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Volatile Organic Compounds in Naturally Fermented Milk and Milk Fermented Using Yeasts, Lactic Acid Bacteria and Their Combinations As Starter Cultures

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Summary

The volatile organic compounds present in 18 Zimbabwean naturally fermented milk (amasi) samples and those produced by various yeasts, lactic acid bacteria (LAB) and yeast/LAB combinations were determined using headspace gas chromatography. The yeast strains used were: *Candida kefyr* 23, *C. lipolytica* 57, *Saccharomyces cerevisiae* 71, *C. lusitaniae* 68, *C. tropicalis* 78, *C. lusitaniae* 63, *C. colliculosa* 41, *S. dairenensis* 32, and *Dekkera bruxellensis* 43, and were coded Y1 to Y9, respectively. The LAB strains used were *Lactococcus lactis* subsp. *lactis* Lc39, *L. lactis* subsp. *lactis* Lc261, *Lactobacillus paracasei* Lb11, and *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1, and were coded B1 to B4, respectively. Some of the volatile organic compounds found in amasi were acetaldehyde, ethanol, acetone, 2-methyl propanal, 2-methyl--1-propanol and 3-methyl-1-butanol. However, the levels of volatile organic compounds in the naturally fermented milk (NFM) samples varied from one sample to another, with acetaldehyde ranging from 0.1–18.4 ppm, 3-methyl butanal from <0.1–0.47 ppm and ethanol from 39.3–656 ppm. The LAB/*C. kefyr* 23 (B/Y1) co-cultures produced significantly ($p<0.05$) higher levels of acetaldehyde and ethanol than the levels found in the NFM. The acetaldehyde levels in the B/Y1 samples ranged from 26.7–87.7 ppm, with *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1 (B4) producing the highest level of acetaldehyde in combination with *C. kefyr* 23 (Y1). Using principal component analysis (PCA), most of the NFM samples were grouped together with single and co-cultures of Lc261, Lb11 and the non--lactose fermenting yeasts, mainly because of the low levels of ethanol and similar levels of 3-methyl butanal. Chromatograms of amasi showed prominent peak of methyl aldehydes and their alcohols including 3-methyl-butanal and 3-methyl-butanol, suggesting that these compounds are important attributes of Zimbabwean naturally fermented milk.

Key words: fermented milk, starter culture, volatile organic compounds, amasi

Introduction

Zimbabwean naturally fermented milk (NFM) is called amasi. It is produced by spontaneously fermented raw milk at ambient temperature in different types of containers including earthenware and metal pots (*1*). Lactic acid bacteria, yeasts and coliforms have been found to be present during the fermentation process (*1–3*) and therefore should contribute to the properties of the fermented product. Like other similar spontaneously fer-

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mented products, the specific microflora in amasi is variable from one fermentation cycle to the next. This in turn results in fermented products that have unstable quality.

Mutukumira (*4*) described amasi as being creamy and highly viscous, with a mildly acid taste. In a separate study, sixteen descriptive terms were developed to organoleptically characterise amasi, which showed that the naturally fermented product is different from products that could be prepared using strains of bacteria and yeasts isolated from NFM (*5*). In addition, the NFM was preferred by people compared to fermented milk made using an imported mesophilic starter culture (*6*). This difference in taste was attributed to the presence of different microorganisms. For future technological development of fermented milk products similar to amasi, it is necessary to relate the production of specific compounds which contribute positively or negatively to the organoleptic properties of the product to particular organisms. The objective of the present study was to detect the types of volatile organic compounds present in NFM and in milk fermented by selected strains of yeasts and lactic acid bacteria, and to establish any similarities between the two types of products.

Materials and Methods

Collection of samples

Eighteen naturally fermented milk samples (200 mL) were collected in sterilized plastic bottles from homesteads and milk collection centres around Harare. These samples were frozen (–30 °C) and then transported in cooler boxes lined with ice packing to the Department of Food Science, Agricultural University of Norway (AUN). On receipt at the AUN the samples were immediately frozen (–30 °C), until they were used for determination of volatile organic compounds.

