Production of Exopolysaccharides in the Kenyan Fermented Milk, *Mursik*

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Abstract: *Exoplysaccharides (EPS) produced by Lactic acid bacteria (LAB) have attracted a lot of interest for application in production of healthy food products since LAB are food grade bacteria and their EPS are natural products. This research aimed to establish whether Mursik, a Kenyan traditional fermented milk product, contained Exopolysaccharides (EPS) and EPS producing Lactic acid Bacteria (LAB). Samples of Mursik were randomly collected from 39 selected homesteads in Njoro Kenya. EPS levels were isolated using alcohol precipitation method and quantified by phenol-sulphuric acid method. EPS producing LAB were screened using sucrose agar and identified by phenotypic and biochemical methods. The data obtained was analysed for variance and means separated by least significance difference. The findings indicate that EPS levels in traditional Mursik differ between households significantly (P< 0.05), and ranged between 17.86 - 59.77 mg/L glucose equivalent (Glu.equiv), average being 44.83 mg/L Glu.equiv. A total of 122 EPS producing LAB were isolated and characterized into five genera namely, Lactococcus (76%), Lactobacillus (11%), Enterococcus (7%), Leuconostoc (5%) and Weissella (1%). Their EPS yields in whey ranged between 44.3 and 449.9 mg/L Glu.equiv. Based on these findings it was concluded that Mursik contains EPS and a variety of EPS producing LAB, predominated by Lactococcus lactis species. It was recommended that Mursik should be developed and utilized as a functional food. EPS producing LAB from Mursik could be developed as starter cultures for improving rheological and functional properties of other fermented milk products.*

Keywords: Exopolysaccharides, Lactic acid bacteria; Functional food; *Mursik*

1. Introduction

In Kenya, traditional fermented milk products are mainly produced by pastoral communities such as the Maasai, Borana, Kalenjins, Gusii and Somali. They are mainly produced by spontaneous fermentation of the milk in traditional containers such as gourds and skin bags. Some of these fermented milks have been reported to have health beneficial properties such as *Kule naoto* produced by the Maasai[1], *Mursik* produced by the Kalenjin [2] and *Ambere amaruranu* produced by the Gusii [3]. *Mursik* is mainly produced by the Kalenjin community in Kenya, through spontaneous fermentation of cow milk in a traditionally prepared gourd [2] [4], referred to as '*Sotet*' in Kalenjin. It forms a major part of the Kalenjin diet due to its delicious taste and belief that it improves health [2] . The Kalenjin also value it as a special drink that is shared in special occasions to symbolize success of certain activities such as successful marriage negotiations and weddings, victory in athletics among other events. Studies by[2][5] established that *Mursik* culture consist of lactic acid bacteria (LAB) species which include *Lactococcus lactis* subsp*. lactis, Leuconostoc mesenteroides* subsp*. dextranicum, Lb. curvatus, Leuc. paramesenteroides* and *Lb. planturum*. According to [2], some of the LAB have antimicrobial properties against pathogenic bacteria species such as *Staphylococcus aureus* and *salmonella typhimurium*.

Figure 1: Traditional *Mursik*: A; *Senna didymobotrya* ('*Senetwet*' in Kalenjin), a plant species which its stems are used for charcoal application in the inner surface of *Mursik* fermentation gourds; B, Traditional gourd used for *Mursik* fermentation; C, Ready to drink *Mursik* after fermentation

Exopolysaccharides (EPS) are polysaccharides that are secreted by bacteria onto their cell walls or into the growth medium [6]. They are categorized as capsular EPS and ropy EPS depending on whether they are attached or unattached on the bacterial cell wall respectively[6][10][41]). They are also categorized based on their monomer composition as homopolysaccharides and heteropolysaccharides [6] [7]. Homopolysaccharides consist of the EPS that have only one type of monomer in their polymer such as glucan (with only glucose) and fructan (with only fructose), whereas heteropoysaccharides have several different types of monomers such as glucose, fructose and rhamnose on one polymer [6][7]. EPS play an important role in improving rheological properties of fermented milk products such as

yoghurt and cheese, especially the low fat varieties ([8] [9] [10]). Their application in food as natural biothickeners has been received so positively by consumers since LAB have a GRAS (Generally Recognized as Safe) status, and their EPS are regarded as natural food additives [11]. Therefore they are good alternatives for the commonly used plant and algae-based food biothickeners, especially those that are chemically modified and those that are obtained from non-food grade microorganisms such as xanthan [12]. However, the major limitation for use of LAB EPS as bioingredients in food is the low production yields by the LAB [12][13]. Most LAB produce below 1g/Liter EPS, whereas the non-food grade microorganisms such as *Xanthomonas campestris* (source of xanthan biothickener) produces between 30 and 50 g/Liter. For economical production of EPS biothickener, a microorganism should produce 10 to 15g/L [12]

