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Weissella Cibaria Nn20 Isolated from Fermented Kimere Shows Ability to Sequester AFB1 Invitro and Ferment Milk with Good Viscosity and Phin Comparison To yogurt

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Research Article

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Abstract

Fermented foods prove many benefits for human health. The purpose of this study was to determine if these strains sequestered aflatoxin B1 (AFB1) *invitro.* We isolated 300 gram positive lactic acid bacteria (LAB) from Kimere a fermented food product from Eastern Kenya. Sixteen strains were identified for further investigation. The maximum survival in gastric juice, captured by measuring optic densities spectrophotometrically at 600nm was for *Weissella cibaria* NN20 isolates in comparison to positive control probiotic *Lactobacillusrhamnosus* GR1 and negative control *Escherichia coli* GR12 (1.515±0.132, 1.459±0.085, 1.442±0.047 respectively). There was similar survival of *Weissella cibaria* NN20 compared to *Escherichia coli* GR12. Binding with AFB1 was found to be slightly better for *Weissella cibaria* NN20 (43.7±2.3 %) than *Lactobacillus rhamnosus* GR1.The final pH, viscosity and general organoleptic acceptance compared to fermented milk with traditional yogurt starter cultures (P>0.05).

Keywords: *Weissella cibaria* NN20; yogurt; Kimere; AFB1

Introduction

The consumption of fermented food dates back to at least Hippocratic Corpus of Ancient Greece [1]. In South Sahara Africa consumption is second to Europe [2]. In Kenya, fermented foods have played a significant role in the diet, with traditional cereal product fermentation used to reduce the microbial contamination of porridges [3].Fermentation provides bio-enrichment of the final food product, and diversity of aromas flavors and texture,

along with enhanced shelf life, and improved nutrient bioavailability [4-6].

Traditionally, fermentation of food products in Kenya has been through a simple spontaneous process. This has been regarded as advantageous among the rural communities where equipment is rudimentary and the system requires limited space [5]. Spontaneous fermentation has been integral to numerous food products across the world [7]. From Eastern Kenya, Kimere joins the long list of these products. Kimere is a fermented dough made by wet milling of millet then either wrapped with green banana leaves or put in calabash and left to ferment for 12 hours before consumption (Figure 1). It constituted a staple food before introduction of maize. Studies have shown that spontaneous fermentation of cereals in tropic takes place mainly through lactic acid bacteria (LAB) activities [8], organisms that provide many health benefits, including reducing uptake of carcinogenic aflatoxins present in the food supply in Eastern Kenya [9,10]. Cereals can contain molds that produce secondary toxic metabolites called mycotoxins. These include fumonisins, ocratoxin A, zearalenone and aflatoxins [11]. Detoxification of cereals by use of LAB therefore adds great value [11-13]. The capacity to detoxify varies amongst bacterial strains [14,15].

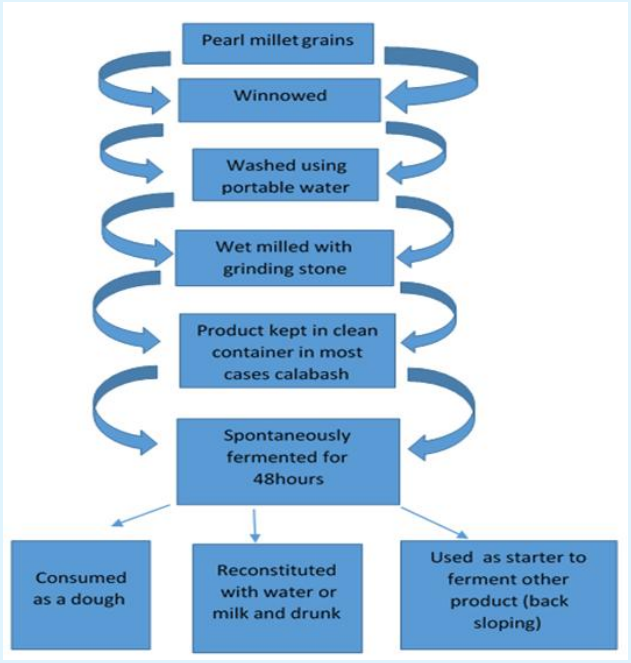

Figure 1: Preparation of Kimere spontaneously fermented and made from pearl millet.

Some of the LAB have been of importance in fermentation of pearl millet, particularly useful in early age due to the nature of biomass transformation during the fermentation process [16]. In addition, fermentation has increased bioavailability of important elements, such as iron and zinc, due to degradation of phytates in cereals, and this has been valued for food safety [17,18]. Furthermore, isolates from spontaneously fermented products have been utilized to prepare fermented food product either by back sloping or through pure isolates [19-20].

