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Isolation, biochemical characterization and safety screening of potential probiotic lactic acid bacteria from spontaneously fermented cereal products from Western Kenya

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The global demand for non-dairy beverages has sky rocketed especially so during this Covid-19 pandemic for potential health benefits. Development of probiotic strains from fermented cereal and legumes with the ability to grow well and adapt to gastrointestinal conditions at the same time possess high therapeutic ability will be a great achievement. This study aimed at isolating and screening probiotic potential Lactic Acid Bacteria (LAB) involved in traditional fermentation of cereals (maize, sorghum and millet). A total of ten isolates were obtained from the cereals out of which five isolates that met preliminary attributes of probiotic bacteria were selected for further investigation. Two isolates SPU2 and FPU1 were found to survive a low pH which is a desirable attribute for the survival of probiotic bacteria in the gut. MPU1, FPU1 and SPU2 are possible thermophiles and can survive at low pH and moderate high salt concentration. The enzymes DNase and gelatinase used to test pathogenicity of a microorganism were not produced by all the isolates in this study. The isolates recorded a high susceptibility to the eight antibiotics. This study also revealed that the tested isolates have the ability to grow well even at the minimum tested pH of 1.0 for 1 and 2 h of incubation, respectively. Most isolates were resistant to 0.3% bile concentration with over 92% survival. FPU1 was more resistant at bile concentration of 1% than all the rest while MPU1 was most resistant at 2% bile salt. Traditionally fermented cereals are potential sources of safe bacteria that can be tried in the production of functional foods.

Key words: Probiotic potential LAB, bile concentration, sodium chloride, pH, cereals.

INTRODUCTION

The love for functional foods has increased as a result of factors such as health awareness and a lot of research information emphasizing the interconnection between food, health and diseases (Küster-Boluda and Vidal-Capilla, 2017). This knowledge is causing abrupt change from foods of animal origin to those originating from

plants (Brigitta and Ágoston, 2019). Whole grain cereals contain phytochemicals such as phytic acid, lignans and phenolic substances which are good for health and disease prevention (Călinoiu and Vodnar, 2018). To improve the nutritional properties of cereals several technologies have been employed but fermentation is the most outstanding (Nkhata et al., 2018). Fermentation improves the nutritional value of food, stability, safety and organoleptic properties (Kawaljit et al., 2017). This process therefore could be a potential technology for producing new bioactive compounds from natural food raw material. Malnutrition continues to be a great challenge and burden in our country today. To improve food security and nutritional value of food consumed among the Kenyan households, intentional efforts and appropriate strategies need to be adopted to guarantee affordable healthy meals. Development of non-dairy probiotic products from cereals could lead to commercialization of products of unique flavour, taste and of nutritional adequacy. Cereal grains are rich in carbohydrates, calories, proteins, vitamins and minerals and are therefore good substrates for the development of probiotics which can be used in probiotic foods (Achi and Asamudo, 2019). Cereal products may have a range of bioactive substances with potential health benefits. Fermentation of food raw material generally involves LAB (Kawaljit et al., 2017; Bintsis, 2018). The natural presence of LAB in cereals is of great interest in producing fermented cereal products (Tsafrakidou et al., 2020). Fermented cereal beverages are reported to be among the most active functional foods (Bansal et al., 2016). Continuous consumption of such functional foods can ensure overall good and prevent diseases. Probiotics are living microorganisms in foodstuffs which, when consumed at certain levels in nutrition, stabilizes the gastrointestinal tract microflora thereby conferring health benefits to the consumer (Markowiak and Śliżewska, 2017). Probiotics also carry active biological substances in reasonable quantity that influence good health (Terpou et al., 2019). The potential health benefit of a given probiotic depends on its profile characteristics (Shi et al., 2016). The most common probiotics in the market are *Lactobacillus* and *Bifidobacterium* (Tsafrakidou et al., 2020). In general, most probiotics are Gram-positive, usually catalase-negative, rods with rounded ends, and occur in pairs, short, or long chains (Peyer et al., 2016). They are non-flagellated, non-motile and non-spore-forming, and are intolerant to salt (Elshaghabee et al., 2017). Most probiotics LAB have optimum growth temperature at about 37°C while other strains at 30°C and pH optimum for initial growth is normally in the range from 6.5 to 7.0 (Peyer et al., 2016). The identification of specific microflora involved in indigenous cereal-legume fermentation is needed to amplify and control positive factors as well as to minimize or prevent negative factors such as growth and metabolism of pathogenic and toxicogenic bacteria (Enujiugha and Adebanjo, 2017). This study aimed at biochemically characterizing and safety screening of probiotic potential LAB isolated from spontaneously fermented cereal products from Western Kenya.

