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Isolation and Characterization of Probiotic Lactic Acid Producing Bacteria in Kenyan Traditionally Fermented Milk

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Abstract

In industrial fermented products, Lactic Acid Producing Bacteria (LAB) are used as starter cultures in products like yoghurt. For most of these communities fresh and fermented milk is a staple part of their diet. Kenyan communities such as the Maasai and Kalenjin ferment their milk adding different substances which differ between communities. This study aimed at isolating and characterizing LAB found in traditionally fermented milk from communities around Kenya and to identify similarities with artificially-added LAB. Different methods were used to identify lactic acid producing probiotic bacteria in fermented milk including gram staining, catalase tests, sodium chloride tolerance test. Strains of LAB isolated were from Family Lactobacilli and Family Streptococci. The quantity differed between communities with lactobacilli being the dominant isolate from the Maasai culture and streptococci being more dominant in the Kalenjin isolate. This proved that traditionally fermented milk offers a wider range of LAB than industrial starter cultures.

Keywords: Lactic acid producing bacteria; traditionally fermented milk; Kenyan communities; industrial starter cultures

Introduction

Fermented foods have been used as a source of lactic acid producing bacteria (LAB) by different communities all over the world. LAB is of great importance in the human body. They have been classified into various families which include; Lactobacillaceae, Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Leuconostocaceae, and Streptococcaceae. These bacteria have several importance in the human body like acting as probiotics. Probiotics are live micro-organisms, when administered in adequate amounts confer a health benefit on the host [1,2]. The word probiotic comes from the Greek word 'pro' meaning promoting and 'biotic' meaning life. Probiotics were discovered in the early 20th century, when Ellie Metchnikoff, known as the 'father of probiotics' had observed that rural dwellers of Bulgaria lived to very old ages despite extreme poverty and harsh climate [3]. His theory was that health could be improved by manipulating host friendly bacteria in sour milk. The International association for probiotics and prebiotics (ISAPP) in conjunction with other medical and scientific experts were also able to differentiate between foods which contained live cultures and those which contained probiotics [4]. Live cultures are any foods with fermentation microbes which need no specific research is required to prove their existence. Probiotics are supported by convincing evidence on their benefit to man and have clearly defined strains.

The human body naturally contains normal flora and harmful bacteria. A balance between the two is necessary and when it is disrupted (dysbiosis) can cause diseases like ulcerative colitis, irritable bowel syndrome amongst others [5]. For probiotics to take effect it requires a prebiotic. This is a non-digestible carbohydrate that acts as food for probiotics and other bacteria. A prebiotic helps a probiotic by combining to form a synergetic effect known as symbiotic. Most probiotics identified are oligosaccharides and are resistant to human digestive enzymes. They go through the gastral-intestinal system without being digested and get fermented in the lower colon to form short

chained fatty acids that nourish the microbes in the colon. Probiotics can be identified in a variety of natural sources including bananas, barley, garlic, honey, milk, Mustard, soybean, sugarcane, tomato and wheat amongst others. In Kenya, fermented milk (mala) is a common supplement for fermented milk lactic acid producing bacteria strains. Two samples will be used for the study; from the Maasai community, and the Kalenjin community to individual diets. With the cultural diversity of the various tribes, fermented milk has developed distinct characteristics because of the different additions.

Materials and Methods

Materials

Milk was obtained from the Maasai and Kalenjin community. The following analytical grade chemicals were used (all from Sigma-Aldrich, Germany): sodium acetate, sodium hydroxide, yeast extract, glucose broth, Tween-80.

Methods

1. Sampling and study size: The milk samples came from the cows were obtained by simple random sampling method. Two samples were required from each community to enable the comparison between the lactobacillus. The study size of this experiment covered two Kenyan communities; the Maasai and the Kalenjin. These two tribes are well known for the unique ways in which they locally ferment their milk with different additives to give the milk a distinct taste and texture.

2. Isolation: Isolation involved creation of a media that was able to effectively sustain sample LAB cultures. An enriched nutrient agar media was used. It was enriched using; Sodium acetate, Sodium hydroxide, Yeast extract, Glucose broth, and Tween 80. This media was weighed out and the optimum quantities were obtained which supported the growth of the cultures. After preparation of the media each was inoculated from the three different samples. Growth of the colonies was then observed at intervals of 24 and 48 hours. When the colonies were formed, isolates were obtained for pure culture so as to obtain pure isolates for morphology observation and further analysis.