Exopolysaccharides from LAB have been shown to impart several health beneficial effects to consumers. Several LAB have been shown to have the ability to stimulate growth of gut microbiota as prebiotics and these include fructooligosacchride kestoses and nystoses produced by *Lactobacillus sansfrancisience* [14][15], glucooligosaccharides from *Leuconostoc mesenteroides* M2860[16], and β-glucan from *Pediococcus parvulus* 2.6 [17]. Increased population of health beneficial microbiota such as *Bifidobacteria* species in the gut inhibits growth and adherence of pathogenic microorganisms [18][19]. LAB EPS have also been reported to reduce blood cholesterol levels, prevent cancer development [20], prevent tumor development and regulate certain immune response activities [21][22][23] in the gut. Also, some LAB EPS enhance colonization of the gut by probiotics by increasing the residence time of the fermented foods in the digestive system and acting as factors for their attachment and adaptation to the gut environment [17][24]. A study by [24] *Lactobacillus Rhamnosus* GG produce long galactose rich EPS which protects it from innate immunity (non-selective antimicrobial system) which protects the gut from pathogens, hence enabling it to persist and colonize the gut. In immunomodulation, bacterial polysaccharides including peptidoglycan and EPS have been reported to induce immunological response system in the gastrointestinal mucosa [21][23]. EPS from LAB such as *Lactobacillus gasseri, Lactococcus lactis* subsp.*cremoris, Lb.rhamnosus, Lb.delbrueckii.*subsp.*bulgaricus* and *Leuconostoc mesenteroides* susp.*meseintroides* have been reported to induce immunological response in macrophages and T-cells, and stimulate mitogenic response in B-cells [21][23]. According to [21], EPS from *Lactobacillus rhamnosus* RW-9595M elicit immunosuppression activity in the gut by increasing production of the cytokine IL-10 which prevent development of inflammatory conditions in the gut. Also, a study by [22] established that the cell-bound EPS produced by *Lactobacillus acidophilus* 606 express antitumour activity against colon cancer cells (HT-29) through autophagic cell death (dynamic protein degradation) mechanism. *Mursik* is widely consumed in Kenya due to the belief that it enhances immunity against common diseases and that it makes one strong [2]. However, the mechanisms in which they impart these

health beneficial effects have not been established. Therefore, the objective of this study was to establish production of EPS in *Mursik*, and hence establish whether they are responsible for the claimed health beneficial effects. This will positively have an impact on its functional properties of *Mursik* and its application as a source of EPS producing LAB for application in other foods as functional LAB or starter cultures for improving rheological properties in other fermented milk products.

2. Materials and Methods

2.1 Study Site

The study was carried out in Njoro District, Kenya. Njoro District is found within Nakuru County in the Rift valley region, bordering the complex Mau Forest on the Eastern edge, between Longitudes 35° 45′ and 35° 46′ (East) and Latitudes 0° 16′ and 1° 10′ (South).

2.2 Sampling

Samples of *Mursik* were collected from 39 randomly selected households in three locations, namely Mauche, Njoro and Mugungugu Divisions, where the Kalenjin communities are mainly settled. Prior to sampling, the household owners were requested to prepare the *Mursik* traditionally so as to be ready after three days and sampling was done on the fourth day. One liter *Mursik* samples were collected from every household using sterile screw capped bottles, and 40 ml sub-samples were drawn from each into 100 ml sterile screw caped bottles for microbiological analysis. The samples were then transported in a cool box to Egerton University, Department of Dairy and Food Science and Technology, in within 2 hours where they were analyzed immediately or stored at 4 - 5 °C for not more than 24 hours before analysis.

2.3 Experimental Design

The experiment was carried out in a Completely Randomized Design (CRD). The samples of *Mursik* (treatments) were collected from thirty nine randomly selected households and analyzed for Exopolysaccharides (EPS) yields and presence of EPS producing LAB. The data (EPS yields) obtained was used to compute for Analysis of Variance (ANOVA) using SAS statistical package (SAS system for windows, v.6.12, USA) and least significant difference (LSD) test was used to determine whether there were significant differences between the treatments at 5 % level of significance. The screened EPS producing Lactic acid bacteria (LAB) were identified by phenotypic and biochemical characterization methods.

2.4 Enumeration of Lactic acid bacteria in Mursik

The LAB in *Mursik* were enumerated using M17 agar (Himedia) for *Lactococcus* and MRS agar (Himedia) for *Lactobacillus* and *Leuconostoc*. Briefly, each *Mursik* sample was shaken vigorously for 1 minute to

homogenize, and then 10 ml was drawn and transferred into 90 ml 2% Sterile Bacteriological peptone (Himedia) solution to make 10^{-1} dilution. From the 10^{-1} dilution, Serial dilutions were further made by transferring 1 ml sample into 9 ml 2% sterile bacteriological peptone solution subsequently up to 10^{-8} dilution level. The last three dilutions (10^{-6}, 10^{-7} and 10^{-8}) were propagated to enumerate LAB using pour plate method. In brief, I ml sample was transferred from each dilution into sterile Petri-dishes in duplicate, then the appropriate selective media was added at 45 ℃ (molten), allowed to solidify for 5 to 10 minutes and then incubated appropriately. Both *Lactobacillus* and *Lactococcus* species were enumerated in each sample. M17 agar plates were incubated aerobically at 30 °C for 48 to 72 hours whereas MRS agar plates were incubated anaerobically in 3.5 Liter anaerobic jars each with a 3.5 Liter anaerobic gas production kit (AnaeroGas Pack, Himedia) at 35 °C for 48 to 96 hours. The LAB counts were determined by counting the colony forming units (cfu) in the plates with between 30 and 300 cfu. Average counts of the duplicate plates were computed and each multiplied by the reciprocal of its dilution factor to obtain the LAB counts in colony forming units per milliliter (cfu/ml). The LAB counts (cfu/ml) were then converted into Logarithm$_{10}$ values (Log cfu/ml) for convenience during data analysis.

