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Alcohol and acetaldehyde in African fermented milk *mursik* – A possible etiological factor for high incidence of esophageal cancer in western Kenya

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Abstract

Background—Esophageal cancer is unusually frequent in western Kenya, despite the low prevalence of classical risk factors such as heavy drinking and tobacco smoking. Among Kenyans consumption of fermented milk is an old tradition. Our hypothesis is that alcohol and acetaldehyde are produced during the fermentation process and that their carcinogenic potential contributes to the high incidence of esophageal cancer.

Methods—Eight samples of *mursik* milk starter cultures were collected from different Kalenjin families in the Rift Valley province, Western Kenya. A protocol provided by the families was used for milk fermentation. Ethanol and acetaldehyde levels were measured by gas chromatography. The microbial flora in starter cultures was identified by 16S and 18S sequencing.

Results—7/8 starter cultures produced mutagenic (>100 µM) levels of acetaldehyde and 4/8 starter cultures produced >1000 µM of acetaldehyde. The highest alcohol levels (mean 79.4 mM) were detected in the four fermented milks with highest acetaldehyde production. The mean number of microbial species in the starter cultures was 5 (range 2–8). Yeasts were identified in all starter cultures (mean 1.5 species/milk) but their proportion of the total microbial count varied markedly (mean 35%, range 7–90%). A combination of yeast and lactobacilli, especially *Candida*

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Conflict of interest:

Mikko Salaspuro is a board member and medical adviser of Biohit Oyj. Biohit Oyj. has several patents and patents pending aimed for the reduction of acetaldehyde exposure. Other authors have no conflicts of interest to declare.

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Conception and design: MTN, NDF, MS, RR

Development of methodology: MTN, LNF, MS, RR

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krusei with *Lactobacillus kefir* with the exclusion of other species, seemed to correlate with higher acetaldehyde and ethanol levels.

Conclusions—Significant levels of ethanol and acetaldehyde were produced during *mursik* fermentation.

Impact—When ingested several times daily the repeated exposure to carcinogenic levels of acetaldehyde may contribute to esophageal carcinogenesis.

Keywords

Candida; carcinogenesis; ethanol; fermented milk; lactobacilli

INTRODUCTION

In developed countries, alcohol drinking and smoking are the principal known risk factors for upper alimentary tract cancer. Evidence derived from epidemiological, genetic, biochemical and microbiological studies strongly suggests that acetaldehyde (ACH), derived either from ethanol or tobacco, is a common denominator in the pathogenesis of these cancers (1–5). Accordingly, the International Agency for Research on Cancer (IARC/WHO) recently concluded that ACH present in alcoholic beverages, as a congener or formed endogenously from ethanol, is a Group 1 carcinogen for humans (6).

Esophageal squamous cell cancer (OSCC) is unusually frequent in southern and eastern Africa, including in western Kenya, where it is the most common malignancy in both men and women (7, 8). Of special interest is the common occurrence of OSCC in young people (<30 year of age) in this area (9, 10), especially among members of the Kalenjin tribe, despite the low prevalence of classical risk factors such as heavy drinking and tobacco smoking (11).

In general, fermented food products constitute a major portion of the daily diet in Africa (12, 13). The use and production of fermented milk is an old tradition among Kenyans. Every tribe has their own unique methods of producing fermented milk, and the differences between fermented milks are based on the location of tribal communities and the different production processes. In general, whole milk is first boiled and poured in a gourd, and left-over fermented milk acting as a starter culture for the new fermentation process is added. These components are mixed well before the gourd is sealed and left to ferment for 2–7 days in room temperature.