When Kimere consumption was part of the staple diet in Eastern Kenya, there were fewer reported cases of aflatoxicosis [10]. The objective of the present study was to assess the ability of LAB isolated from Kimere, for sequestration of aflatoxins.

Materials and Methods

Kimere sampling

Thirty samples of spontaneously fermented kimere were collected from 30 different homestead in three sub-counties of Eastern Kenya. The samples were put in sterile screw caps tubes, rapidly cooled using dry ice parks, transported in cool boxes, and stored below -4°C until microbiological analysis.

Isolation of LAB

Serial dilutions of the fermented food were incubated in MRS agar (Hi media laboratories Pvt Ltd, Kenya) for 24 hours at 37°C. Colonies were grouped according to their appearance: shine, shape, size and colour. The colonies were then streaked on the MRS agar incubated at 37°C for 24 hours and Gram stained. Three hundred Gram positive, rod-shaped, non-motile, non-budding isolates with negative endospore test were selected for further study, stored at -80°C.

Survival of Strains in AFB1solution

Eight ppm solution of aflatoxin B1 (AFB1) was prepared (sigma ALdrich A6636-1mg). Strains concentrations were standardized in PBS to a consistent optical density (OD) in a 96 well plate, with one strain in three wells per plate. The ODs were captured every 30min for a period of 18 hours.

Acid Tolerance Test

We modified the methods of [20] and [21] to test for survival of strains in low acidity, with PBS, pH adjusted to pH2, pH3 and pH7. With strains concentration

standardized by taking their ODs and concentration adjusted following the formula C1 x V1=C2 x V2 where C1 is OD of known concentration of strain, V1 is the known volume of the strain, V2 is unknown final volume after adjusting and C2 is the desired standard OD .The bacterial strains at pH2, pH3, pH7 were transferred in a 96 micro wells plate, with each strain in three wells per plate per pH. The ODs were captured after every 30 minutes for 18 hours using multi scan machine (Thermal Electron Corporation) at 600nm [23].

Bile Tolerance Test

The MRS broth was prepared then pH adjusted to 3, using 0.1m hydrogen chloride (HCl). The oxygall bile (Difco) was added according to manufacturer specification at 3%v/v. The ability of the bacteria to survive was tested in triplicate in a 96 micro well plate. The ODs were captured at 600nm for every intervals of 30 min for a period of 16 hours.

Isolate Identification

Strains for DNA analysis were extracted using the QIAamp DNA stool mini kit (Qiagen). Strains were vortexed in1 mL buffer ASL before removal and addition of 0.1 mm zirconia/silica beads (Biospec Products) with 2, 30 seconds rounds of bead beating at full speed with cooling on ice in between (Mini-Bead Beater; Biospec Products).We used the forward primer (CCATCTCATCCCTGCGTGTCTCCGACTCAGCWACGCGARG AACCTTACC) and reverse primer (CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT ACRACACGAGCTGACGAC) for sequencing. Amplification was carried out in 42 mL with each primer present at a 10 mL (3.2 pMol/mL stock), 20 mL GoTaq hot start colorless master mix (Promega) and 2 mL extracted DNA. The PCR protocol was as follows: initial activation step at 95uC for 2 minutes and 25 cycles of 1 minute 95uC, 1 minute 55uC and 1 minute 72uC. PCR products were quantified by use of gel electrophoresis. At the London Regional Genomics Centre (LRGC, www.lrgc.ca, London, Ontario, Canada) samples were treated with an Ion OneTouch System (Life Technologies) and sequenced on an Ion Torrent Personal Genome Machine sequencer on a 316 chip (Life Technologies) [24].

Master Mix Preparation

50 ul of different reagent was added to 10 ul instaGene product to prepared 60 ul of master mix. Thus 20.4ul of distilled water (dH20) was added first in a 100ul vials. This followed 8.4 ul of dinitrogen phosphates (dNTPs) of 1.25mM. Then 5ul of 10x PCR buffer was added. 2.5 ul magnesium chloride (MgCl2) of 50mM concentration was added. 1.6 ul of HAD-1 and HAD-2 of 20uM strength forward and reverse primers were added respectively. Finally Taq (5ul/ml) according to manufacturer specification was added before the master mix was mixed thoroughly and inserted into PCR master cycler.