2.5 Isolation and Quantification of Exopolysaccharides From Mursik

Exopolysaccharides (EPS) in the *Mursik* were isolated as per the method described [25] with a few modifications. In brief; Each *Mursik* sample was first homogenized in a high speed homogenizer (Janke and Kunkel, Ultra-Turrax) for 1 minute and then 50 ml samples were transferred into clean centrifuge tubes in duplicate. To each 50 ml sample, 17% TCA (80% m/v) (Lobachemie, 99% purity) was added, mixed and allowed to stand for 20 minutes to precipitate the protein. They were then centrifuged at 4930 x g (mLw T23D centrifuge) for 20 minutes. Clear supernatant was extracted from the centrifuged samples by decantation into clean beakers and then EPS isolated by two-step alcohol precipitation method as follows; to the clear supernatant, twice the volume (100 ml) of chilled absolute ethanol (Scharlau, Ethanol analytical grade, Spain) at -10 °C or below was added and then kept in the fridge at 4 ℃ for overnight (12 - 16 hours). The mixture was then centrifuged at 4930 x g for 20 minutes. The EPS extract (precipitate) was obtained by carefully decanting off the supernatant. The EPS extract was re-dissolved in 5 ml distilled water and then the EPS extraction process repeated as described above. Finally, the EPS extract was dried at 45 ± 2 °C for 48 hours and quantified by phenol sulphuric acid method as described below.
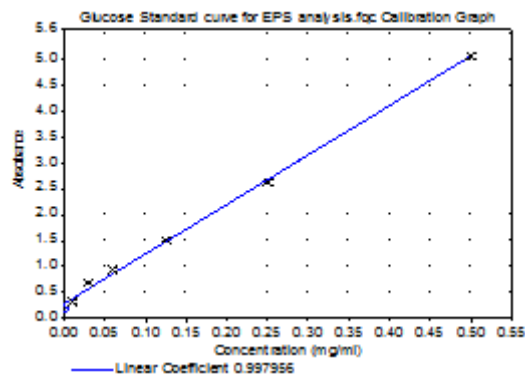

Figure 2: Glucose standard Calibration Curve used for Quantification of EPS isolated from *Mursik*.

The EPS extracts from *Mursik* were analyzed for total carbohydrate content using Phenol-sulphuric acid method as follows; 1 ml of 5% phenol (Panreac, analytical grade, Spain) solution was added to each EPS extract, followed by addition of 5 ml fast running concentrated sulphuric acid (using a pipette with a cut tip) to ensure immediate mixing. The solution was allowed to stand at room temperature for 10 minutes, then shaken and incubated at 30 °C for 20 minutes. The carbohydrate content was determined by immediately measuring the absorbance of the samples at 490 nm in a UV-Visible Spectrophotometer (Thermo-Scientific, *Evolution 300*^{TM} *UV-Vis)* with a Vision pro software (Thermo-Scientific, Vision *pro*^{TM} software) and interpreting using a glucose standard calibration curve with a linearity of 0.99 (see Fig.2). The amount of EPS in every sample was expressed as milligrams per liter glucose equivalent (EPS mg/L Gluc Equiv).

2.6 Screening and Characterization of Exopolysaccharides Producing Lactic Acid Bacteria from Mursik

The LAB from *Mursik* (earlier cultured in M17 and MRS agar in part 3.5) were screened for EPS production as follows; For every sample, 5 - 6 LAB colonies were randomly selected, each purified by streaking on its original selective agar (M17 and MRS agar) and incubating at their optimum growth temperatures (aerobically at 30 ℃ for M17 agar isolates and anaerobically at 35 ℃ for MRS isolates) for 24 hours. The pure isolates were then confirmed to be LAB by catalase, cytochrome-*e*-oxidase and gram stain tests as per the method [26]. The isolates which tested catalase negative, cytochrome-*e*-oxidase negative, and gram positive were confirmed to be LAB. Also by gram stain, the LAB isolates were further characterized based on cell morphology (cell shape) and cell arrangement. The LAB which appeared ovoid (spherical) were referred to as cocci and those that appeared rectangular shaped were rods. The cocci and the rods were characterized as single, paired or chained cells based on their cell arrangements. The confirmed LAB were then screened for EPS production by streaking on Sucrose Hiveg agar (Himedia) and incubating at their optimum growth temperatures as earlier done for 48 hours. EPS positive LAB developed large or small mucoid (see figure 3) or ropy colonies on the sucrose agar [26] [6]. The EPS positive LAB were

streaked on their appropriate selective agar (M17 or MRS agar) and further characterized as shown below.

Figure 3: Mucoid colonies for an EPS positive LAB, *Leuconostoc species* (MH1 (4) M17) Isolated from *Mursik* when grown on Sucrose agar

The EPS producing LAB isolates were characterized to the genera and species level where possible by phenotypic and biochemical tests as follows; Production *of gas in MRS broth was tested* by transferring each isolate into MRS broth with inverted Durham tubes as described by [27][28] and incubating at their optimum growth temperatures as earlier described. The Gas positive isolates (Heterofermenters) produced gas which partially or fully displaced the broth from the Durham tubes, whereas gas negative (Homofermenters) LAB did not produce gas (no gas in the Durham tubes). *Tolerance to 4 % and 6% Sodium chloride (Salt)* was tested as per the method described by [29]. The isolates grew (colonies observed) in the salt concentration which they tolerated. *Temperature Tolerance* was done as per the method described by [27][28], where *Lactococcus* species (M17 isolates) were tested for growth in M17 broth at 10, 30, 40 and 45 ºC for 48 hours (aerobically), and *Lactobacillus* species were tested for growth in MRS broth at 35 and 45 ºC for 48 hours (anaerobically). The isolates grew (MRS broth became cloudy) in the broth incubated at the temperatures which they tolerated and vice versa. *Arginine hydrolysis test* was done by growing the LAB in Arginine Dihydrolase Hiveg^{TM} Broth (Himedia) at their optimum growth conditions as earlier described. Arginine positive LAB grew and changed the colour of the Arginine Dihydrolase Broth from clear red to purple whereas arginine negative bacteria did not change the colour of the broth. The isolates which changed the colour of Arginine Dihydrolase Hiveg^{TM} Broth to purple were confirmed to be Arginine positive using Nessler's test [26]. Arginine positive isolates developed a brown colour upon addition of nessler's reagent into their culture. *Vancomycin resistance test* was done by streaking each isolate on M17 or MRS agar (for *Lactococcus* and *Lactobacillus* isolates respectively), then placing a 30 micro-gram (mcg) Vancomycin disc (SD045-5CT, Himedia) on the agar where the culture was heavily streaked and incubating them at their optimum growth conditions as earlier described for 24 - 48 hours. The cultures were observed for presence of inhibition zones around the 30 mcg vancomycin discs, where the Vancomycin resistant isolates did not have any inhibition zones and Vancomycin susceptible isolates had inhibition zones (0.8 – 1.5 cm radius) around the discs. *Litmus milk Test* was done by growing the test organisms in litmus