Preparation of concentrated yeast cultures

Actively growing cultures of yeasts previously isolated and identified from Zimbabwean NFM (*2*) were inoculated into malt extract broth (Merck, Damstardt, Germany) (200 mL) in a 250-mL screw capped bottle and incubated in a water bath at 25 °C for 72 h. The yeast strains used were: *Candida kefyr* 23, *C. lipolytica* 57, *Saccharomyces cerevisiae* 71, *C. lusitaniae* 68, *C. tropicalis* 78, *C. lusitaniae* 63, *C. colliculosa* 41, *S. dairenensis* 32, and *Dekkera bruxellensis* 43, coded as Y1 to Y9, respectively. The cultures were then centrifuged at 5 000 × g in a refrigerated (4–10 °C) centrifuge (Sorval 5RB, Du Pont Instruments, Delaware, USA). The pellet was resuspended in sterile reconstituted (10 %) skimmed milk (20 mL) containing 10 % glycerol and then stored at –80 °C. This was later used as a Direct Vat Set (DVS) culture for inoculating UHT milk. The concentrated culture contained about 7 log (CFU/mL) of yeasts.

Preparation of concentrated lactic acid bacteria cultures

Concentrated cultures of the lactic acid bacteria (LAB) strains *Lactococcus lactis* subsp. *lactis* Lc39, *L. lactis* subsp. *lactis* Lc261, *Lactobacillus paracasei* Lb11 and *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1, (coded B1, B2, B3, B4, respectively), also previously isolated from NFM (*5,7*), were prepared. The LAB were inoculated into M17 broth (250 mL) (Oxoid Unipath Ltd, Hampshire, England) and incubated at 30 °C for 24 h. The cultures were then centrifuged in a similar way to the yeast culture, resuspended in reconstituted (10 %) skimmed milk (25 mL) and stored at –80 °C. These were later used as a Direct Vat Set (DVS) cultures for inoculating UHT milk. The concentrated cultures contained about 9 log (CFU/mL) of LAB.

Inoculation of UHT milk with DVS cultures

Small volumes of each of the thawed concentrated yeast and LAB cultures, calculated to contain about 5 and 7 log (CFU/mL) of yeast and LAB, respectively, were added to 40 mL of UHT milk (Tine Norske Meierier AS, Oslo, Norway). The yeast-LAB combinations are shown in Table 1. The inoculated milk samples were incubated at 25 °C for 48 h.

Determination of volatile organic compounds

Volatile organic compounds in the NFM, LAB and yeast-LAB fermented milk samples were determined using headspace gas chromatography (HS-GC) as described by Narvhus *et al.* (*8*). Briefly, a portion (10 g) of each sample was weighed into a headspace vial (20 CV, Chromocol, Welwyn Garden City, UK) and sealed with a PTFE-coated septum and aluminium ring (20-CBT3 and 20-ACB, Chromocol). The samples were analysed using a DANI HSS automatic headspace sampler (DANI, Monza, Italy) connected to a Carlo Erba 5300 gas chromatograph (Carlo Erba Instruments, Milan, Italy) fitted with a flame ionisation detector. The volatile organic compounds were separated on a Supelco SPC-1 GC column: 30 m × 0.53 mm i.d., film thickness 5 μm (Supelco, Bellefonte, PA,

Table 1. The lactic acid bacteria (LAB) and LAB/yeast cultures used to simulate the characteristics of naturally fermented milk

LAB strain	Yeast strain								
	Y1	**Y2**	**Y3**	**Y4**	**Y5**	**Y6**	**Y7**	**Y8**	**Y9**
B1	B1/Y1	B1/Y2	B1/Y3	B1/Y4	B1/Y5	B1/Y6	B1/Y7	B1/Y8	B1/Y9
B2	B2/Y1	B2/Y2	B2/Y3	B2/Y4	B2/Y5	B2/Y6	B2/Y7	B2/Y8	B2/Y9
B3	B3/Y1	B3/Y2	B3/Y3	B3/Y4	B3/Y5	B3/Y6	B3/Y7	B3/Y8	B3/Y9
B4	B4/Y1	B4/Y2	B4/Y3	B4/Y4	B4/Y5	B4/Y6	B4/Y7	B4/Y8	B4/Y9