In the Kalenjin tribe the fermented milk, *mursik* is produced in specific calabash gourds, also known as *sotet*. Some days before the milk is treated, a small branch of an Ite tree (*Senna didymobotray*) is debarked and allowed to dry. One end of this stick is first burned in a fire and then rubbed on the inner surface of a cleaned gourd. This is repeated several times until the gourd is fully coated inside with charcoal dust. This process reduces the porosity of the gourd and improves the flavour. Some Kalenjin groups also add small quantities of blood obtained from prickling a vein in the neck region of a healthy bull, and from which fibrin has been removed by gentle stirring. Addition of blood can impact the microbial metabolism, as iron is an important cofactor for a number of essential cellular processes. A thick bluish layer forms on the surface when *mursik* is ready. It is shaken well before drinking, to ensure that a uniformly thick emulsion is formed. In some Kalenjin households fermented milk is consumed several times daily.

Fermentation and all of these additions to the process are used to improve the odour, the taste and the flavour of fresh milk. In an interview with farmers, Mureithi et al. noted that

farmers were of the opinion that fresh milk smells and tastes like cow urine and had to be improved before it can be consumed (14). Also, lack of refrigeration, and the need to store milk for the dry season (when milk production decreases due to a lack of pasture) required that excess milk be stored for a longer time. For example, the Pokot developed *chekha mwaka*, a specially treated milk that could be stored for over a year without getting spoiled (14).

Yeast and lactic acid bacteria (LAB) have been reported to be the dominant microbial species in African fermented milk (15). The most commonly isolated yeasts are *Candida* and *Saccharomyces* spp. (16). *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Enterococcus* spp. are the most frequently isolated bacteria (15). Interaction of the microbial flora affects the properties of the fermented milk, such as its consistency and flavour. ACH is one of the most important flavour compounds in milk products, especially yoghurts (15).

Fermented milk products may contain up to 3.8% w/v (0.8 M) ethanol and mutagenic (>100µM) concentrations of ACH (17–20). In addition, oral microbes are able to produce marked amounts of ACH even from very low (0.01–0.02 M) amounts of ethanol present in saliva after alcohol intake (21). ACH production locally in the mouth is enhanced when ethanol concentrations increase (22, 23). Also, ACH present in alcoholic beverages blends readily into the saliva (22, 23). Presently, only beverages containing over 2.8% ethyl alcohol are classified as Group 1 carcinogenic alcoholic beverages. However, fermented foods may expose the upper digestive tract mucosa to ethanol and ACH levels equivalent to those of alcoholic beverages (1, 5).

Our hypothesis is that regular consumption of fermented *mursik* milk contributes to the particularly high incidence of esophageal cancer in the Kalenjin tribe through its ethanol and ACH content. As an initial test of this hypothesis, we first determined the levels of ethanol and ACH produced in the fermentation process. Second, we identified the microbial flora in *mursik* milk responsible for the fermentative process to determine whether it was compatible with production of ACH.

MATERIALS & METHODS

Sample collection

Eight samples of *mursik* milk starter cultures were collected from different Kalenjin families in the Bomet area, Rift Valley province, Western Kenya by investigators from Tenwek Hospital (Bomet, Kenya). The samples were stored in blue-capped plastic tubes for transportation. The samples were first transported via baggage at room temperature to the Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland, USA and then to the Research Unit on Acetaldehyde and Cancer, University of Helsinki, Finland in dark glass tubes by courier; the total transportation time was four weeks. In the laboratory, the samples were anonymised and stored at 4–6°C until used for milk fermentation and analysis.

Milk fermentation

A protocol provided by the Kalenjin families was used for milk fermentation. Finnish whole milk (Arla-Ingman, Finland) was used in the process. It was first briefly boiled and allowed to cool before mixing 0.1 mL of each starter culture with 9.9 mL of the milk. This suspension was prepared in sextuplicate, each to be analysed at different time points. The suspensions were sealed and left to ferment at room temperature (22–25 °C) for 0h, 24h, 48h, 4d, 7d, and 14d. The pH of the fermenting milks was measured at each time point. The milk fermentation was performed twice.