milk as per the method [26]. The EPS producing isolates from 24 hour culture broths were each inoculated into Litmus milk tubes in duplicate using a sterile wire loop and incubated as follows at their optimum growth conditions as earlier described for up to 14 days. The cultures were examined on daily basis to establish the various reactions (changes) in the Litmus milk tubes. The reactions were interpreted as follows; Pink colour indicated Acid production (A); Blue colour indicated Alkali production (Alk); White colour indicated reduction of the litmus indicator (R); Presences of clot (C) indicated coagulum formation; and No Change (NC) of the litmus milk indicated negative litmus test (original blue colour retained).

2.7 Isolation and Quantification of Exopolysaccharides from the Exopolysaccharide Producing Lactic Acid Bacteria from Mursik

Whey was used as a growth medium for testing EPS production by the EPS producing LAB isolates obtained from *Mursik*. This was done as per the method described by [25] with a few modifications as follows; Sweet cheese whey was prepared at Guildford Dairy institute (GDI), Department of Dairy and Food Science and Technology, Egerton University, by following the GDI procedure for the manufacture of Gouda cheese. Good quality raw milk, meeting GDI minimum acceptable quality specifications was selected, then pasteurized at 63 °C /30 minutes and cooled to 30 - 32 °C. Active Mesophillic starter culture (CH-22, Chr Hansen) was added at the rate of 2% and ripened for 30 minutes. Rennet (CHY-MAX® Powder Extra NB, Chr Hansen) was added to the milk as per the manufactures instructions (3 g per 100 liters milk) and allowed to set for 45 minutes. The curd was then cut into 5 mm cubes, allowed to heal for 10 minutes and then the whey/curd mixture was gently stirred while raising the temperature gradually to 35 - 38 °C in within 30 – 60 minutes. The curds were separated from whey, filtered using a muslin clothe and centrifuged at 4930 x g for 15 minutes to obtain clear whey. The whey was distributed into falcon tubes, each 90 ml, sterilized in the autoclave at 121 °C for 15 minutes, cooled to room temperature and then used for inoculation. Prior to inoculation of the EPS producing LAB cultures into the whey, the culture was prepared according to the method described by [30] with a few modifications. The method was slightly modified by using MRS broth instead of Modified Exopolysaccharide Selective Medium as the growth medium for the inoculums. The isolates were first reactivated by streaking on M17 and MRS agar (for M17 and MRS isolates respectively) and incubated at their optimum growth conditions as earlier described for 48 hours. From the M17 and MRS agar plates, each culture was transferred to 10 ml MRS broth tubes (1st MRS broth) using a sterile wire loop and incubated at their optimum growth conditions for 24 hours. From the 24 hour MRS culture, the isolates were again transferred (one loop-full) into 10 ml MRS broth tubes (2nd MRS broth) and incubated as earlier described for 24 hours, after which the cultures were then ready for use as inoculums in the whey media. Each 10 ml MRS broth cultures (2nd MRS broth) was wholly transferred into 90 ml whey medium and incubated

at 30 °C and 35 °C (M17 and MRS isolates respectively) for 30 hours. Immediately after incubation, the cultures were transferred into a water bath with boiling water for 10 minutes to inactivate potential EPS polymer degrading enzymes, and then cooled to room temperature before EPS isolation. EPS was isolated from each whey culture as per the method described by [25]. Briefl; 10 ml samples from each culture were transferred from a well mixed whey culture into 100 ml falcon tubes in duplicate. Proteins and cells were precipitated by adding 17 % TCA (80 % w/v), allowed to stand for 20 minutes, and then centrifuged at 4930 x g for 20 minutes. A clear supernatant was obtained by decanting the clear liquid from the centrifuged sample. EPS was precipitated from the supernatant by addition of two volumes (20 ml) of chilled ethanol (Scharlua, AR Spain) at below -10 °C and allowed to precipitate in the fridge at 4 °C for overnight. The EPS extract was harvested by centrifuging the ethanol-EPS mixture at 13000 x g (Eppendorf Centrifuge, 5804) for 25 minutes. The extract was obtained by carefully decanting off the clear liquid (supernatant) and then drying the EPS extract at 45 °C in a hot air oven for 48 hours. The EPS was quantified by Phenol-Sulphuric acid method as earlier described in part 3.6.

2.8 Preservation and storage of exopolysaccharide producing lactic acid bacteria

The EPS producing LAB from *Mursik* were preserved by freezing in MRS broth with 20% glycerol at - 18 to - 20 ℃. To avoid loss of the isolates, they were reactivated and preserved after every 6 months. Short term preservation was done using Litmus milk at 2 - 4 °C with monthly reactivation and repeated preservation.

3. Result and Discussion

Table 1: The mean counts of Lactic acid bacteria in *Mursik* Sampled from Njoro District.