Key: LAB strains: B1 *Lactococcus lactis* subsp. *lactis* Lc39, B2 *L. lactis* subsp. *lactis* Lc261, B3 *Lactobacillus paracasei* Lb11, B4 *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1; yeast strains: Y1 *Candida kefyr* 23, Y2 *C. lipolytica* 57, Y3 *Saccharomyces cerevisiae* 71, Y4 *C. lusitaniae* 68, Y5 *C. tropicalis* 78, Y6 *C. lusitaniae* 63, Y7 *C. colliculosa* 41, Y8 *S. dairenensis* 32, Y9 *Dekkera bruxellensis* 43

USA) using nitrogen as a carrier gas at a flow rate of 5 mL/min. The temperature programme was as follows: 53 °C for 1 min, increase at a rate of 15 °C/min to 70 °C for 2 min, increase at 22 °C/min to 130 °C for 3 min. Samples were equilibrated for 45 min at 50 °C before headspace samples were taken. Detector response was monitored by Turbochrom Ver. 4.1 (Perkin Elmer, Shelton, Connecticut, USA). The analysis was externally calibrated using standard solutions prepared in sterile reconstituted (10 %) skimmed milk of the following compounds: acetaldehyde (Fluka, Buchs, Switzerland), ethanol (Vinmonopolet, Oslo, Norway), acetone (Tokyo Kasei, Japan), diacetyl (Sigma-Aldrich, St Louis, MO, USA), acetoin (Merck), 3-methyl butanal, 3-methyl-1-butanol, 2-methyl butanal, 2-methyl-1-butanol, 2-methyl pentanal, 2-methyl-1-pentanol (Sigma-Aldrich Chemie, Germany). Raw milk was used as a blank. All the determinations were done in duplicate.

Statistical analysis

Principal component analysis (PCA) (UNSCRAMBLER® 7.01, Camo A/S, Trondheim, Norway) was performed on the data in order to group the samples according to the types and levels of volatile organic compounds (VOC). Interpretation of the data was made by inspection of the scores and loadings bi-plots.

Results and Discussion

The levels of VOC in the naturally fermented milk are shown in Table 2. The VOCs varied greatly from sample to sample. For example, the levels of acetaldehyde ranged between 0.1 and 18.4 ppm and ethanol ranged between 39.3 and 656 ppm. Since the NFM samples were not standardised, the variation in VOCs was expected. However, the wide range suggests greater diversity in terms of the microflora and fermentation conditions. For example, it has been reported that some households in Zimbabwe remove serum (whey) over a prolonged period of time in order to obtain a product with thicker consistency (*4*), probably resulting in higher levels of VOCs. The chromatograms in Fig. 1 suggest that acetaldehyde, ethanol, acetone, 2-methyl propanal, 2-methyl-1-propanol, 3-methyl butanal and 3-methyl-1-butanol are important compounds in the flavour profile of NFM. A number of unidentified peaks for NFM were also observed. Tables 3 and 4 show the levels of some of the volatile organic compounds produced by the pure LAB, yeast and LAB/yeast cultures. The LAB/Y1 cultures had significantly ($p<0.05$) higher levels of ethanol and acetaldehyde than the NFM, while the levels produced by the other LAB/yeast combinations were within the same range as those in NFM. Raw milk had negligible levels of some of the volatile organic compounds, indicating that the levels recorded in the fermented milks were produced during the fermentation process.

Volatile organic compound data from 18 NFM samples and 49 LAB, yeast/LAB and single yeast cultures were clustered using principal component analysis (PCA) (data not shown). It was observed that most of the NFM samples (66 %) were grouped together with most of the pure yeast and yeast/LAB cultures, specifically B3, B3/yeast, B2, and B2/yeast. The PCA could not resolve these samples, suggesting that they were the same in most respects.