3. Gram staining: This test was done to authenticate the presence of LAB in the culture. Gram positive bacteria turns violet blue due to the presence of a thick peptidoglycan in their cell wall. Gram negative bacteria stains red due to a thinner peptidoglycan in their cell [6]. The procedure involved; preparing a heat fixed sample, adding the primary stain (crystal violet blue) followed by Gram's iodine after washing of the unbound primary stain. The slide was then rinsed in acetone or alcohol followed by use of running distilled water. The secondary stain safranin was then added before incubating for one minute. Observations were done to determine the presence of LAB.

4. Sodium chloride tolerance: Isolated samples of lactobacilli were grown in enriched media in several test tubes each containing different concentrations of sodium chloride from 1-9%. After autoclaving the media for 15 minutes in 15lbs pressure at 121°C, the test tubes were then inoculated with 10 microliters of overnight culture of Lactobacillus and then incubated anaerobically at 37°C for 24 hours. The bacterial growth was measured afterwards [7].

5. Catalase test: Catalase test was done by aseptically mixing 1ml of hydrogen peroxide in a test tube and adding 1ml of culture. For positive results, there was a formation of bubbles to indicate evolution of gas [8].

Morphological analysis

After culturing, various qualities of the colonies including; the shape, the color, and the size of the colony was observed. Cell morphology was also observed upon further investigation to confirm the shape, on whether it was rod shaped or cocci of individual bacterial cells. Likewise, it was observed to indicate on whether they occurred in pairs or individually, with clear understanding on the spore formation and size.

Data analysis

Data collection was done through experimentation and observation methods. The experiments done provided a data base for analysis and comparison of the results obtained from the different sources.

Results

Isolation of LAB

Table 1: MRS Media vs Enriched Media.

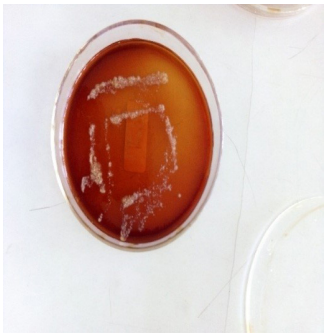
MRS Media	Enriched Media
Beef extract	Beef extract (NA)
Peptone	Peptone (NA)
Yeast extract	Yeast extract
Glucose	Glucose
Sorbitan monooleate	Tween 80
Magnesium sulphate	-
Ammonium citrate	-
Manganese (ii) sulphate	-
Sodium acetate	Sodium acetate
-	Sodium hydroxide

Morphology

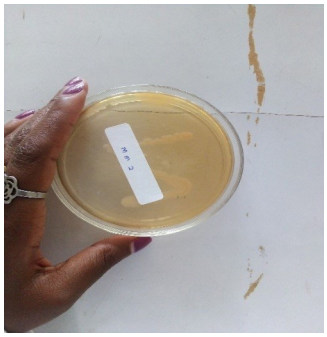
Colony Morphology

Table 1: Colony morphology of cultured lactic acid producing bacteria.

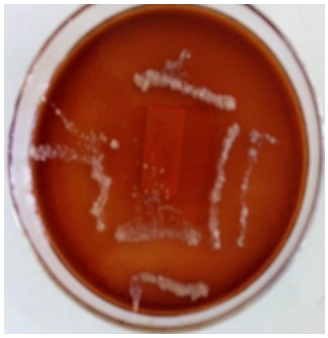
Sample	Color	Shape	Margin	Elevation
MS_1	White	Rounded	Smooth	Flat
MS_2	White	Rounded	Smooth	Aerial
MS_3	Creamy	Oval	Smooth	Aerial
KS_1	Creamy	Oval	Smooth	Flat
KS_2	White	Rounded	Smooth	Flat
KS_3	White	Oval	Smooth	Flat
CS	White	Rounded	smooth	Flat



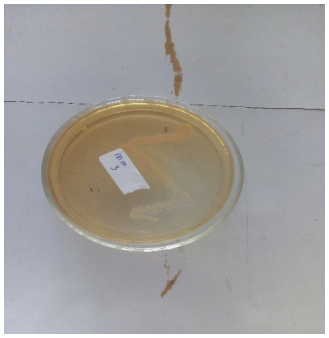
A Colony MS1



B colony MS2

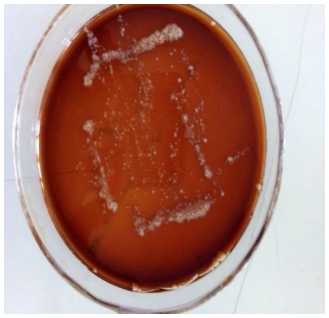


C Colony TS1



D colony MS3

E



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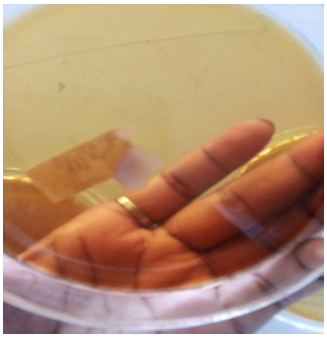
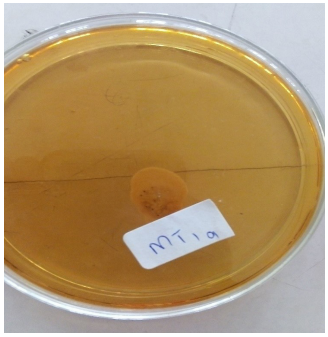
Cell morphology

Table 2: Cell morphology of cultured lactic acid producing bacteria.