Division	Lab Genus Lactococcus		Lab genus (Lactobacillus /Leuconostoc)	
	Mean Counts (Log/ml)	Mean Counts(SD)	Mean Counts	Mean Counts
Mauche (n = 18)	9.121 [a]	± 0.5138	7.107 [c]	± 0.7589
Njoro (n = 13)	9.124 [a]	± 0.424	8.516 [a]	± 0.5064
Mugungugu (n = 8)	9.099 [a]	± 0.2727	8.074[b]	± 0.3175

Table 2: The Mean Counts of Lactic Acid Bacteria in *Mursik* from Different Locations in Njoro District

Genus	Mean Counts	
	Log/ml	SD
Lactococcus	9.12[a]	±
Lactobacillus/Leuconosto	7.79[b]	±

Note: The Means in the same column with the same letter are not significantly different at *P*< 0.05 (LSD =0.2742 (Mauche vs. Njoro); LSD = 0.3344 (Njoro vs. Mugungugu); LSD = 0.3191 (Mauche vs Mugungugu).

3.1 Lactic Acid Bacteria Count in Mursik

The LAB counts in the *Mursik* from Njoro District are shown on Table 1 and 2. There was a significant difference between the *Lactococcus* and *Lactobacillus/Leuconostoc* counts at 5 % level of significance (*P*< 0.05) as shown in Table 1. The LAB of the genus *Lactococcus* were dominant over the genus *Lactobacillus/Leuconostoc,* the average *Lactococcus* count being 9.12 Log cfu/ml and the average *Lactobacillus/Leuconostoc* count being 7.79 Log cfu/ml as shown in Table 2. Also, the mean counts for *Lactococcus* species were not significantly different (*P*< 0.05) in all the samples obtained from the three locations (Mauche, Njoro and Mugungugu), whereas those for *LactobacilluslLeuconostoc* were significantly different as shown in Table 2.

3.2 Exopolysaccharide Levels in the Mursik Samples

The EPS levels in *Mursik* per household in Njoro District are presented in Table 3. There was a significantly different between the mean EPS levels in the *Mursik* obtained from the different Households (*P*< 0.05). The mean EPS level which significantly differed based on LSD test are shown in Table 3, and the yields ranged between 17.86 and 59.77 mg/L glucose equivalents (Gluc.Equiv), the average being 44.83 mg/L Gluc.Equiv. The mean EPS levels in the *Mursik* also differed between Locations (*P*< 0.05) as shown in Table 4. The mean EPS level of the *Mursik* obtained from Njoro Division was not significantly different from that from Mugungugu Division, but both were significantly different from that from Mauche Division (see Table 4). The mean EPS level for Mugungugu was the highest, with 52.56 mg/L Gluc.Equiv, followed by that of Njoro and Mauche, with 45.9 and 35.41 mg/L Gluc.Equiv respectively.

Table 1: Average Exopolysaccharides levels (mg/L glucose Equivalents) in *Mursik* per household in Njoro District.

Sample (Household)	Mean EPS (mg/L glucose Equivalents)	Sample (Household)	Mean EPS (mg/L glucose Equivalents)
MH39	59.77[a]	MH19	50.38[cde]
MH37	58.62[ab]	MH25	48.41[de]
MH14	58.54[ab]	MH32	46.21[ef]
MH36	58.06[ab]	MH22	39.65[fg]
MH29	58.01[ab]	MH11	36.03[gh]
MH27	56.64[abc]	MH33	35.89[gh]
MH20	56.33[abc]	MH13	33.46[gh]
MH38	55.92[abc]	MH24	32.35[ghi]
MH31	54.66[abcd]	MH16	32.09[hi]
MH28	54.38[abcd]	MH21	25.49[ij]
MH34	53.35[abcde]	MH23	22.89[jk]
MH12	51.79[bcde]	MH15	18.24[jk]
MH26	51.59[bcde]	MH18	17.86[k]

Note: The Means with the same letter are not significantly different at P< 0.05. LSD = 16.0178 (Mauche vs. Njoro, and Njoro vs. Mugungugu); LSD = 13.8618 (Mauche vs. Mugungugu).

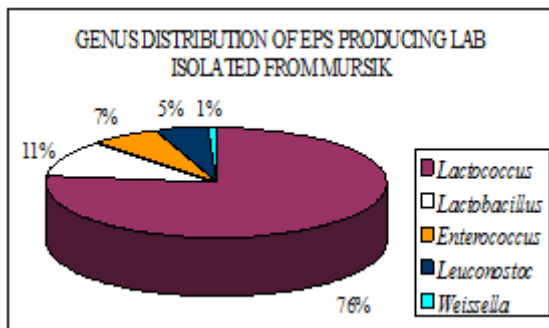## 3.2 Composition of Exopolysaccharide Producing Lactic Acid Bacteria Screened From Mursik

Figure 4: Genera Distribution for the EPS producing LAB isolated from *Mursik*

A total of 122 EPS producing LAB were isolated from *Mursik.* Based on phenotypic and biochemical characteristics (see Table 5), they were classified into five genera as follows, *Lactococcus* (76%), *Lactobacillus* (11%), *Enterococcus* (7%), *Leuconostoc* (5) and *Weissella* (1%) as shown in figure 4. Upon further characterization, 88 *Lactococcus* isolates were identified as *Lactococcus lactis subsp.lactis* while 6 Lactococci isolates were not identified to the species level. All the *Lactobacillus* isolates were homofermentative and were not characterized to the species level. Also, the *Enterococcus* and *Weissella* isolates, were not identified to the species level. These results clearly show that *Mursik* in this region have a variety of EPS producing LAB, but the predominant species are *Lactococcus lactis susp.lactis* (76%). This could be because the predominant LAB species in the *Mursik* were *Lactococcus* (as seen in Table 1 and 2).