Acetaldehyde and ethanol analysis

The ACH and ethanol concentrations in the fermented milks were measured at three time points (0, 24h, 48h) by gas chromatography (Perkin Elmer Headspace sampler HS 40XL, Perkin Elmer Autosystem Gas Chromatograph equipped with Ionization Detector FID, USA) as described previously (24). These time points were selected based on the microbial growth curves, which reached a plateau by 48h. Aliquots of 500 µl were pipetted into 20 mL gas chromatography vials and closed airtight. The fermentation process was stopped by injecting 50 µl of perchloric acid (PCA) through the rubber septum of the vial. To measure artifactual acetaldehyde, boiled whole milk without starter culture kept at room temperature in sealed vials alongside those with starter culture, were analysed. Acetaldehyde and ethanol levels measured in these control samples were subtracted as background. The measurements were done in triplicate twice. The means of two triplicate analyses are presented.

Culture and identification of microorganisms

Microbial growth was enumerated (CFU/mL) at 0h, 24h, 48h, 4d, 7d and 14d time points by dilution plating on lysed blood agar (BBL 211047, BD, USA) and incubation for 48h at 37°C. The microbial flora in starter cultures was determined by using selective and non-selective media in aerobic and anaerobic conditions. Fastidious anaerobe agar (FAA; (LAB-M, LAB 90, UK)) supplemented with 5% v/v horse blood was used to enumerate the total cultivable bacteria. Lysed blood agar (BA; Trypticase soy agar (BBL 211047, BD, USA)) supplemented with 5% v/v horse blood was used for the enumeration of total aerobic bacteria. Neomycin-vancomycin blood agar (NV; blood agar and neomycin sulphate (Sigma N-1876, Sigma-Aldrich, USA)) supplemented with vancomycin (7.5 µg/mL), menadione (0.5 µg/mL) and 5% v/v sheep blood was used to enumerate anaerobic gram-negative bacteria. Cysteine-, lactose- and electrolyte-deficient agar (CLED; C.L.E.D medium (BBL 212218, BD, USA)) was used to select for aerobic gram-negative fermentative rods. To detect yeasts, Sabouraud Dextrose agar (SP (Lab M, UK)) supplemented with penicillin (100,000 iu/mL) and streptomycin was used. The BA, CLED and SP plates were incubated at 37°C for 48h and the FAA and NV plates were incubated under anaerobic conditions at 37°C for 5 days. From these primary plates multiple subcultures of bacterial and yeast colonies were prepared after incubation. The pure cultures were Gram-stained and identified by 16S or 18S PCR gene-sequencing. In addition, yeast isolates were identified by API 32C auxanographic strips (bioMerieux, France) following the manufacturer's instructions.

16S and 18S sequencing of isolates

Yeast isolates were identified by sequencing using ITS1 and NL4 primers spanning the ITS1-5.8S, rRNA-ITS2 and 26S rRNA regions (25, 26). The MycXtra DNA extraction kit (Myconostica, UK) was used to isolate DNA. A modified CTAB method was used (27) for those isolates from which DNA was not extracted successfully using the MycXtra kit. Bacterial isolates were sequenced using PA and PH primers spanning the 16S rRNA region (28). Initially DNA extraction was performed using Chelex 100 beads (29). If this was unsuccessful, a modified CTAB method was used (27). Sequencing was performed at the University of Manchester DNA Sequencing Facility by using an ABI Prism 3100 Genetic Analyser (16 capillary instrument) and by Beckman Coulter Genomics sequencing service (Essex, United Kingdom). Sequences were compared and aligned with sequences available in GenBank using the NCBI-BLASTn matching tool optimised for highly similar sequences (30). Definitive identification based on sequence similarity in the ITS-5.8S-ITS2 region was based on a thorough BLAST match as defined by Nilsson et al (31).

Statistical analysis

Data was analysed using Graph Pad Prism version 5.00 (GraphPad Inc. San Diego, California, USA). To analyse the correlation between acetaldehyde and ethanol levels, the means of triplicates measured for each milk at each time point were calculated and plotted. Spearman's rho (r_s) with a 95% confidence interval was used for the correlation analysis. P values of less than 0.05 were considered statistically significant.

