Technological Features of Selected *Kivuguto* Strains during Milk Fermentation

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Abstract  *Kivuguto* milk is a traditional fermented milk of Rwanda. A previous study allowed for the selection of three bacteria involved in the fermentation process. The aim of the present work is the technological characterization of *kivuguto* strains for its production in the dairy industry. Acidification, proteolysis, the flavor compound profile, rheology and sensory analyses of fermented milks were assessed as important indicators of the starter culture formulation. Acidification showed that *kivuguto* milk ferments in 14 hours at 19°C with a titratable acidity of 73°D. The samples of CWBI-B1466 *Lactococcus lactis* and CWBI-B1470 *Leuconostoc pseudomesenteroides* had fermentation times of 14 h and 20 h, respectively. All samples were viscoelastic fluids, and the most important flavor compounds found were two alcohols, one ester and two furan derivative compounds. Proteolysis revealed low values ranging to 3.04-5.45 mg.L⁻¹, which is very interesting in terms of taste acceptability. The three strains showed positive technological properties for *kivuguto* starter culture development and the data are fully in agreement with the preliminary results of the technological analyses. Acidification showed similarities between the formulated *kivuguto* and the traditional *kivuguto* as recognized by a tasting panel in a discrimination test. Ultimately, this study allowed for the formulation of *kivuguto* milk using three bacteria, prior to studying the stability of these properties during storage under refrigeration, which is the last stage before industrial production of *kivuguto* milk can begin.

Keywords  Technological Features, *Kivuguto*, Milk Fermentation, Acidification, Rheology, Proteolysis, Aroma Compounds, Sensory Analysis

1. Introduction

Fermented milks are manufactured throughout the world, and approximately 400 generic names are applied to traditional and industrialized products [1]. To reach the industrial scale, the selection of starter cultures involved in the fermentation process must be performed. Thus, a long study where biochemical and technological properties are well understood may allow for starter culture development.

In milk fermentation, the major biochemical reactions are the metabolism of residual lactose, lactate and citrate, the liberation of free fatty acids (lipolysis), associated catabolic reactions, the proteolysis of milk proteins (mainly caseins) into peptides and free amino acids, and the catabolism of free amino acids [2]. Acidification is an important technological property in milk fermentation. It is performed by microbial starters used for that purpose and must stay within an acceptable concentration during storage for quality and stability. Fermentative starters reduce lactose to lactate and the pH decreases leading to several physicochemical changes. From pH 6.6 to 4.6, casein micelles are broken down by proteolysis into smaller peptides, changing the texture of milk and leading to coagulation.

Some starter cultures also produce exopolysaccharides which increase the viscosity and texture and decrease susceptibility to syneresis [3]. This phenomenon leads to gelation, reached at pH 4.6. In dairy technology, knowledge of the rheological properties is necessary in order to characterize the fermented milk quality in terms of texture. In practice, the change in rheological properties affects the acceptance of the product by the consumer [4,5], either positively or negatively. Moreover, the rheological properties influence the design and the sizing of the process for shear stress control [4,6,7]. More specifically, they influence the engineering calculations of the process, such as the flow, selection of pumps, determination of load loss in the tubes [4,5,8,9], as well as during heat exchange. Acidification also generates flavor compounds; this process is strain dependent. Aroma perception is one of the foremost criteria for the evaluation of fermented milk...
because of its influence on consumer acceptance [10]. According to Thomas [11], consumer acceptance and the preference for milk as a beverage is influenced by its flavor more than any other attribute. The typical flavor of fermented milks is derived from lactic acid and many carbonyl compounds produced by starter cultures. Lactic acid is responsible for the sharp, refreshing taste of these products [12]. Another source of flavor compounds is the thermal degradation of lipids, lactose and proteins during the heat treatment of milk before fermented milk manufacture e.g. aldehydes, ketones, alcohols, lactones, and sulfur compounds [13].

Kivuguto milk is manufactured by spontaneous fermentation or by backslipping in Rwanda. The milk is more appreciated by local consumers than modern fermented milks produced on the Rwandan market. To date, no investigations have been conducted on the manufacturing techniques, neither on the physicochemical and organoleptic characteristics, nor on any other technological parameter. In a previous study [14], three strains were selected for kivuguto starter formulation: CWBI-B1466 Lactococcus lactis registered under Genbank accession number JF313446, CWBI-B1465 Leuconostoc mesenteroides subsp. mesenteroides, accession number JF313445 and CWBI-B1470 Leuconostoc pseudomesenteroides, accession number JF313454.