Table 3: The mean exopolysaccharide levels (mg/ml glucose equivalents) in *mursik* per location in njoro district

Location	EPS mean	
	Mg/L Glucose equivalents	SD
Mauche (N = 7)	35.41[b]	± 15.37
Njoro (N = 12)	45.90[ab]	± 12.60
Mugungugu (N = 7)	52.56[a]	± 8.66
Overall mean 44.83		

3.3 Exopolysaccharides Yields from the Exopolysaccharide Producing Lactic Acid Bacteria Screened from Mursik

The EPS levels produced by the EPS producing LAB isolates varied between both genera and species as shown in figure 5. Seventy four (74) *Lactococcus lactis species* were tested and found to produce between 55.7 and 449.9 mg/L Gluc.Equiv as shown in figure 5(a). The majority (48%) of these isolates produced between 101 and 200 mg/L Guc.Equiv and very few (5%) produced over 400 mg/L Gluc.Equiv. Twelve (12) *Lactobacillus* isolates

were tested and they produced between 44.3 and 298.4 mg/L Gluc.Equiv EPS as shown in figure 5 (b). The majority (67%) produced between 201 and 300 mg/L Gluc.Equiv. Seven (7) *Enterococcus* isolates were tested and they produced EPS levels between 62.2 and 345.9 mg /L Gluc.Equiv as shown in figure 5(c). The majority (43%) produced between 101 and 200 mg/L Gluc.Equiv. EPS production by the *Leuconostoc mesenteries* and *Weissella species* were each tested on one isolate which produced193.55 mg/L and 244.5 mg/LGluc.Equiv.
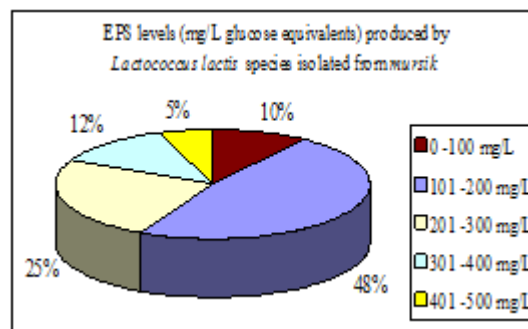
Figure 5: Production of EPS by *Lactococcus lactis* species
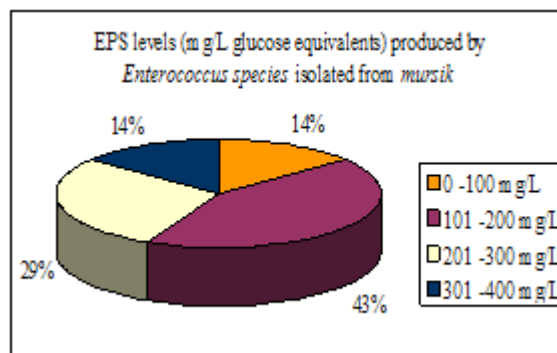
Figure 6: Production of EPS by *Lactobacillus species*
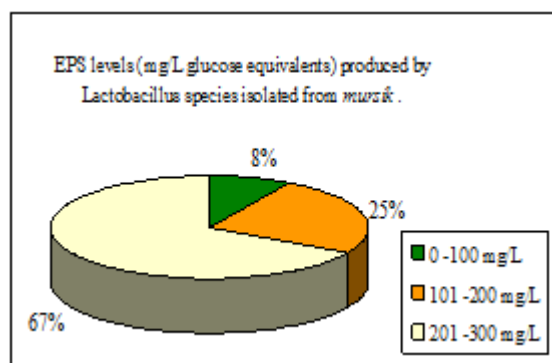
Figure 7: Production of EPS by *Enterococcu*s species.

Figures 5, 6 and 7: Production of EPS by the EPS producing LAB isolated from *Mursik.*

4. Discussion

A. Production of Exopolysaccharides in Mursik

Production of EPS by LAB is important in fermented milk products since they improve their rheological properties, mainly the texture, water holding properties, viscosity, gelling properties, and melting properties[10][12]. They

also impart health beneficial effects to the consumers of fermented milks. However, a key limitation for application of these LAB EPS as a source of biothickener in food is their low yields [7][12][13]. In this study, it was established that the traditional *Mursik* contained EPS which ranged between 17.86 to 59.77 mg/L Gluc. Equiv, the average being 44.83 mg/L Gluc.Equiv. The low EPS yields in *Mursik* may be attributed to several factors which include the composition of EPS producing LAB in *Mursik*, growth medium composition and growth conditions. Although *Mursik* production technology is generally similar among the Kalenjins, different households have different household practices for *Mursik* fermentation. Household variations may arise from the methods for gourd preparation, *Mursik* incubation conditions, the type of plant species used for application of charcoal fines in the gourds, and hygiene and cleaning practices for the gourds. This results in variation in the LAB profile and/or composition of the EPS producing LAB that will be in the *Mursik*. The method used for ripening the gourd prior to its use as a fermentation vessel is very important since it determines the type of LAB which becomes established on its inner surface, hence the LAB culture in *Mursik*. These cultures therefore determine the quality of the final *Mursik* that is produced using the gourd. Cleaning of the gourd is also important since it affects its microbiological quality. Poor cleaning and storage of the gourd results in establishment of non-desirable microorganisms such as yeast and moulds on its inner surface, and eventually become contaminants in *Mursik*.