As shown in Tables 2 and 3, these samples contained relatively low levels of most of the VOCs. How-

Table 2. Volatile organic compounds detected in Zimbabwean naturally fermented milk samples collected from homes and milk collection centres

Sample*	*w*(volatile organic compounds)/ppm									
	Ethanol	Acetaldehyde	Acetone	Acetoin	2-methyl propanal	2-methyl propanol	3-methyl butanal	3-methyl butanol	2-methyl butanol	Diacetyl
A1	57.3	0.4	2.5	14.3	0.04	0.65	0.07	2.44	0.04	0.24
A2	656.0	0.1	0.9	98.4	0.07	0.16	nd	0.39	0.01	4.62
A3	139.3	0.4	2.9	140.2	0.04	0.16	0.04	0.85	0.07	0.39
A4	59.7	0.1	7.6	20.3	0.01	0.07	0.01	0.18	0.03	0.14
A5	144.8	0.7	0.3	35.5	0.02	0.07	0.01	0.21	0.02	1.23
A6	149.2	0.1	0.6	11.2	0.01	0.01	nd	0.06	0.01	0.13
A7	192.6	0.2	0.4	7.6	0.01	0.21	0.02	0.37	0.05	0.30
A8	79.2	0.2	1.3	11.0	0.02	0.12	0.04	0.77	0.08	nd
A9	162.8	0.5	2.6	88.4	0.03	0.13	0.07	0.89	0.07	1.59
A10	228.6	0.5	1.1	7.1	0.25	0.71	0.47	2.03	0.11	0.20
A11	39.3	18.4	2.3	59.1	0.53	1.17	0.03	3.37	0.38	1.15
A12	170.8	0.1	1.0	9.9	0.01	0.09	nd	0.12	0.03	0.19
A13	113.6	2.3	0.5	8.0	0.01	0.07	0.01	0.15	0.04	0.08
A14	140.4	0.2	0.6	26.0	nd	0.23	0.01	0.85	0.18	0.11
A15	287.2	0.4	10.1	52.3	0.01	0.17	nd	0.39	0.08	0.58
A16	73.0	0.5	3.6	24.8	0.05	0.35	0.04	0.94	0.09	0.56
A17	76.2	0.5	2.3	32.1	0.02	0.25	0.04	0.55	0.12	0.77
A18	100.6	0.8	1.6	31.1	0.15	0.63	0.42	1.30	0.25	0.31