The present paper deals with the main technological properties used to characterize kivuguto milk using these strains. Additionally, a sensory analysis was performed by an untrained panel as a validation test of similarities between the kivuguto produced by the selected starter bacteria and the traditional one. Ultimately, the purpose of this work is the industrial production of this milk. To the best of our knowledge, no study has been conducted before with an investigation into the technological properties of this traditional milk. Moreover, our approach uses the best methodology for fermented milk formulation, as it analyzes four main technological properties after microbial selection.

2. Materials and Methods

2.1. Bacterial Strains and Milk Fermentation

The selected microorganisms for fermenting kivuguto were preserved at -80°C at the CWBI Collection (Walloon Center of Industrial Biology) and registered in the Genbank database. The three strains were CWBI-B1466 Lactococcus lactis, registered under Genbank accession number JF313446, CWBI-B1465 Leuconostoc mesenteroides subsp. mesenteroides, accession number JF313445 and CWBI-B1470 Leuconostoc pseudomesenteroides, accession number JF313454. Prior to this experiment, assays were performed with many fractions of each strain to find a fermented milk similar to kivuguto; one formulation was retained for further analyses. Attention was paid to the enumeration data obtained during the selection, as a reference proportion. The incubation temperature was fixed to 19°C. The flasks were then inoculated until a pH of ≈4.5 was reached and thereafter stored at 4°C before analyses. The preparation of samples was made using four milks: 1° M1: whole milk fermented with the mixed kivuguto starters composed of three selected strains CWBI-B14166 Lactococcus lactis, CWBI-B1465 Leuconostoc mesenteroides and CWBI-B1470 Leuconostoc pseudomesenteroides at a ratio of 40%, 35% and 25%, respectively. The pre-culture was inoculated at $10^6$ cells.g$^{-1}$ as the initial concentration. This inoculum was used to culture at 4% (vol/vol) in 2 L of UHT milk in a 5 L flask.

2° M2: whole milk fermented with strain CWBI-B1466 Lactococcus lactis at $10^6$ cells.g$^{-1}$ as the initial concentration for pre-culture and used as an inoculum at 4% (vol/vol) in 2 L of UHT milk (ultra high temperature) in a 5 L flask.

3° M3: whole milk fermented with strain CWBI-B1470 Leuconostoc pseudomesenteroides at $10^6$ cells.g$^{-1}$ as the initial concentration for pre-culture and used as an inoculum at 4% (vol/vol) in 2 L of UHT milk in a 5 L flask.

4° M4: UHT milk used for fermentation. Tests were carried out without inoculation.

2.2. Acidification and Enumeration

Milk cultures of each sample were inoculated at ambient temperature and the pH (WTW pH351i pH meter, Weilheim, Germany) and titratable acidity (°D) were measured after incubation time by titrating a 10 mL sample with NaOH (1/9N) using phenolphthalein as the indicator. The titratable acidity was measured in Dornic degrees (°D); under such conditions, 1°D is equivalent to 0.1 mL of NaOH, i.e. 0.1 g lactic acid per kg of milk. For the biomass count, suitable dilutions were made and plated on MRS agar medium. Results were the average of three independent measurements.

2.3. Rheological Properties

The rheology characterization of the kivuguto milk was performed by a description of its viscoelastic behavior using an oscillatory model rheometer. The rheological parameters storage modulus (G') and loss modulus (G'') were followed as a function of time at 10°C using a high resolution Bohlin CVO 120 rotational rheometer (Malvern Instruments, Worcestershire, UK). The measuring geometry employed was a rotating upper cone and a fixed lower plate ($\alpha=4°$, $\Omega=40$ mm). The oscillation frequency was 1.0 Hz and the shear stress was 1 Pa, which was found to be within the linear viscoelastic region of fermented milk samples according to Stern et al. [15]. Three replicates were assessed.