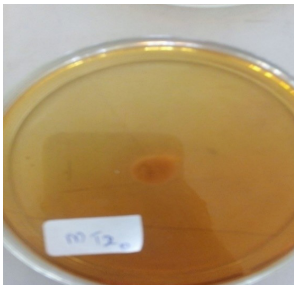
sample		Shape	Gram stain	Salt tolerance	Catalase test	Spore formation
MS_1	A	Rod (short)	Positive	3% positive	-	None
	B	Rod (pairs)	Positive	4% positive	-	None
	C	Rod (short)	Positive	3% positive	-	None
MS_2	A	Rods (rounded)	Positive	2% positive	-	None
	B	Rod (rounded)	Positive	3% positive	-	None
	C	Rod (pairs)	Positive	3% positive	-	None
MS_3	A	Rod (rounded)	Positive	2% positive	-	None
	B	Rod (short)	Positive	4% positive	-	None
	C	Ro d short)	Positive	2% positive	-	None
KS_1	A	Rod (pairs)	Positive	4% positive	-	None
	B	Spherical	Positive	3% positive	-	None
	C	Rod (short)	Positive	4% positive	-	None
KS_2	A	Spherical	Positive	3 % positive	-	None
	B	Spherical	Positive	2% positive	-	None
	C	Spherical	Positive	3 % positive	-	None
KS_3	A	Rods (rounded)	Positive	2% positive	-	None
	B	Rod (pairs)	Positive	3% positive	-	None
	C	Spherical	Positive	3% positive	-	None
CS		Rods	Positive	4% positive	-	None



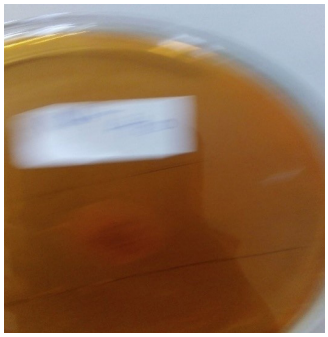
G –Kalenjin sample pure culture 1



H –Maasai sample pure culture 1



I –Maasai sample pure culture 2



J-Kalenjin sample pure culture 2

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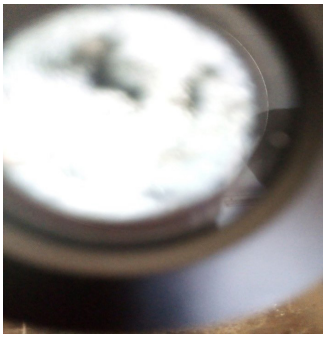

K-microscopic observation of cultured isolates

Discussion

Isolation

Isolation of LAB requires specific conditions which are provided by the ideal media; De Man Ragosa and Sharpe (MRS). In the absence of this media a substitute was established using nutrient agar media which was enriched using various compounds to make it enriched and selective. This media had to provide conditions which optimize growth of LAB. This condition is pertinent for industrial starter cultures used in diary production using LABs and bifidobacteria [9].

The media created was able to be selective only allowing the growth of the LAB that was inoculated. The beef extract found in the nutrient agar, glucose and the added yeast extract provided nutritional value to the bacteria. Sodium acetate made the media selective and allowed only the growth of LAB colonies. Sodium hydroxide stabilized the pH of the media to make it conducive for culture. The pH of the media ranged between 6.5-7.2. In the Maasai samples, growth was observed with the optimum growth being between 36-48 hours. This duration is convenient enough for most of the diary production processes. In the traditional and contemporary Maasai and Kalenjin milk preparation norms, the milk would take a similar range of time (36-48 hours) to commence fermentation [10]. Faster retention periods were obtained when higher temperatures were used. The inoculates were then isolated for a pure culture. The Kalenjin sample also yielded results in the selective media and growth was observed between 36-48 hours. The control yielded growth of the colonies of LAB found in the sample.