Composition of LAB and /or EPS producing LAB species affects the EPS yield in fermented milks. According to [31] EPS productions by LAB vary with genera, species and strains, and that thermophillic LAB generally produce more EPS than mesophillic LAB. A study by [32] and [33] also established that the EPS yields by *Streptococcus thermophilus* species were affected by the composition of EPS producing strains in the culture. LAB species vary in their ability to produce EPS due to variation in their genetic potentials[31]. EPS biosynthesis is a complex process which involve activities of different enzymes, mainly non-EPS specific (housekeeping) and EPS specific (*glysosyltranferases*) enzymes [7][12] Housekeeping enzymes catalyze the production of sugar nucleotides (EPS precursors) whereas the *glycosyltranferases* assemble of the sugar nucleotides into EPS polymers [7][12]. EPS production phenotype in a given LAB is encoded by *eps*-genes that are either located in the plasmid DNA in most mesophillic LAB, or in the chromosomal DNA in most thermophilic LAB [12][34]. Each *eps*-gene encodes for a specific *glycosyltransferases,* which catalyzes biosynthesis of a particular type of EPS [12][34]. *Glycosyltranferases* also determine the monomer composition and structure (type of linkages and the side chains) of EPS to be produced [7], and their concentration determines the amount of EPS (yield) to be synthesized. In this study, *Mursik* Culture was found to consist of Mesophillic LAB, the predominant species being *Lactococcus lactis*. Also, the EPS producing LAB in the *Mursik* were predominated by *Lactococcus lactis* species. Except for few strains, most *Lactococcus lactis species*

generally produce low EPS yields. *Lactococcus lactis supsp cremoris* have been reported to produce between 25 and 600 mg/L under controlled conditions. There are no much report previous studies on the EPS production capacities for *Lactococcus lactis subsp.lactis* [12][34].

Production of EPS by LAB is affected by the growth condition such as pH, temperature, incubation period and oxygen potential [32][33][35]. EPS yields have been shown to be lower when cultures are propagated under un-controlled pH system compared to when they are propagated under controlled pH system [32][33]. Traditional *Mursik* fermentation is done under un-controlled pH conditions, and hence this may contribute to low EPS yields. Also, fermentation of *Mursik* is mainly done at ambient temperatures (20 ±1 ℃), which may not be optimum for EPS production by some of EPS producing LAB. EPS production by Mesophillic LAB mainly occurs occur when the rate of LAB growth is low [7][35][36][37]. This is because in these LAB, biosynthesis of EPS and cell wall polymers (peptidoglycan, teichoic acid, lipoteichoic acid and others) occur in almost similar processes, where both require sugar nucleotides, housekeeping enzymes and isoprenoid lipid carriers for assembly and production of the polymers [7][35][36][37]. Therefore, during active cell growth, the rate of EPS biosynthesis is lower since most of the sugar nucleotides and enzymes are directed towards biosynthesis of the cell wall polymers for the new cells [7][35][36][37]. Based on this, EPS Production by Mesophillic LAB mainly occur at sub-optimal conditions because due to low bacterial cell growth, hence most of the sugar nucleotides, housekeeping enzymes and isoprenoid lipid carriers are more diverted towards EPS biosynthesis [36][37].

Growth medium composition, mainly the carbon and nitrogen sources also affect EPS production by LAB [32][33] [38]. LAB species vary in their abilities to utilize different carbon sources or sugars for EPS production [39][40]. The major source of carbon (sugar) in the milk is lactose. This may not been a good carbon source for EPS production by some of the EPS producing LAB (genera, species or strains) in *Mursik*. Carbon source may also be a limiting factor for EPS biosynthesis during active cell growth since it is also utilized for energy generation in the glycolysis system as well as for production of sugar nucleotides required for biosynthesis of cell wall polymers for the new bacterial cells [7].

Production of EPS degrading enzymes (*glycohydrolases*) by some LAB affects their EPS yields. *Glycohydrolases* hydrolyze the already formed EPS polymers in the culture resulting in reduction of yields [36]. This has been established to occur mostly during prolonged incubation periods and due to induction by certain environmental conditions such as pH [36] [37]. The method for isolation of EPS from *Mursik* may have also contributed to the low EPS yields observed in *Mursik*. EPS may have been lost during the isolation process either with the retentate as co-precipitates with protein during precipitation with Trichloroacatic acid (TCA) or with the supernatant during EPS extract recovery from chilled ethanol after

centrifugation. It has been reported that substantial amounts of EPS are lost during the isolation process due to the complexity of the isolation and purification method [36][37][6]. According to [6], precipitation of protein with TCA from the culture results in up to 50%l losses of EPS as co-precipitates with the protein. An alternative method for protein precipitation involves the use of proteases such as pronase [6]. However, after protein digestion with proteinases, the culture must be subjected to a heating process to inactivate the proteinases and this also interferes with EPS isolation process [6]. There are other methods for EPS isolation which include membrane filtration, ultra filtration and diafiltration, but precipitation with TCA and chilled ethanol is more preferred in studies since it results in EPS with fewer impurities [6] EPS purification is mainly done by dialysis using membranes of different cut-off (1000 – 12000 Da). In this study, EPS isolation process involved to use of 17% TCA with 80% m/v concentration for protein precipitation and EPS precipitation using chilled absolute ethanol. Although the EPS levels in the *Mursik* samples were generally low, this is in line with the reported low EPS levels by most LAB culture. However, EPS from LAB have been reported to have excellent physico-chemical properties even at low levels [7][13], and the threshold levels required to impart health beneficial effects on consumers has not been established.