2.4. Proteolysis

The peptides/free amino acids (FAAs) accumulated in
milk after the incubation time as a consequence of the proteolytic activity of the tested strains were determined using the o-phthalaldehyde (OPA) method [16]. This method is based on the reaction of OPA and 2-mercaptoethanol with the α-amino groups released during the hydrolysis of milk proteins. They form a complex which absorbs strongly at 340 nm. The OPA solution is obtained by combining the following reagents and completing a volume of 50 mL with water: 25 ml of 100 mM sodium tetraborate (Sigma Aldrich, Diegem, Belgium), 2.5 ml of 20% SDS (Merck, Darmstadt, Germany), 40 mg OPA (Sigma Aldrich, Diegem, Belgium) dissolved in 1 mL of methanol and 100 µL of 2-mercaptoethanol (Sigma Aldrich, Diegem, Belgium). This reagent should be prepared on the same day as the assay. To measure the proteolytic activity, with milk protein as the substrate, a 150 µL aliquot (of a 1:25 dilution of milk in 1% SDS) was added to 3.0 mL of the OPA reagent. The solution was stirred by inversion and incubated for 2 minutes at room temperature, and the absorbance is measured at 340 nm using a Genesys 10S VIS spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA).

The results from triplicates were calculated by a standard curve obtained from the dilution of leucine in distilled water and expressed in leucine equivalents (mg.L\(^{-1}\)) (Sigma Aldrich, Diegem, Belgium) in milk.

2.5. Aroma Compounds

**Headspace sample preparation.** Headspace (HS) samples were prepared manually. A 10 g milk sample was introduced in a 20-mL HS vial (Filter Service, Eupen, Belgium), hermetically sealed with a polytetrafluoroethylene-coated rubber septum and an aluminum cap (Filter Service, Eupen, Belgium). The sample was kept at 4°C after fermentation for a short time before analysis. Otherwise, samples were kept at -20°C and put at 4°C the day before analysis. Samples were equilibrated for 65 min at 70°C prior to analysis and the volatile compounds trapped in the headspace region of the vial (2000 µL) were collected with a microsyringe (Filter Service, Eupen, Belgium) and analyzed by gas chromatography (GC) using direct gas injection.

**Gas chromatography.** Milk sample volatiles (2000 µL) were injected into an Agilent Technologies 7890A GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a 30 m x 250 µm x 0.25 µm VF-WAX polar column (Agilent Technologies) was used for the study. Helium was used as the carrier gas, at a flow rate of 1.5 ml. min\(^{-1}\); splitless mode was used. The following temperature program was used: 50°C for 6 min, increased to 180°C for 5 min at a rate of 8°C.min\(^{-1}\) and held 10 min at 15°C.min\(^{-1}\) from 180 to 250°C. The injector and detector temperatures were 220°C and 250°C, respectively.

**Mass spectrometry analysis.** The volatile compounds were identified by mass spectrometry (MS) using an Agilent Technologies 5875C with Triple-Axis Detector coupled to 6890 GC System (Agilent Technologies). MS was carried out in electron ionization (EI) mode, with an ionization potential of 70 eV and ionization current of 2 A. The ion source temperature was 200°C, the resolution was 1000 and the mass range was 30 to 450 m/z.

**Chemical identification.** Compounds were identified by comparing recorded mass spectra with the Wiley 275L mass spectra library (Scientific Instrument Service, Ringoes, NJ, USA), the NIST MS Library (NIST, Gaithersburg, MD, USA), the PAL 600K mass spectral library (Palisade Corporation, Ithaca, NY, USA) and those in the literature, as well as a comparison of their retention times with authentic standards of a saturated \(n\)-alkanes standard solution (C\(_7\)-C\(_{30}\) alkanes) (Sigma Aldrich, Diegem, Belgium) as external references under the same chromatographic conditions, allowing for the calculation of the Kovats index [17] of the separated volatile compounds [18].

**Standard solutions and quantification.** Aqueous solutions of acetic acid, pentan-1-ol, and methyl benzoate as external standards were prepared from high purity chemicals (>99%) purchased from Sigma-Aldrich (Diegem, Belgium). 40 µL of each standard was accurately weighed and diluted in 100 mL in double distilled water and thereafter mixed at a ratio of 1:1. The prepared solution was hermetically sealed in 20 mL headspace vials at -20°C until they were used. Quantification of compounds was calculated by the external standard technique. The mean results of three assays were used to calculate the response factors corresponding to mean peak area for each standard compound, and the amount of each compound in the sample was calculated according to the known amount standard and its peak area. Moreover, the response factors of pentan-1-ol and methyl benzoate were used to calculate the concentrations of 3-methylbutan-1-ol and furanmethan-2-ol and 1,7,7-trimethylbicyclo[2.2.1] hept-2-yl acetate and furan-(2H)-one, respectively.