*Naturally fermented milk samples; nd=not detected

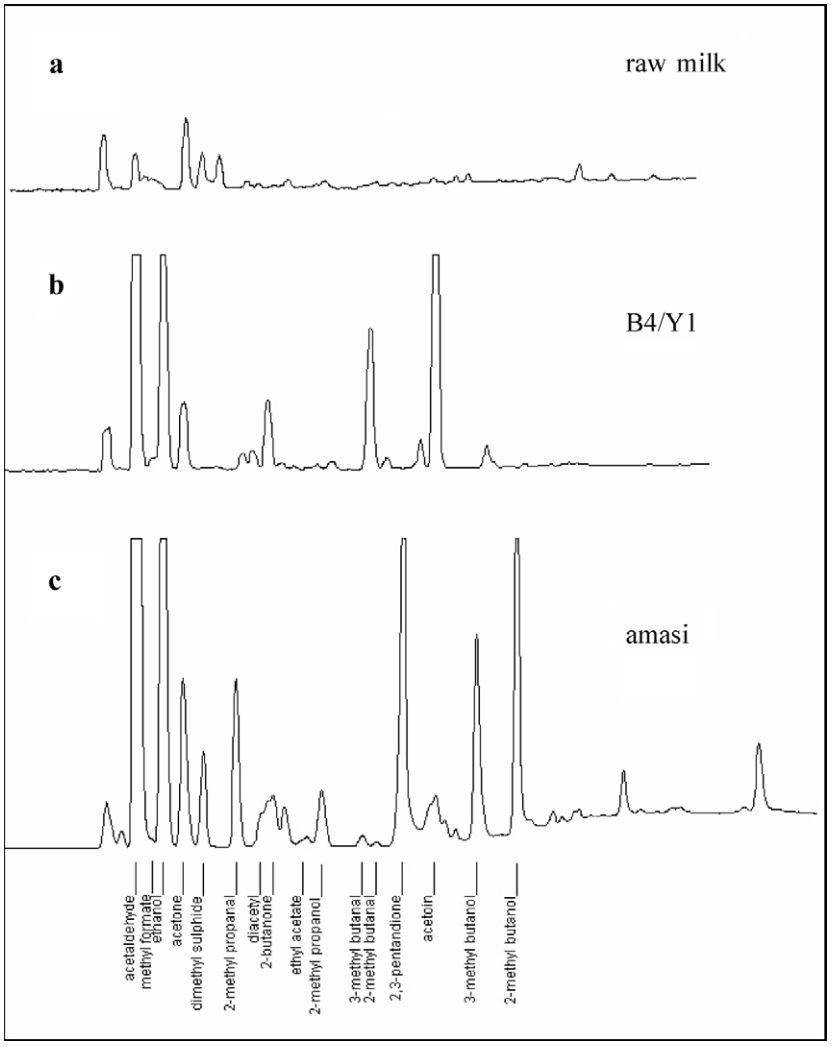

Fig. 1. Gas chromatograms showing the volatile organic compound profiles of raw milk, amasi and milk fermented using a combination of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* C1(B4) and *Candida kefyr* 23 (Y1)

ever, they had levels of 3-methyl butanal ranging between 0.01 and 0.26 ppm. Aldehyde 3-methyl butanal has a lower taste threshold than the alcohol and therefore this compound could be an important characteristic in NFM. Amasi showed the presence of many more volatile compounds when compared to the B4/Y1 fermented milk (Fig. 1), and the malty aldehydes and alcohols showed prominent peaks in most NFM samples and the yeast/ /LAB combinations (Fig. 1, Tables 2 and 3).

All samples that contained B1 (*L. lactis* subsp. *lactis* Lc39) had high levels of methyl aldehydes and their respective alcohols, confirming earlier reports on this strain (*9*). In addition, the yeast *C. kefyr* 23 resulted in the production of high levels of acetaldehyde and ethanol, and the products containing this strain were different from the NFM.

Therefore, it can be deduced from these observations that 3-methyl butanal and the other malty compounds were important in characterizing NFM and could be responsible for imparting a malty taste to the fermented milk. This confirms earlier observations by Narvhus and Gadaga (*10*) that a mild malty taste is characteristic of many African NFM products. These malty compounds are formed from branched chain amino acids by strains of *Lactococcus* and *Lactobacillus* (*8*,*11*,*12*).

Although the presence of yeasts in fermented milk is important because they produce carbon dioxide and ethanol that give a desirable freshness to the fermented milk, high levels produced by the lactose-fermenting *C. kefyr* 23 gave a totally different product compared to the NFM. Because of the frequent co-occurrence of yeasts and LAB in NFM, other interactions that are beneficial to product characteristics may be more important (*9*). In an earlier work, coliforms and some pathogens were also isolated from Zimbabwean NFM (*3*), suggesting that their metabolites may also influence the flavour profile of the fermented milk.

In previous studies, the *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1 strain had shown promise as a possible starter culture that can produce desirable fermented milk similar to NFM (*8*,*9*), principally because it is a fast acid producer. From the current study, however, it is proposed that a mixed strain culture, which produces relatively low levels of the malty compounds, acetaldehyde, ethanol and acetoin, and possibly containing *L. lactis* subsp. *lactis* biovar. *diacetylactis* C1, should be deve-