Several studies have shown that there is a wide variation in EPS production by different LAB. The EPS yield, EPS molecular mass and EPS monomer composition vary with different LAB species and strains [31][38]. In this study, *Mursik* culture was established to consist of a variety of EPS producing LAB consisting of *Lactococcus* species (76%), homofermentative *Lactobacillus* species (11%), *Enterococcus* species (7%), *Leuconostoc* species (5%) and *Weissella* species (1%). Their EPS production capacities varied between genera and within the species, their yields ranging between 44.3 - 449.9 mg/L glucose equivalents. Majority (48%) of the *Lactococcus* lactis species produced between 101 and 200 mg/L glucose equivalents and very few (17%) produced above 300 mg/L glucose equivalents. Generally, this are low yields compared to those for non-food grade microorganisms [12][36][37]. However, some LAB species such as *Lactobacillus rhamnosus* have been reported to produce higher yields, up to 2.7g/Liter [12], although they are still far much below the recommended level for economical production of EPS bio ingredient [12]. Low EPS yields have been reported in different LAB genera and species including *Lactobacillus, Enterococcus, Leuconostoc* and *Weissella*, although a few high yielder have also been reported [12][31]. The diversity in EPS production by the EPS producing isolates from *Mursik* is mainly attributed to their genetic diversity (species and strain genetic variation), their variation in growth medium and growth condition requirements. Generally, LAB of the genus *Lactococcus* produces low EPS yields [36][37]. Some LAB have been shown to produce different EPS yields and EPS composition from different carbon sources [7][40]. The growth medium that was used for the analysis of EPS production by the EPS producing LAB from *Mursik* was whey, whose major carbon source is lactose.

Therefore, lactose as a carbon source may have been a limiting factor for production of EPS by some of the isolates. Growth condition (temperature, pH) may have also been a limiting factor for EPS production since different LAB strains have different optimum temperature and pH conditions at which they produce EPS [33][38]. Higher EPS levels are produced in pH-controlled systems than in pH un-controlled systems. In this study, propagation of the EPS producing isolates from *Mursik* was done under un-controlled pH condition, hence may have contributed to the observed low yields. Production of EPS degrading enzymes (*glycohydrolases)* by some LAB isolates may have also their affected the EPS yield.

5. Conclusion

From this study, it was concluded that the traditional *Mursik* produced in Njoro District contains Exopolysacharides (EPS) and EPS producing Lactic acid bacteria (LAB). The EPS producing LAB in *Mursik* are composed of a variety of LAB genera but predominated by the *Lactococcus* species. The EPS levels in *Mursik* are generally low and this is attributed to the low potential for EPS production by the *Lactococcus* species which are the predominant species. The EPS levels by the EPS producing LAB isolates are also low and they vary with genera and species*.*

6. Recommendations

The finding of this study indicated that *Mursik* contains EPS and EPS producing LAB, responsible for observed health benefits of *Mursik. Mursik* should therefore be developed as a functional fermented milk product by developing a defined *Mursik* culture which can be preserved and used for larger production of *Mursik.* This will also enable production of *Mursik* using modern fermentation technologies which guarantee good quality and product safety. Production conditions such as temperature, pH, growth media composition and incubation periods for optimum EPS production in *Mursik* should be established in order to optimize its functional properties. The EPS producing LAB from *Mursik* should be developed for application in production of other fermented food products as functional starter cultures as well as agents for improving food rheological properties. In future, studies should be done to establish their requirements for optimum EPS production and hence establish their applicability in food production processes.

7. Acknowledgement

8. Future Scope of the Study

Mursik is a source of a variety of Exoploysaccharides (EPS) producing LAB which can be developed for application in the dairy, food and other industries as starter culture or source of EPS synthesizing enzymes. In future, further studies should be done to establish optimum growth requirements for EPS production by the individual EPS producing Lactic Acid Bacteria in order to establish their best economical use. Also further studies should be done in future to characterize the EPS polymers produced by the various EPS producing LAB. Traditional production technology for Mursik production should be modified to adopt modern production technologies where a purified culture is used as a starter culture. This will assist in preservation of the Mursik culture, adoption of commercial production process in Mursik production, as well as enable application of production technologies that will improve on EPS production during processing.

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Table 5: Phenotypic and Bioche

Genus		Cocci			Rods / Coccobacilli				
		Lactococcus lactis	*Pediococcus*	*Enterococcus*	*Lactobacillus* (Homofermentative)	*Lactobacillus* (Heterofermentative)	*Weissella* Arginine Negativ	*Weissella* Arginine positive	*Leuconostoc)*
No of isolates		94	0	8	13	0	0	1	6
Cell morphology		Cocci	Cocci	Cocci	Rod	Rod	Rod	Coccobacilli	Coccobacilli
Cell arrangement		Single, paired, chained cells	paired tetrads	Single cells, paired, chains	Single cells, paired, chains	Single cells, paired, chains	Single cells, paired, chains	Single cells, paired, chains	Single cells, paired, chains
Phenotypic Characteristics									
1. Gas production from MRS broth		-	-	-	-	+	+	+	+
2. Arginine hydrolysis		+[a]	+	+	±	+	-	+	-
3.Growth Temperature	10 ºC	+	± (Most -)	+	±	±	±	±	+
	30 ºC	+	+	+	+	+	+	+	+
	35 ºC	+	+	+	+	+	+	+	+
	40 ºC	+	+	+	+	+	+	+	±
	45 ºC	-	± (Most +)	±	-[b]	±	±	+	-
4.Tolerance to Sodium Chloride (NaCl)	4%	+	+	+	+	±	±	±	±
	6.5%	±	±	±	±	±	±	±	-
5. Vancomycin resistance (30 mcg disc): S-susceptible R- resistant		S	R	S	R/S	R/S	R/S	R	R
6. Litmus milk Test: A-acid production, R-reduction of litmus colour, C- coagulum formed		+ (ARC)	+(ARC)	(+)ARC	± (ARC)	± (ARC)	± (ARC)	± (ARC)	± (ARC)

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