2.6. Sensory Analysis

A modified triangle test allowed eight panelists who were untrained but familiar with kivuguto milk to differentiate between samples and to identify the sample most similar to kivuguto according to de Lacharlerie et al. [19] in a discriminative test. The test was sought to determine between two products, A and B, which were similar to kivuguto, one of the two being kivuguto. Samples were presented in three-digit coded cups with two samples per set given to each subject. The principle is that subjects receive three samples. Two are the same product (A or B), while the third is different. Therefore, there are two options for the three samples: AAB or ABB. The randomized presentation order uses the six possible combinations of the triplet on each plate: AAB, ABA, BAA, BAB, BBA, ABB. Two sets were consecutively evaluated by the panelists and the first set was kivuguto (M1) versus the reference milk.
sold on the Rwandan market, whereas the second set was kivuguto (M1) versus yogurt, also sold on the Rwandan market. The UHT milk used to ferment the milk also came from Rwanda. Results were calculated using a table of critical values for the triangle test for differences, with the table built on a binomial distribution with EXCEL IV for the estimation of statistical significance in the sensory evaluation.

3. Results & Discussion

3.1. Cultures and Fermentation

Fermentations assays were performed with single or mixed kivuguto starters to ferment raw sterile milk. Both the mixed or single cultures started with an initial inoculation of $10^6$ freeze-dried cells in a pre-culture made in a 250 mL flask. The best mixture was composed of 40% Lactococcus lactis, 35% Leuconostoc mesenteroides subsp. mesenteroides and 25% Leuconostoc pseudomesenteroides. Single and/or mixed fermentations were carried out in 2 L (4% vol/vol) of UHT milk. Acidification data are presented in Table 1.

The fermentation time in hours ended at the time when the acidification reached pH $\approx 4.55$. The data show that the single cultures ended the fermentation at 14 h and 20 h for Lactococcus and Leuconostoc, respectively. For the mixed fermentation (kivuguto), the fermentation time was 14 h. The maximum velocity $V_m$, i.e. the lactic acid produced per liter per hour after milk inoculation, was equal for kivuguto and the best acidifier (Lactococcus), i.e. 0.26 and 0.25 g.L$^{-1}$.h$^{-1}$ respectively. This data is an indication of a positive interaction of Leuconostoc cells in milk with Lactococcus, as the percentage of the inoculum of Leuconostoc cells is 60% in kivuguto, whereas in the single fermented milk of Lactococcus the inoculum was 100%. This means that the acidifying activity of 40% Lactococcus in kivuguto is equally balanced by 60% Leuconostoc. The $V_m$ of Leuconostoc pseudomesenteroides was 0.17 g.L$^{-1}$.h$^{-1}$. Note that the lag phase was 4 h for kivuguto and Lactococcus milks, and 7 h for Leuconostoc pseudomesenteroides milk, meaning that Lactococcus is more active than Leuconostoc after inoculation. Juillard et al. [20] found the same result when culturing milk with mixed thermophile lactic acid bacteria. In addition, the lactic acid produced in mixed cultures was greater than that in single cultures (7.3 g.L$^{-1}$ for kivuguto, 7.2 g.L$^{-1}$ for Lactococcus milk and 7.0 g.L$^{-1}$ for Leuconostoc milk). Oliveira et al. [21] found two-fold a many lactic acid bacteria produced by mixed cultures compared to single culture milks using Bifidobacteria cells. The cell count at the end of fermentation ranged from 3.3 $10^9$ to 4.2 $10^9$ cfu.mL$^{-1}$ for the three samples.

3.2. Rheological Analyses

Small deformation oscillatory testing was applied for the evaluation of kivuguto milk rheology. The $G'$ and $G''$ data evolution over time are presented in Figures 1, 2 and 3.

Table 1. Acidification data of milk fermented by selected kivuguto strains at 19°C

| Culture                      | $T_L$ (h) | $T_f$ (h) | $V_m$ (g.L$^{-1}$.h$^{-1}$) | pH$_f$ | $A_f$ (°D) |
|------------------------------|-----------|-----------|-----------------------------|--------|------------|
| kivuguto                     | 4         | 14        | 0.26                        | 4.5-4.6| 73±4       |
| Lactococcus lactis           | 4         | 14        | 0.25                        | 4.5-4.6| 72±5       |
| Leuconostoc pseudomesenteroides | 7         | 20        | 0.17                        | 4.5-4.6| 70±4       |

$h=$hour; $T_L=$ lag phase; $T_f=$ fermentation time at pH$_f$ = 4.5 (final pH), time required for the pH to reach 4.5; $V_m=$ maximum acidification velocity at pH$_f$ in g of lactic acid per liter per hour; $A_f=$titratable acidity in °D at pH$_f$. 