Table 3. Volatile organic compounds detected in UHT milk fermented using lactic acid bacteria (LAB) and LAB/yeast cultures previously isolated from Zimbabwean naturally fermented milk. The LAB were B1 (*Lactococcus lactis* subsp. *lactis* Lc39), B2 (*L. lactis* subsp. *lactis* Lc261), B3 (*Lactobacillus paracasei* Lb11) and B4 (*L. lactis* subsp. *lactis* biovar. *diacetylactis* C1). The yeast cultures used were Y1 (*Candida kefyr* 23), Y2 (*C. lipolytica* 57), Y3 (*Saccharomyces cerevisiae* 71), Y4 (*C. lusitaniae* 68), Y5 (*C. tropicalis* 78), Y6 (*C. lusitaniae* 63), Y7 (*C. colliculosa* 41), Y8 (*S. dairenensis* 32) and Y9 (*Dekkera bruxellensis* 43)

Sample*	*w*(volatile organic compounds)/ppm									
	Ethanol	Acetaldehyde	Acetone	Acetoin	2-methyl propanal	2-methyl propanol	3-methyl butanal	3-methyl butanol	2-methyl butanol	Diacetyl
B1	59.0	0.8	1.3	2.0	0.65	1.17	2.82	14.75	0.70	0.20
B1/Y1	3393.7	28.7	0.6	3.6	0.38	1.70	0.02	17.19	1.22	nd
B1/Y2	48.4	1.0	1.5	5.8	0.12	2.76	0.73	11.19	0.38	nd
B1/Y3	223.1	7.4	1.4	5.0	0.13	3.04	0.24	12.84	0.44	nd
B1/Y4	48.5	0.6	1.3	5.7	0.02	2.88	0.05	13.11	0.34	0.29
B1/Y5	58.5	1.3	0.5	6.0	0.06	2.57	0.25	10.98	0.29	0.17
B1/Y6	51.4	0.6	1.3	5.0	0.02	2.79	0.06	12.89	0.39	0.25
B1/Y7	200.2	6.5	1.4	5.1	0.14	3.25	0.25	13.66	0.47	nd
B1/Y8	211.9	7.0	1.4	4.9	0.13	3.20	0.25	13.57	0.35	nd
B1/Y9	241.0	9.4	1.3	5.8	0.12	3.30	0.26	13.44	0.46	nd
B2	44.5	0.3	1.7	6.2	nd	nd	0.01	0.03	0.02	nd
B2/Y1	4361.3	26.7	2.8	3.8	0.37	2.85	0.03	4.47	0.81	nd
B2/Y2	49.0	0.8	1.5	3.8	0.01	nd	0.06	0.06	0.03	nd
B2/Y3	263.9	9.9	1.5	3.0	0.05	0.15	0.11	1.12	0.05	nd
B2/Y4	59.2	0.7	1.7	7.6	0.03	0.06	0.16	0.42	0.02	0.33
B2/Y5	61.9	1.3	0.5	3.7	0.03	0.11	0.04	0.18	0.03	0.27
B2/Y6	56.1	0.5	1.5	3.8	0.02	0.06	0.13	0.22	0.01	nd
B2/Y7	259.5	9.0	1.5	3.2	0.06	0.27	0.13	1.60	0.07	nd
B2/Y8	247.0	10.1	1.4	3.1	0.07	0.37	0.14	1.76	0.14	nd
B2/Y9	254.3	11.6	1.4	3.9	0.07	0.57	0.13	1.49	0.14	nd
B3	7.1	2.5	1.1	5.3	nd	0.01	0.01	nd	nd	0.59
B3/Y1	8854.0	51.0	12.0	14.5	1.18	8.58	0.08	10.60	2.32	nd
B3/Y2	59.9	2.0	1.2	7.0	0.03	0.05	0.06	0.13	0.04	0.20
B3/Y3	86.7	3.4	1.1	5.4	0.02	0.06	0.02	0.11	0.04	0.77
B3/Y4	33.7	0.7	1.0	5.3	0.01	0.08	nd	0.05	nd	0.17
B3/Y5	46.5	1.2	0.3	3.9	0.01	0.15	nd	0.10	0.05	0.05
B3/Y6	39.0	0.8	1.0	4.8	nd	0.07	0.01	0.04	nd	0.10
B3/Y7	96.9	3.4	1.1	6.1	0.02	0.10	0.02	0.12	0.04	0.57
B3/Y8	91.5	3.4	1.1	4.7	0.02	0.13	0.02	0.15	0.06	0.67
B3/Y9	104.4	4.1	1.0	4.0	0.03	0.41	0.03	0.25	0.06	0.47
B4	30.0	1.6	1.7	86.2	0.10	0.25	0.65	1.70	0.13	1.37
B4/Y1	5297.0	88.0	7.0	58.1	0.87	5.40	0.08	8.37	1.14	0.50
B4/Y2	28.6	1.4	2.4	53.7	0.04	0.21	0.57	1.35	0.09	0.19
B4/Y3	390.0	30.0	1.7	66.8	0.22	0.62	0.48	4.10	0.24	0.24
B4/Y4	41.8	2.5	1.6	66.8	0.14	0.27	0.51	2.35	0.17	0.28
B4/Y5	50.6	0.8	0.6	46.6	0.07	0.34	0.15	1.78	0.17	0.19
B4/Y6	43.8	2.6	1.5	64.6	0.16	0.30	0.62	2.48	0.16	0.24
B4/Y7	393.0	35.0	1.6	64.9	0.19	0.90	0.24	5.09	0.33	0.23
B4/Y8	407.0	28.0	1.7	71.4	0.18	0.97	0.26	5.06	0.33	0.24
B4/Y9	357.0	34.0	1.7	74.8	0.19	1.19	0.27	4.75	0.37	0.23