RESULTS

Microbial counts

During the fermentation process the microbial counts increased exponentially up to 48h (Figure 1a). The highest counts were detected at this time point. The counts ranged from $6.63 \pm 0.34 \log_{10}$ CFU/mL (at 0h) to $10.42 \pm 10.21 \log_{10}$ CFU/mL (at 48h). After 48h the counts declined slowly to $9.54 \pm 0.43 \log_{10}$ CFU/mL at the 14-day timepoint. The pH of the fermented milks declined linearly from 6.59 ± 0.01 at 0h to 3.46 ± 0.04 at 14d. There were no significant differences in the total microbial counts or the pH between the different starter cultures used (data not shown).

Microbiota of the starter cultures

The mean number of microbial species identified in the *mursik* milk starter cultures was 5 (range 2–8) (Figure 2). Yeasts were identified in all starter cultures (mean 1.5 species/milk) but their proportion of the total microbial count varied markedly (mean 35%, range 7–90%, Figure 1b). A total of three *Candida* species and one *Saccharomyces* species were identified (Figure 2). The most common yeast species isolated from the *mursik* milk samples was *Candida krusei* (5/8 milks, Figure 2). *C. kefir* and *C. sphaerica* were the second most common yeasts found (3/8 milks). *Saccharomyces fermentati* was identified in one milk.

The dominant bacterial findings in the fermented milks were *Lactobacilli* present in six of eight milks (mean 37%, range <1 – 70 % of total colonies) (Figure 1b). A total of eight *Lactobacillus* species were identified (Figure 2). The most frequent species of *Lactobacillus* was *L. kefir*, which was isolated from six milks. In six of the eight milks *Lactobacilli* were found in combination with yeasts. In five milks *L. kefir* was found in combination with *C. krusei*. The next most common *Lactobacillus* spp. were *L. casei*, *L. paracasei* and *L. rhamnosus*. Other species were less prevalent (mean 27%, range <1 – 93%, of total colonies). Of these, *Bacillus* spp. were most common (5/8 milks, Figure 2).

Acetaldehyde and ethanol analysis

Seven of the eight starter cultures produced mutagenic (>100 μ M) levels of acetaldehyde into the milk by the 24h time point (Figure 1c). The highest level of ACH ($1808.7 \pm 20.1 \mu$ M) was detected in milk 1 at 48h. Four of the eight starter cultures produced over 1000 μ M ACH, ranging from 1146.2 to 1808.7 μ M, at the 48 h time point. Four starter cultures produced lower although potentially mutagenic levels of ACH by the 24 and 48h timepoints (74.8 – 547.4 μ M). Minimal levels of ACH and ethanol were detected in samples 7 and 8 at 48h (6.84 μ M and 7.01 μ M; and 0.44 mM and 1.04 mM, respectively). The highest levels of ethanol were detected in the four fermented milks with the highest acetaldehyde production (mean 79.4 mM, range 61.8 – 106.2 mM). Other milks contained 0 – 50 mM of ethanol (Figure 1d). The levels of ethanol produced during the fermentation process correlated well with the levels of acetaldehyde produced ($r_s=0.88$, CI=0.7331 to 0.9485, $p<0.0001$).

Correlation of microbial findings with acetaldehyde and ethanol levels

The high levels of acetaldehyde were detected in samples where a combination of three to five species of yeast and *Lactobacilli* were identified (Figure 1c and Figure 2). Presence of a higher number of other species, especially *Bacillus* and *Staphylococcus* spp., resulted in lower levels of acetaldehyde and ethanol. Also, low levels were measured in samples where *Lactobacilli* were absent (Figure 1 and Figure 2).

DISCUSSION

Esophageal squamous cell carcinoma is a particularly frequent cancer in both males and females in southern and eastern Africa, including western Kenya (7, 11). Formal epidemiologic studies have not yet been performed to determine the dominant risk factors for OSCC in western Kenya, but classical risk factors such as heavy alcohol drinking and tobacco smoking do not appear to explain the high rates of OSCC in this area.