Figure 1. Elastic moduli and loss moduli evolution (time in seconds) of the strain CWBI-B1470

The results show an increase up to 10 min for both $G'$ and $G''$. First, the experiment was applied with single strains of kivuguto milk, i.e. fermentative strains of CWBI-B1466 Lactococcus lactis and CWBI-B1470 Leuconostoc pseudomesenteroides. The results show in two cases that $G'>G''$ and never $G''>G'$, meaning that no liquid form was described by the two milks. Instead, the two milks showed an increase up to 10 min of $G'$ and $G''$, with a higher value of $G'$ in the milk fermented with CWBI-B1470 Leuconostoc pseudomesenteroides than that with CWBI-B1466 Lactococcus lactis.
characterized of G' and G'' of G''. The data from these experiments show that the values CWBI-B1466 B1470 G'>G'' and the two types of milk fermented with single strains is that characterize the non-fermentative strain CWBI-B1465 storage moduli and loss moduli in Lactococcus lactis and the firmer the gel. The data given by the two strains the fermentation of a mixture of the two strains with a third pseudomesenteroides was similar to CWBI-B1466 Leuconostoc kivuguto starter. The behavior of the storage moduli and loss moduli in kivuguto was similar to the two types of milk fermented with single strains is that G'>G'' and the kivuguto storage moduli G'< to G' of CWBI-B1470 Leuconostoc pseudomesenteroides, but > to G' of CWBI-B1466 Lactococcus lactis milk. This is the same for G''. The data from these experiments show that the values of G' and G'' of kivuguto were slightly below those of CWBI-B1470 Leuconostoc pseudomesenteroides and characterized kivuguto as a viscoelastic milk.

It is known that the rheological properties of yogurt depend essentially on the ratio of total solids to the milk composition (protein, salts), to milk homogenization, to the type of culture, to acidity (pH), to proteolysis and to the heat treatment undergone by the milk [23,24]. This was the same for G''. Previous studies have shown that lactic acid bacteria produce exopolysaccharides in milk, especially dextrans [15].

Additionally, the incorporation of cultures producing exopolysaccharide into dairy foods can provide viscosifying, stabilizing and water binding functions [25]. The Leuconostocs are good producers of exopolysaccharides (EPS), especially dextrans, which increase the viscosity of milk. The two Leuconostocs composing the kivuguto starter culture are EPS producers, as underlined in previous studies.

Leuconostocs also produce other EPS, like α-glucooligosaccharides (GOS) from maltose or isomaltose, which can be used as thickeners or texturizers in cultured milk or as stabilizers [26,27]. Glycolysis by the strain of Lactococcus reduced the pH and consequently the coagulation of milk by modification of the casein structure. Very interestingly, CWBI-B1470 Leuconostoc pseudomesenteroides is involved in three catabolic pathways: acidification by lowering the pH, dextran production by increasing viscosity and aroma generation through citrate metabolism, whilst CWBI-B1465 Leuconostoc mesenteroides subsp. mesenteroides is only a dextran and aroma producer.

According to Kristo et al. [22], the higher G' and lower the tan δ values, the more solid-like the character of the gel, and the firmer the gel. The data given by the two strains cultured alone show that the milk with the highest value of tan δ was the CWBI-B1470 Leuconostoc pseudomesenteroides milk rather than the CWBI-B1466 Lactococcus lactis milk. Second, the experiment assessed the fermentation of a mixture of the two strains with a third non-fermentative strain CWBI-B1465 Leuconostoc mesenteroides subsp. mesenteroides, as the three strains characterize the kivuguto starter. The behavior of the storage moduli and loss moduli in kivuguto was similar to the two types of milk fermented with single strains is that G'>G'' and the kivuguto storage moduli G'< to G' of CWBI-B1470 Leuconostoc pseudomesenteroides, but > to G' of CWBI-B1466 Lactococcus lactis milk. This is the same for G''.