Gel Electrophoresis and PCR Products Quantification

To establish quality and quantity of PCR products, gel electrophoresis was performed. To prepare the gel, 0.5 g of agarose was weighed into a conical flask; 50ml of 0.5x TBE was added and swirled to mix, then microwaved for 1 minutes to dissolve agarose and thereafter allowed to cool to 60°C. Then, 1ul of ethidium bromide (10mg/mL) was added before pouring slowly into the tank. Finally, 0.5x TBE buffer was poured into the gel tank to submerge the gel to 2-5mm depth.

10ul of PCR products were transferred to a fresh microfuge tube well labelled to identify the lanes on the gel photograph. 2ul of loading buffer (bromophenol blue, Sigma B8026)-to give the colour and density to the sample to make it easy to load into the well- was added into 10ul sample. After the marker dye had run ¾ of the tank the gel was stopped and results taken by X-ray photo in UV light box.

Fermented Milk Yogurt-Like Product Developments.

Streptococcus thermophilus and respective bacterial strains were used at the ratio of one is to one during inoculation. Cultivation of the LAB strains was performed in MRS broth before harvesting after 24 hours incubation at 37 °C anaerobically. The *S. thermophilus* were grown in demane 17 (M17) broth. The yogurt was produced with pasteurized cow's milk, and taste, flavor, colour, ph and viscosity were determined organoleptically.

AFB1 Binding by the Strains in Milk Fermented Products

Prepared skim milk was heated at 90°C for 5 min and then cooled to 42°C. A stock solution of AFB1(Sigma ALdrich A6636-1mg) was collected from 5 µg/ml AFB1 in ben-zene/acetonitrile derived from stock, and evaporated by heating in a water bath at 80°C. AFB1 residue was dissolved in 2 ml methanol. A volume of 0.08 ml was transferred from the contaminated methanol to 20 ml of skim milk, resulting in milk containing 10 ng/ml AFB1. Then, 20 ml milk was inoculated with 2% cells of *S. thermophilus* and one LAB strain at 1:1. Cell-free reconstituted milk contaminated with AFB1was used for

positive control with only *Streptococcus thermophilus*, while fermented products made from uncontaminated reconstituted milk was used for negative control. The samples were incubated at 42°C for 4 hours. Fermented milk products were then centrifuged and unbound AFB1 in the supernatant was determined by High Performance Liquid Chromatography (HPLC). Each sample for the HPLC analysis was diluted 1:125 in PBS. 1.5ml of this solution was transferred into Eppendorf safe lock vials for aflatoxin binding analysis using high performance liquid chromatography technique [25]. The percentage of AFB1 bound to the cells was calculated as the difference between the total AFB1 the amount of free AFB1 in the resultant solution. Data from triplicate experiments were integrated and recorded using a Millennium chromatography manager Software 2010 (Waters, Milford, MA) as appropriate. In this study all assays were performed three times and both positive and negative controls were included. At the same time fermented milk products with uncontaminated milk were tested for viscosity and pH. Yogurt prepared using *Lactobacillus delbreuckii* subsp*. bulgaricus* and *S. thermophilus* at the ratio of 1:1 was used as a control.

Results

Of 300 isolates from Kimere, 16 strains were selected for further experiments based on morphologically gram positive rods, ability to survive in low acidity, catalase test negative, non-motile and endospore test negative (Figure 2).

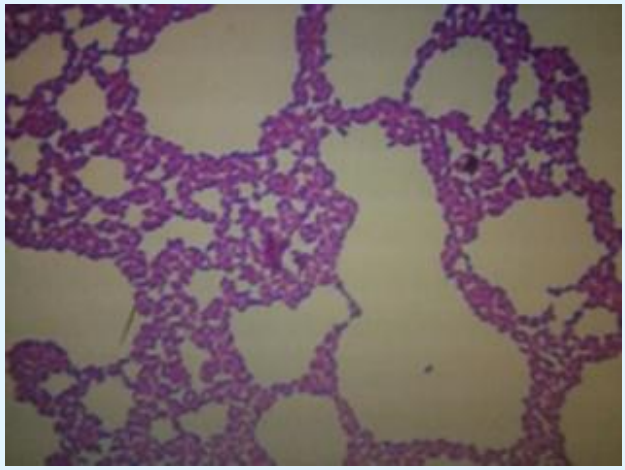

Figure 2: Microscopy and gram stain characteristic of *Weissella cibaria* NN20 isolate.

The strains showed significance sequestration of AFB1 ($P<0.05$ compared to the negative control). The highest sequestration was 43.7%±2.3 of available AFB1, for strain NN20, almost four times compared to negative control which sequestered 10.9%±1.5 (Table 1).