Acetaldehyde has been shown to be a mutagenic carcinogen in both animal and *in vitro* models at concentrations as low as 100 μM (32–34). The mutagenic effects of ACH are mediated via binding to DNA, adduct formation, cross-linking and chromosomal aberrations (32). During and after alcohol drinking the majority of acetaldehyde is formed in the oral cavity by oral microbes (35). Certain yeast and bacterial species representing normal oral flora, such as *Candida*, *Streptococcus* and *Neisseria* spp., are capable of producing significant amounts of acetaldehyde from ethanol *in vitro* (24, 36, 37).

The current study provides evidence that high levels of dietary ethanol and acetaldehyde are probably present in the traditional diet of many people in western Kenya. Marked levels ($>100\text{ mM}$) of ethanol and exceptionally high levels ($>1800\text{ }\mu\text{M}$) of ACH were detected during the fermentation process of the traditional *mursik* milk. This is in line with earlier studies reporting that fermented milk products may contain up to 3.8% w/v (0.8 M) ethanol and mutagenic concentrations of ACH (17–20). The acetaldehyde levels detected in *mursik* were as high as those found in many alcoholic beverages (34, 38, 39). Marked differences between different starter cultures were seen (6.84 – 1808.7 μM at 48h). This is in line with the wide variations reported between different alcoholic beverages, where levels from 0 to 25000 μM of ACH have been reported to be dependent on the microbes and technology used for fermentation. Acetaldehyde levels detected in the present study were over four times those detected in commercially available yoghurts (20). In particular, monoculture of *Candida kefir* has been shown to produce high amounts of ACH in fermented milk, and when incubated together with lactic acid bacteria (LAB) the amount of ACH produced in the fermentation process can be significantly increased (16). Unlike yeasts, LAB are able to break down lactose into glucose and galactose, essential sources of energy for yeasts. And under the hypoxic conditions present in a bowl of milk (0.001% O_2), yeast can produce energy through ethanol fermentation, thus increasing the production of ACH (40).

In the Kalenjin tribe, *mursik* milk is ingested daily by most family members irrespective of age. The proportion of esophageal cancer cases occurring in patients 30 years of age or younger is exceptionally high amongst the Kalenjins, being 45-fold that seen in the US (11). Repeated exposure of the esophageal mucosa to high levels of ACH may lead to mutagenic changes and finally to carcinogenesis. Regular consumption of home-fermented *mursik* milk from an early age could provide one explanation for the exceptionally high incidence of esophageal cancer in this tribe, and amongst the younger age groups in particular.

In the present study, ACH production in the milks significantly correlated with ethanol levels, reflecting the dynamic balance maintained by the ADH enzymes of the fermenting microbes. Up to 106 mM ethanol was detected in the *mursik* milks analyzed in our study.

Previous *in vivo* studies have shown that as low as 10 – 20 mM ethanol concentration in saliva results in over 100µM salivary ACH levels (21). This is in line with *in vitro* findings, which show that many oral commensal *Candida* species are able to produce carcinogenic amounts of ACH (>100 µM) *in vitro* when incubated in clinically relevant levels of ethanol (11mM) (24).

The microbiota in *mursik* milks consisted mainly of lactobacilli and yeasts. This is in accordance with previous studies (15). The most common combination of species was *C. krusei* together with *L. kefir*. *C. krusei* has been previously identified in African fermented milks (15, 16). However, other yeast species, such as *C. kefir* and *Saccharomyces sp.*, were also identified in these studies. In another study, five species of *Lactobacilli* were reported from a slightly larger set of fermented milks (*kule naoto*) produced by Maasai tribe members in Kenya (41). In our material eight different species of *Lactobacilli* were identified in the *mursik* milks and only two species were the same as in the Mathara study (38). However, molecular methods were not used for identification in their study. In addition, a number of other species such as *Bacillus* and *Staphylococcus* spp were isolated in our study. The presence of these probable contaminants is not surprising as the fermentation process is neither industrial nor controlled. These species have also been previously reported from Kenyan and Sudanese milk products (42, 43).