The proteolysis of milk protein was assessed using the rapid and simple OPA-based spectrophotometric assay [16]. Measurements were performed at inoculation and at the end of fermentation. The data are presented in Table 2 and show the differences for each fermented milk.

A sample of the sterile milk M4 (not inoculated) was also analyzed (results not shown). In this study, the hydrolysis of milk protein by the mixed cultures of kivuguto (Milk M1) released 3.04 mg/L of peptides/FAAs after the fermentation time, whereas the milk from single fermentation M2 (Lactococcus) and M3 (Leuconostoc) gave 3.11 and 5.45 mg/L, respectively. The proteolysis analysis revealed greater production in the mixed milk than in the single fermented milks. This may be due to the third strain Leuconostoc mesenteroides which is not fermentative when cultured alone. These data show low concentrations of peptides for all samples, meaning low proteolysis activity. This is very important because high proteolysis values are an indication of bitter peptide production. These values are very similar to those reported by other authors in various fermented products.

Thivierge [28] found an increase in milk proteolysis ranging from 0.33 to 64.58 mg/L with 26 isolates of Lactococcus spp. More analyses allowed the selection of only strains with proteolysis values of 0.33 to 7.70 mg/L as presumptive cheese starter cultures. Canas et al. [29] obtained an increase of 6.50 mg/L in total amino acids during spontaneous malolactic fermentation of wine by...
yeasts and *Oenococcus oeni*. Production of extracellular peptidases and proteases, which are secreted by some strains of *Oenococcus oeni* [30], could have contributed to this increase.

| Milk | Concentration of peptides/free amino acids at the fermentation time T (mg/L) |
|------|-----------------------------------------------------------------------------|
| M1   | 3.04 ± 0.73                                                                |
| M2   | 3.11 ± 0.30                                                                |
| M3   | 5.45 ± 0.16                                                                |
| M4   | < 0                                                                          |

M1 mixed culture; M2 Lactococcus culture, M3 Leuconostoc culture and M4 without culture

**Table 2.** Proteolysis of milks fermented by *kivuguto* strains

*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* have been reported to express cell-surface proteinases. However, the free amino groups found in milk were very low compared with those of the sample made with the addition of a protease [31]. Prior to performing analyses, Abdel Rahman et al. [32] filtered camel milk samples using Whatman filter paper and found about 60-fold of our results. Moreover, the amount of free amino acids was higher in a mixed starter cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* than in the corresponding single cultures. Fortina et al. [33] obtained low proteolytic activities for most strains of *Lactococcus garvieae* to be used as a dairy starter culture. The mean value was 42.7 g.L⁻¹.