Strain	ug/ml average AFB1 based on St. curve	ug/ml Corrected average AFB1	St dev	%free AFB1	Bound %AFB1*
NN1	5.18	3.21	2	62	38
NN3	4.92	2.84	2.3	57.8	42.2
NN6	5.06	3.04	1.3	60.1	39.9
NN7	5.23	3.28	7.8	62.8	37.2
NN8	5.16	3.18	3.7	61.7	38.3
NN9	5.05	3.03	5.7	60	40
NN11	5.29	3.37	5.3	63.7	36.3
NN13	5	2.95	6.4	59.1	40.9
NN14	5.05	3.03	2.9	60	40
NN16	5.01	2.97	2.3	59.3	40.7
NN18	3.33	1.93	1.2	58	42
NN19	5.14	3.15	6.4	61.3	38.7
NN20	4.83	2.72	2.3	56.3	43.7
NN21	5.04	3.01	0.3	59.7	40.3
NN22	5.04	3.01	6.9	59.8	40.2
NN26	5.02	2.98	2	59.4	40.6
GR28	4.99	2.94	2	58.9	41.1
GR1	5.22	3.27	3.6	62.7	37.3
GR12	5.62	5.01	1.5	89.1	10.9
Blank	6.29	6.27	8.6	99.84	0.16

Table 1: LAB strains binding to AFB1 after being fermented in milk.

The figures for strain binding representing binding after correction with the brank reading.

All the strains were identified to species level after DNA amplification using polymer chain reaction method followed by DNA quantification by gel electrophoresis methods (Figure 3).

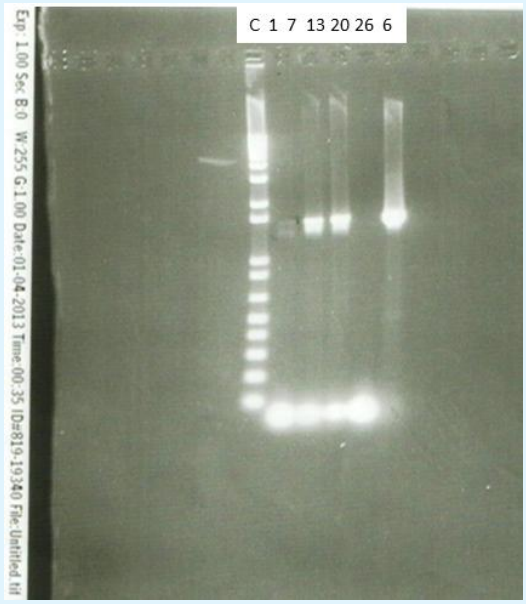

Figure 3: Photo of PCR products quantification of selected strains by gel electrophoresis Shows ladder (C), and strainsNN1, NN7, NN13, NN20, NN26 as indicated of the 16 strains, 25% were related to *Lactobacillus fermentum*; 68.75% *Weissella cibari*; and one was *Weissella confuse* (Table 2).

Strain/code	Possible Isolates Identity	% identified characteristics
NN1	*Lactobacillus fermentum*	98.90%
NN3	*Weissella cibaria*	99.9
NN6	*Weissella cibaria*	100
NN7	*Lactobacillus fermentum*	99
NN8	*Weissella cibaria*	100
NN9	*Weissella cibaria*	99.9
NN11	*Weissella cibaria*	99.9
NN13	*Lactobacillus fermentum*	99.1
NN14	*Weissella cibaria*	99.9
NN16	*Weissella cibaria*	99.9
NN18	*Weissella cibaria*	99.9
NN19	*Weissella cibaria*	100
NN20	*Weissella cibaria*	99.9
NN21	*Weissella cibaria*	99.9
NN22	*Weissella confusa*	99.9
NN26	*Lactobacillus fermentum*	99.1
GR1	*Lactobacillus rhamunosus*	99.9
GR12	*Posted non similarities*	Not applicable

Table 2: Molecular identification of the isolates.

All the 16LAB strains had the ability to ferment milk but *Weissella cibaria*NN20 produced the best quality product with viscosity and pH similar to traditional yoghurt (P>0.5), as assessed blindly by three subjects (Figures 3, 4).