*UHT milk cultured with lactic acid bacteria (B) and lactic acid bacteria+yeast (B/Y); nd=not detected

Table 4. Volatile organic compounds detected in milk fermented using pure yeast cultures previously isolated from Zimbabwean naturally fermented milk. The yeast cultures used were Y1 (*Candida kefyr* 23), Y2 (*C. lipolytica* 57), Y3 (*Saccharomyces cerevisiae* 71), Y4 (*C. lusitaniae* 68), Y5 (*C. tropicalis* 78), Y6 (*C. lusitaniae* 63), Y7 (*C. colliculosa* 41), Y8 (*S. dairenensis* 32), and Y9 (*Dekkera bruxellensis* 43)

Sample*	*w*(volatile organic compounds)/ppm									
	Ethanol	Acetal-dehyde	Acetone	Acetoin	2-methyl propanal	2-methyl propanol	3-methyl butanal	3-methyl butanol	2-methyl butanol	Diacetyl
Y1	6604.0	18.0	5.4	2.9	0.46	1.78	0.02	8.40	2.18	nd
Y2	11.6	0.5	1.1	3.5	0.01	0.01	0.04	0.11	0.02	nd
Y3	69.4	1.5	1.0	4.7	0.01	0.04	0.02	0.19	0.08	nd
Y4	52.8	0.9	1.0	5.5	0.01	0.02	0.01	0.05	0.04	nd
Y5	61.7	1.0	0.3	0.3	0.01	0.10	nd	0.09	0.05	nd
Y6	48.7	1.0	1.0	4.2	nd	0.05	0.01	0.06	0.03	nd
Y7	67.9	2.6	1.1	1.4	0.02	0.10	0.01	0.10	0.04	nd
Y8	59.7	2.2	1.0	0.0	0.02	0.15	0.01	0.12	0.06	nd
Y9	62.6	2.1	1.0	1.5	0.02	0.41	0.01	0.27	0.08	nd

*UHT milk cultured with pure yeast cultures (Y1–Y9); nd=not detected

loped. In future work, it is important to study the taste threshold of 3-methyl butanal in fermented milk.

Conclusions

This study has shown that the presence of malty compounds, especially 3-methyl butanal, is an important attribute of NFM and can be used to characterise amasi. High levels of acetaldehyde and ethanol are not typical of NFM. This suggests the need to further select strains that give milder characteristics in order to produce fermented milk similar to NFM. Suitable starter culture strains should produce the malty compounds, in addition to diacetyl, acetoin and ethanol.

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