### 3.4. Aroma Compound Analysis

Seven compounds were identified by the comparison of their mass spectra with those described in three libraries, with data from the literature and with Kovats index (KI) of standard compounds run under similar conditions [34]. The results (Table 3) are reported together with their relative area and estimated concentrations: 3-methylbutan-1-ol, pentan-1-ol, acetic acid, methyl benzoate, furanmethan-2-ol, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl acetate, and furan-2(5)H-one were clearly identified. Other compounds were also detected, but their signal/noise ratios and their very low concentrations prevent unambiguous identification. Two compounds (namely methyl benzoate and 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl acetate) belong to the UHT milk as they were found in the four samples used for analyses, and are therefore not discussed in this work. The three strains produce aroma compounds: CWBI-B1466 *Lactococcus lactis* produced pentan-1-ol and 3-methylbutan-1-ol; CWBI-B1470 *Leuconostoc pseudomesenteroides* also produced pentan-1-ol and 3-methylbutan-1-ol; CWBI-B1465 *Leuconostoc mesenteroides* subsp. *mesenteroides* contributed with the rest of the molecules found in *kivuguto*, i.e. acetic acid, furanmethan-2-ol, furan-2(5)H-one. This consequently showed a high potential as a flavoring strain which is what it had been selected for; we were sure that it was not a fermentative strain, but might have a huge technological impact on *kivuguto* milk production. Our results show that *kivuguto* is characterized by a typical profile of volatile components from various sources like acids, alcohol, lactone, esters, etc., resulting from the various metabolic pathways of the strains of *Lactococcus* and *Leuconostoc*. Considering the *kivuguto* flavor profile, we can suggest that the selected *kivuguto* strains have their own amino acid convertases, which matches with their ability to produce unusual flavors [35]. The esterification of 3-methylbutan-1-ol/pentan-1-ol from acetic acid gives isoamyl acetate/amyl acetate (pentyl acetate), which smell like banana. According to Arcander [36], acetic acid has an acid taste perceptible well below 1% in water. It has a vinegary, pungent aromatic note with a perception threshold of 22 to 54 ppm in water [37,38] and 3 to 7 ppm in butter [37, 39-41]. Also, lactones are known as a source of pleasant milk flavor. Gadaga et al. [42] evaluated *amasi* milk and found acetaldehyde, ethanol, acetone, 2-methyl propanol, 2-methylpropan-1-ol, 3-methyl butanal and 3-methylbutan-1-ol as important compounds in the flavor profile of this naturally fermented milk of Zimbabwe. Among these molecules, the most important were ethanol and 3-methylbutan-1-ol. Note that the *amasi* starter contains nine yeasts and four lactic acid bacteria. Our results are likely in accordance with Ayad et al. [35] who reported that wild strains of *Lactococcus lactis* are able to produce flavors different from those produced by industrial strains, mostly methyl alcohols and methyl aldehydes. 3-methylbutan-1-ol/pentan-1-ol can be derived from the reduction of the aldehydes formed via Strecker degradation from the amino acids: alanine, valine and leucine [43]. These free amino acids are catalyzed by casein proteolysis. Thereafter, acetaldehyde quickly oxidizes into acetic acid in the presence of aldehyde dehydrogenase (AL-DH) via nicotinamide-adenine dinucleotide (NAD) [44]. 3-methylbutan-1-ol/pentan-1-ol can also be derived from lactose fermentation. Alonso & Fraga [10] used the same method to analyze yogurt flavor compounds and obtained acetaldehyde, acetone, butanone, butan-2,3-dione (diacetyl), 3-hydroxybutanone (acetoin) and acetic acid. These compounds are the result of the catabolism of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as strains used to ferment yogurt. Apart from work on yeasts, there is little information on the production of furanones by microorganisms, although there is a suggestion that some species of *Lactobacillus* can produce the two forms of furanones [45]. Furanones provide the aroma found in Emmental cheese. According to Slaughter [46], two food-derived furanones have antioxidant activity comparable to that of ascorbic acid.
Table 3. Headspace analyses of volatile compounds (VCs) in *kivuguto* milk

| CAS Number | IUPAC Name                        | Identification | VCs in milk | Sample RI | Reference RI | Relative area (±SD, n=3) | Estimated concentration (mg/kg±SD, n=3) | Observed mass spectrum (m/z (%)) for >10% |
|------------|-----------------------------------|----------------|-------------|-----------|-------------|-------------------------|----------------------------------------|------------------------------------------|
| 8.46       | 123-51-3 3-methylbutan-1-ol       | MS, STD, RI    | +           | +         | 1202        | 1204<sup>a</sup>        | 11.53±0.64 0.051±0.003                 | 88(<1); 70(15); 69(54); 57(29); 55(100); 55(13); 45(11); 43(51); 41(62); 41(56); 38(25) |
| 8.92       | 71-41-0 pentan-1-ol               | MS, STD, RI    | +           | +         | 1226        | 1244<sup>b</sup>        | 11.53±0.64 0.083±0.001                 | 70(97); 56(29); 56(16); 55(100); 44(11); 43(52); 42(70); 40(71); 38(31) |
| 14.26      | 64-19-7 acetic acid               | MS, STD, RI    | +           | -         | 1473        | 1477<sup>c</sup>        | 4.37±0.38 0.154±0.014                 | 60(67); 45(94); 43(100); 41(19)          |
| 16.61      | 93-58-3 methyl benzoate           | MS, STD, RI    | +           | +         | 1613        | 1635<sup>d</sup>        | 3.99±0.005 0.079±0.001                 | 136(<1); 136(33); 105(100); 77(58); 51(21); 50(10); 42(14) |
| 17.14      | 98-00-0 furanmethan-2-ol          | MS, STD, RI    | +           | -         | 1650        | 1661<sup>e</sup>        | 39.9±1.59 0.177±0.007                 | 98(46); 94(10); 81(59); 69(30); 69(27); 54(12); 53(49); 51(18); 50(16); 42(11); 41(52); 41(42); 38(47); 38(14) |
| 17.91      | 92618-89-8 1,7,7-trimethyl[2.2.1]hept-2-yl acetate | MS, STD, RI | +           | +         | 1699        | 1584<sup>f</sup>        | 5.77±0.005 0.115±0.001                 | 196(<1); 137(14); 136(57); 121(52); 110(29); 109(30); 108(29); 106(12); 95(100); 94(12); 93(66); 92(20); 91(16); 83(14); 81(17); 79(12); 78(12); 77(12); 67(10); 66(26); 55(14); 58(66); 53(14); 44(13); 42(13); 40(47); 38(15) |
| 18.49      | 497-23-4 furan-2(5H)-one          | MS, STD, RI    | +           | -         | 1739        | -            | 15.68±1.03 0.312±0.020                 | 83(60); 54(100); 54(21); 39(21); 36(10) |

