*- The size of the major PCR products are in bold;† - Genus/species as determined by growth at 10°C, 45°C, in 6.5% (w/v) NaCl and by API 50CH kit.;‡ Tentative identification and percent similarity values from Basic Local Alignment Search Tools (BLAST).; § -European Molecular Biology Laboratory (EMBL) Database accession numbers.;‖-Double bands as seen from the profiles; nd - not done

Discussion

Several African traditionally fermented milk products are processed in gourds, which are usually prepared in the same manner, such as use of early lactation milk **(16, 19)**. Use of milk in preparing the new gourds probably contributed to introduction of the LAB, since back-slopping was not done. The undesirable smell and taste of *Kwerionik* made in containers rather than the gourds could be due to lack of the desirable fermentative organisms that colonise the gourds, especially since the other utensils were washed with soap. In addition, it was not easy to completely fill these containers and the headspace probably promoted growth of spoilage microorganisms. According to Kebede *et al* **(20)**, the type of container influenced the microflora type produced and therefore the characteristics of the fermented products. However during this study, the microbiology of the products processed in other containers were not analysed.

Treating the gourds with charcoal or smoking with a burning piece of wood was widely practiced in several African traditional milk fermentation practices **(21, 22)**. Among the tree species employed by the Sabiny, it was *Cassia didymobotyra* and *Euclea divinorum* that had been used elsewhere **(22, 23)**. The tree species used depended on the ecological and biogeographic conditions of the area. The charcoal/smoke was reported to offer prolonged preservation to the milk in synergy with other components, to sterilise the gourds and to add colour and flavor to the product **(24)**.

Considering the production process, *Kwerionik* was a traditional fermented whole-milk product. Other products with similar production processes exist, such as, *Maziwa lala* in Kenya, *Ergo* and *Itutu* in Ethiopia; and *Amasi* in Zimbabwe **(10, 25)**. However, for many of these products, back-slopping was practiced. For several others, such as *Rob* from Sudan and Mashita from Western Uganda, fermentation occurs at higher ambient temperatures and usually involves churning to separate the butter and cheese **(11, 26)**. The differences in the production processes, especially the ambient temperature, influenced the type of microflora involved. The similarity in the production processes for *Kwerionik* and *Itutu* was probably due to the Kalengin group of people, among which belonged the Elgon Kalengin, who were believed to have moved into the Mt. Elgon area from the North, perhaps from an area in Ethiopia, north of Lake Turkana. Hence they shared the traditional way of preserving the milk.

The pH of *Kwerionik* ranged from 3.5 to 4.5. This was a case with several other fermented milk **(16, 27, 28)**. After one month of fermentation despite the continuous removal of whey and addition of fresh milk, the pH of the samples was not significantly different ($p > 0.05$). This was probably due to the relatively small quantities of whey removed and consequently small amount of fresh milk needed to top up during the late stages of fermentation in comparison to that removed during the early stages. In addition the buffering property of milk could have influenced the insignificant pH changes.

The low or non-existence of the coliforms had been experienced with many lactic acid fermented products, pointing to the safety of the product **(11, 29)**. Absence of the coliforms could be attributed to presence of organic acids and lowered pH of the fermented products among other factors; since the alkaline fermented condiments were reported to be contaminated with the coliforms **(30)**.

Significant differences in the mesophilic aerobic counts by during the fermentation process were noted. Similar findings for the mesophilic aerobic counts had been reported **(13, 27, 28)**. These were mainly composed of the *Enterococci* that were capable of growing on PCA under aerobic conditions.

The cocci (*Enterococci*, *Lactococci* and *Leuconostocs*) occurred in high numbers during the first week of fermentation and the Lactobacilli took over after this time similar to what had been reported by several researchers **(11, 31, 32, 33)**. Better survival of *Lactobacillus* species during the

later stages of *Kwerionik* fermentation, where the pH was below 4.0 was due to the differing levels of acid tolerance and regulation of the internal pH by the various LAB **(34, 35, 36)**. The ambient temperature, 20 - 25°C, at which fermentation occurred, was conducive for the growth of the mesophilic lactic acid bacteria and the equipment used, in particular the gourds and the leaves used to clean them, probably served as a source of the organisms.

Enterococcus faecalis was isolated from all the field samples as is often the case with artisanal milk products **(37)**. Some of them did not grow in 6.5% NaCl broth, but such *Enterococci* strains had been reported earlier **(38)**. *Enterococcus faecium* had been reported to be the dominant biotype in some traditional fermented milk products **(32)**. The Enterococci are widely distributed in nature and gain entry into milk and its products during processing and handling **(39)**. Presence of these organisms in all the field samples indicated that they could have probably contributed to fermentation of the milk, although there are high chances of cross contamination between batches, especially during whey removal and addition of milk. The *Enterococci* from various dairy products have been reported to contribute to flavor and to have functional benefits, but some strains are pathogenic and this needs to be ruled out **(40)**.

The *Leuconostocs* were isolated from milk and from the one-week field and laboratory samples. Similar findings had been reported by other researchers where *Leuconostocs* had been found to grow in association with the acid producing *Lactococci* **(33, 41)**. Similar findings where *Lactococcus* species were involved in dairy fermentation but with decreasing levels as the duration increases have been reported. Lactococci are used in the dairy industry for production of lactic acid and some flavor components. They have been found to be safe, but different stress conditions including high acidity affect their viability **(42)**.

Lactobacilus paracasei has been isolated from some traditional fermented milk products but is of more importance in relation to its functional properties **(43, 44)**. *Lactobacillus casei* was encountered only in one of the laboratory trial, but have been isolated from different environments, and some of the strains are used as probiotics in commercial products **(45)**. *Lactobacillus plantarum*, although was the major isolate from *Kwerionik*, it was not normally considered as one of the starter cultures for milk fermentations, but has been found in cheeses mainly as adventitious bacteria **(46)**.

The dominant lactic acid bacteria that were encountered in *Kwerionik* were frequently isolated from the dairy environment, except for *Lact*obacillus *plantarum* which is mostly involved in fermentation of meat, fish, vegetable juices, silage, sourdough and soda crackers **(47)**. In comparison to other African fermented milks, *Kwerionik* was fermented for a longer time, but has LAB similar to that of Senegalese *Mbanik*, a Zimbabwean fermented milk; and and *Maziwa lala* and *Kule naoto* in Kenya **(9, 10, 12)**. However, for the products with the same production processes, minor differences in the microflora noted could be due to the researchers' preference and difficulty in species identification by the phenotypic methods that were employed by some of the researchers.

In conclusion the LAB isolated from *Kwerionik* included *Lactobacillus plantarum*, *Enterococcus faecalis*, *Lactobacillus paracasei* subsp *paracasei*, *Lactobacillus casei* subsp *casei*, *Lactococcus lactis* subsp *lactis*, *Enterococcus hirae*, *Enterococcus faecium* and *Leuconostoc mesenteroides* subsp *mesenteroides*. There was a need to establish the dairy technological properties of the isolates in order to select strains that could be used as starter cultures for industrial and improved production of *Kwerionik*. Some *Lactobacilli* identified as *Lact curvatus* by API and as *Lactobacillus casei* by sequencing of the 16S gene needed to be subjected to further studies to allow proper identification.

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