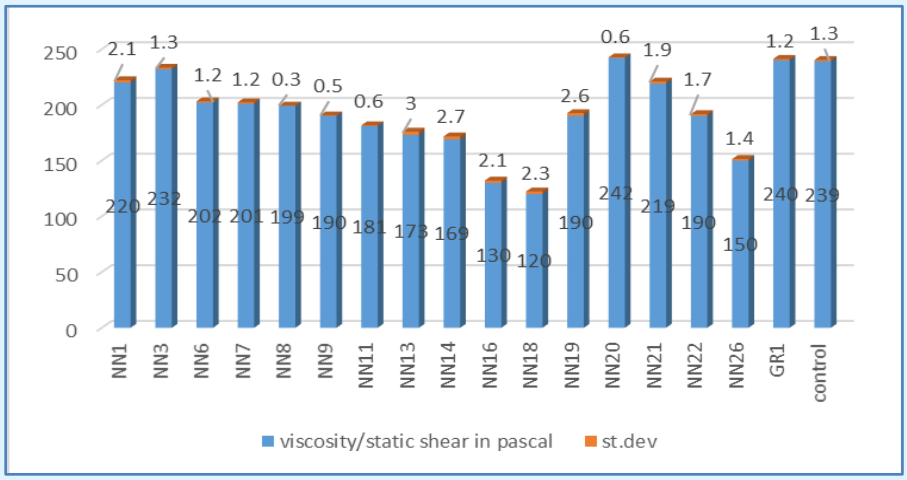

Figure 3: Viscosity of fermented milk products using LAB strains from Kimere compared to traditional yogurt control.

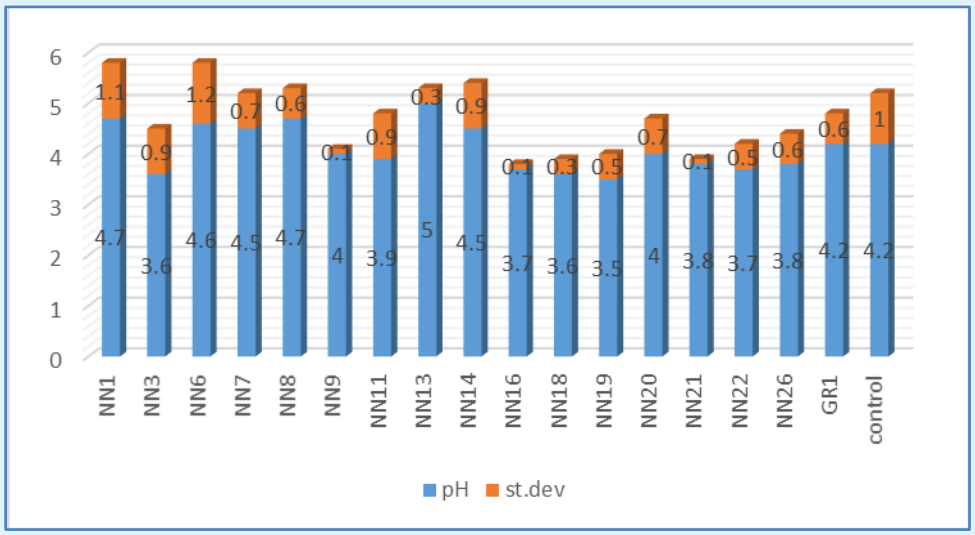

Figure 4: The pH of fermented milk products using LAB strains from Kimere compared to traditional yogurt control.

Discussion

This study showed that Kimere in Eastern Kenya contains LAB which has the capacity to significantly lower AFB1 levels *in vitro.* These strains from different regions had this capacity. The results corroborate studies that used fermented doughs and food products to reduce aflatoxin contamination [26]. The *Weissella cibaria*NN20 strain had marginally the best results for AFB1 sequestration in Kimere as a dominant species, a finding not previously reported for spontaneously fermented food in Africa [27].

The health benefits attributed to *Weissella cibaria* have been reported to include their potential to reduce the risk of cancer, and Sulphur production associated with halitosis [28-30]. It is worth noting that the isolated strains were capable of fermenting milk to produce products with viscosity and pH similar to yogurt, albeit the flavor was weaker. The survival rates of these isolates were similar to the positive control *Escherichia coli* GR12. Kimere is consumed after 48 hours when LABs stabilize in spontaneously fermented products [20] therefore the consumer will receive the high counts of the *Weissella cibaria*

Fermented millet products have been major contributors to the diets of people living in Eastern Kenya. This likely reduced the incidence of aflatoxin poisoning, whose incidence increased with the replacement of Kimere with maize.

Conclusion

This study emphasizes that Kimere contains LAB strains with the potential to confer a range of health benefits, in particular reduced uptake of aflatoxins by unsuspecting children consuming contaminated products. Indeed, some LAB strains can even degrade aflatoxins (Kort et a. unpublished).The study provides support for people in parts of the world consuming potentially aflatoxin-contaminated food, to switch to fermented foods using organisms such as *W. cibaria* and *L. rhamnosus*.

Acknowledgement

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