<sup>(1)</sup> Retention time; <sup>(2)</sup> CAS number of compounds listed in order of elution from a VF-Wax. Source: CAS SciFinder<sup>®</sup> (Chemical Abstracts Service, Columbus, USA); <sup>(3)</sup> Identification methods: MS, comparison of mass spectra with those in NIST08, Wiley275 and PAL 600K libraries; RI, comparison of retention indices with those in literature; STD, comparison of retention time and mass spectra of available standards; <sup>(4)</sup> M1. Milk with three *kivuguto* strains; M2. Milk with *Lactococcus lactis*; M3. Milk with *Leuconostoc pseudomesenteroides*; M4. Raw milk without strains; with such a profile, the third strain CWBI-B1465 *Leuconostoc mesenteroides* subsp. *mesenteroides* may have been responsible for the presence of acetic acid, furanmethan-2-ol and 2(5)H-furanone in *kivuguto* milk. <sup>(5)</sup> Retention indices on VF-Wax column experimentally determined using a saturated C7-C30 alkanes standard solution; <sup>(6)</sup> Kovats indices taken from the literature: <sup>a</sup> Fukami et al. [47] (measured with a TC-Wax column); <sup>b</sup> Umano et al. [48] (measured with a DB-Wax column); <sup>c</sup> Cullere et al. [49] (measured on a DB-Wax column); <sup>d</sup> Ferreira et al. [50] (measured on a DB-Wax column); <sup>e</sup> Wong & Bernhard [51] (measured on a DB-Wax column); <sup>f</sup> Davies [52] (measured on a Carbowax column); <sup>(7)</sup> Concentrations calculated in M1 milk; <sup>(8)</sup> EI, 70 eV, source at 220°C.
3.5. Sensory Analysis

The sensory properties of the samples were evaluated by panelists to find which sample was the closest to kivuguto milk. Samples were the milks made by selected kivuguto strains, yogurt and the commercial fermented milk sold in Rwanda. Panelists were assumed to have consumed the three milks. The first set was composed of a sample of kivuguto with yogurt and the second set of kivuguto and the fermented milk sold in Rwanda. Each set was assayed by the panel and each panelist tasted three cups distributed on a plate. In random order, the panel of eight subjects identified the kivuguto among the other samples with significant differences found for the first set (P=0.05) and the second set (P=0.01). The results show that the panel easily detected differences between samples. Moreover, they were able to recognize which sample was made by the kivuguto starter.

3. Conclusions

The characterization of kivuguto milk and milks fermented by two strains of kivuguto starter in monoculture was studied regarding four technological properties: acidification, rheology, proteolysis and flavor compounds. The results of this study allowed for the evaluation of the acidification level and counts of bacteria in milk made by selected kivuguto starters, as well as the viscoelastic properties. These properties showed how far the kivuguto rheology can be compared with other fermented milks, like yogurt, filmjölk and leben. Assessment of the static headspace by GC coupled to mass spectrometry is a suitable method for the extraction and analysis of volatile compounds in fermented milks and revealed the kivuguto aroma profile. The discrimination test by a sensory panel also detected differences in kivuguto milk compared to other milks. Our findings show that the selection procedure satisfactorily provided a starter culture for manufacturing kivuguto milk.

Acknowledgements

The authors wish to express gratitude to Gembloux Agro-Bio Tech ULG, the CWBI and the CUD for financial support. The authors gratefully appreciate the panelists for their participation in the sensory evaluation. The authors also acknowledge V. Hote, D. Trisman, F. Michels, P. Dzaomuho and S. Filocco for their technical assistance and Proof-Reading-Service (http://www.proof-reading-service.com/guide/index.html) for proof-reading the text.

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