In our material a combination of yeast and lactobacilli, especially *C. krusei* with *L. kefir* with the exclusion of other species, seemed to correlate with higher ACH and ethanol levels. We have previously shown that human commensal *C. krusei* isolates are poor producers of ACH from ethanol in pure cultures (24). *C. krusei* cannot metabolise lactose, but is able to utilise the breakdown products made available by lactose metabolism by Lactobacilli. On the other hand, *C. kefir* can utilise lactose for fermentation independently. In a previous study *C. kefir* was reported to produce high levels of ACH and ethanol in co-culture with lactobacilli (44). Generally, where *Lactobacilli* were absent acetaldehyde production was minimal or very short-lived.

Charcoal treatment of the fermentation gourds may modify the odour, taste and flavour of *mursik* by decreasing the pH of the milk and impacting on the growth and metabolism of the microbes. We used starter cultures, which had been prepared this way, but we were unable to obtain similar gourds to replicate this special treatment for our laboratory experiments. Nevertheless, we measured highly mutagenic levels of ACH from the milks even in the absence of charcoal treatment. Charcoal-derived carcinogens have been considered to be a contributing factor for the high oesophageal cancer incidence in Iran (45). Charcoal can be a source of polycyclic aromatic hydrocarbons, some of which have been identified as carcinogens and could contribute to the carcinogenicity of traditionally prepared *mursik* (46). On the other hand, charcoal could also act in a protective manner by binding toxins and carcinogens in the fermenting milk. In the present study, the gas chromatograph analyses were limited to the 48h time point, the time when *mursik* is ready for consumption. It is highly likely that fermentation continues after this whereby production of acetaldehyde and ethanol in longer fermentations needs to be addressed in future work. However, the impact of longer fermentations has been analysed for kefir starter cultures where acetaldehyde and ethanol levels were shown to remain high beyond 7 days of fermentation (47). The transportation of the *mursik* milk samples from Kenya took four weeks which may have influenced their viability. However, the storage conditions during transportation resembled those used by Kalenjins, who often store their starter cultures at room temperature for long periods of time. Also, it is possible that Kenyan milk may contain fewer carbohydrates than Finnish milk but any such differences are likely minimal.

In conclusion, our study shows that significant levels of ethanol and ACH are produced during *mursik* fermentation. When ingested several times daily, the esophageal mucosa is inevitably exposed to high levels of carcinogenic ACH, which may lead to carcinogenesis. The incidence of esophageal cancer in Kenya is especially high among certain tribes in western Kenya, including the Kalenjin tribe, and the *mursik* milk samples analysed in this study were all collected from Kalenjin families. It is also possible that predisposing genetic factors, such as ADH or ALDH polymorphisms, among closed tribal communities may have an additional role in the high incidence of esophageal cancer. The prevalence of these polymorphisms in different tribal communities in Kenya remains largely unknown, although some initial studies have been done (48). Thus, future studies on genetic factors and molecular mechanisms of ACH-induced carcinogenesis are warranted. Meanwhile, finding alternative fermentation processes and starter cultures leading to minimal ACH production could be a major public health measure in the prevention of esophageal cancer in Kenya.

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Figure 1.

(a) Colony counts measured from milk samples in aerobic conditions in 37°C cultured on blood agar. The highest counts were seen at 48h time point. (b) Percentage of different microbes in *mursik* milk starter cultures (0h). Acetaldehyde (c) and ethanol (d) levels in milk samples at three different time points (0, 24, 48h). Highest levels of acetaldehyde and ethanol were found at the 48h time point. Ethanol production correlated well with acetaldehyde production ($r_s=0.84$, $CI=0.75 - 0.91$, $p<0.0001$).

C: *Candida*; S: *Saccharomyces*; L: *Lactobacillus*; B: *Bacillus*; M: *Micrococcus*;
Staph: *Staphylococcus*

¹ *B. pumilus*, *B. cereus*, *B. subtilis*, *B. thuringiensis*

² *S. epidermidis*, *S. pasteurii*

Figure 2.

Distribution of microbial species among the *mursik* milk starter cultures.