Colony Morphology

1. Maasai milk sample culture: After incubation all samples indicated the presence of LAB. Upon further investigation, where pure culture samples of the colonies were obtained, the morphology of the different colonies was described. In sample 1, the colony appeared white in color citing presence of non-pathogenic yeasts such as lactobacilli and streptococci spp. [11]. It was rounded and had a smooth margin. The bacteria grew horizontally across the media with no elevation. Similar observations were recorded by Adeniyi [12] while studying the antibacterial activities of LABs isolated from cow faeces against potential enteric pathogens. Sample 2 colony presented itself as white in color. The colony was round and had a smooth margin. There was a slight elevation in the colony showing aerial growth. The third isolate was white and the colony was round. It had a smooth margin and aerial growth was also observed. These characteristics are common for most LABs [13].

2. Kalenjin milk sample culture: All the three samples incubated were able to culture LAB at ambient conditions. Azat [14] showed that such LABs have desirable antimicrobial, auto-aggregation and hydrophobic properties. A pure culture of an individual colony was obtained from each. The isolate from the first sample was creamy in color. It had a rounded shape and a smooth margin. There was slight aerial growth of the colony. The second sample isolate appeared white in color. It had a smooth margin and was rounded in shape. Like the Maasai milk sample, the isolate also grew horizontal to the substrate with no elevation. The second colony isolate was creamy and rounded in shape. It had a smooth margin and is flattened along the substrate. The third sample isolate was creamy in color, rounded in shape, had a smooth margin and is flattened along the media surface.

Cell morphology

1. Maasai milk sample: A pure culture from each sample was done and then various tests were carried out on the isolates. The first sample had isolates which were gram positive and upon further observation under the microscope, the cells appeared rod shaped and short. This is a characteristic of LAB under Family Lactobaocillaceae. The second isolate also had gram positive cells. They appeared rod shape and rounded in shape. These cells were in the Family Lactobaocillaceae, the third isolate also had gram positive bacteria. When observed under the light microscope they presented as rod shaped bacteria which were squared. The majority of isolates were LAB of Lactobacillaceae with the exception of a few unidentified species. The round shapes of the LABs have a lot of effect on their motility and mobility [15]. The shapes allow the bacteria to move around easily in numerous directions and avoid potential predators [15].

2. Kalenjin milk sample: After the colony morphology was observed a pure culture was performed to identify and characterize the cells. In the first isolate gram-positive cells were identified. The cells were rod shaped and appeared in pairs. This is a characteristic of family Lactobaocillaceae. The second isolate presented itself as gram positive spherical cells which are common characteristics of LAB from the family Streptococcaceae. The third isolate had gram positive rod shaped which were rounded cells. These are common characteristics of Family Lactobaocillaceae.

Salt tolerance

All the samples did well under low concentrations of sodium chloride ranging between 2% to 6% concentration. These are the optimum standards for LABs to survive in an environment. The findings closely corresponded to those of El-Gendy [16]. This range is sufficient for encoupling between the growth and energy production of LABs. Most L. plantarum strains of LABs can easily reduce the pH of their surrounding media at salt concentrations of less than 8%.

Catalase test

Catalase enzyme neutralize toxic hydrogen peroxide produced during biochemical processes by certain gram-positive bacteria, effectively diluting them to water [17]. All the sample isolates tested negative for catalase activity. This test showed that the isolates were in fact LABs and had little contamination. The test further indicated that the LABs were anaerobic and did not require oxygen to form hydrogen peroxide (a toxic by-product of anaerobic digestion). This was the confirmatory test of LABs in the Kalenjin and Maasai milk samples [18-20].

Spore formation

The isolates were all non- spore producing bacteria. This is also common for LABs. Researchers from different countries have been able to isolate lactic acid bacteria from different fermented foods. These foods are consumed by locals in their countries just as traditionally fermented milk is consumed here in Kenya. Lactobacilli isolates have previously been isolated from curd milk samples. They used the conventional media De Man Ragosa and Sharpe (MRS) agar media and were able to characterize them by the phenotypic characteristics [21-25].

This experiment was able to isolate and characterize LAB using an enriched media. Unlike industrial starter cultures which offer only one variety of microflora, traditionally fermented milk offers a wider array of bacteria. However, these bacteria may not offer a health benefit and might instead cause infection in the body. Only a few strains of the bacteria are beneficial to the host and the remainder may either be infective or not affect the host in any way.

Conclusion

Lactic acid producing bacteria were successfully isolated and characterized from fermented milk samples of two Kenyan pastoral communities (Maasai and Kalenjin). The milk samples tested positive to most of the LAB tests and had a high correlation to conventional industrial cultures. This study indicates that the two milk samples can be used to harness industrial LABs which have a wide spectrum of industrial uses.

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