METHODOLOGY

Cereal (Sorghum, millet and maize) samples (500 g each) were obtained randomly from cereal store traders from Vihiga County, Western Kenya. A total of 25 samples were collected for each cereal. The samples were packaged in kaki bags and taken to the Food and Microbiology Laboratories, Technical University of Mombasa for analysis.

Fermentation of cereal products

Fifty grams of each cereal sample (maize, sorghum and finger millet) were weighed after thorough mixing, sorted and cleaned. A blender was used for grinding the cereal flours with sterilization of the blender after every cereal sample was ground with 70% ethanol. The sample flours were each transferred to fermenting bottles aseptically, two parts water added, mixed and sealed. Fermentation was done by incubating at 30°C for 48 to 72 h. 10 g was drawn from each fermentation aseptically for probiotic potential LAB isolation.

Isolation of LAB

LAB was isolated from traditionally fermented cereals (maize, sorghum and millet). The fermented samples were appropriately suspended and diluted in sterile saline. An initial dilution of (10^{-1}) was obtained after homogenizing 10 ml of each sample with 90 ml of 0.85% (w/v) sterile sodium chloride solution. Dilutions of up to 10^{-7} were serially made for every fermentation. From each of the 10^{-5} to 10^{-7} corresponding dilutions, 1 ml sample was plated out onto (De Man, Rogosa and Sharpe) MRS agar (De Man et al., 1960) supplemented with 0.05 g/L Cysteine-HCL (MRS-CysHcl) and M17 agar plates by spread plate technique in triplicate. Incubation of the inoculated plates was then followed at 37°C for 48 to 72 h anaerobic jars. The plates were keenly examined. The bacteria colonies with distinct morphologies such as color, form, margin, consistency and surface elevation were randomly selected. Purification of selected colonies was performed by sub-culturing twice on MRS agar plates through streaking. MRS broth with 30% glycerol (El-Soda et al., 2003) at +4°C (Patil et al., 2010) was used for maintenance of the pure cereal LAB isolates. Gram staining, cell morphology, catalase test (Sharpe, 1979), carbon dioxide production from glucose and antibiotic susceptibility test were carried out. Five isolates were selected for further screening.

Biochemical characterization of the lactic acid bacteria isolates

In this study, 0.17 g/l bromothymol blue was added to MRS broth as

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pH indicator (pH 7). The MRS broth was filled in universal bottle with screw caps, each carrying 20 ml followed by autoclaving. An overnight culture isolate of each was used as the inoculum. The cells were spun down, normal saline (0.85%) used for re-suspension. From the suspension, a loopful of the mass was inoculated into each of the bottles. The following temperatures were assessed: 15, 37, 45 and 55, the NaCl concentration studied include: 2, 3, 6.5 and 10% (w/v), the following are pH evaluated 2, 3, 4 and 6 (Liong and Shah, 2004). The initial pH of MRS broth was modified using 1 M NaOH and 1 M phosphoric acid. Color change in any test tube implied growth.

Safety assessment of LAB

Antibiotic susceptibility

Zhang et al. (2016) method was followed with slight modification to determine antibiotic susceptibility of the cereal LAB isolates. Eight antibiotics include: Gentamicin, Ofloxacin, Nitrofurantoin, Cefaclor, Nalidixic Acid, Augmentin, Minocycline and Cefuroxime. Antibiotic-impregnated discs (Abtek Biological Ltd, England) were placed on plates streaked with isolates. Any sign of clearance or zone of inhibition along the tips after an overnight growth at 37°C was taken to mean the isolates were susceptibility to the antibiotics.

Test for hemolysis

The method of Linaje et al. (2004) was followed in determining hemolytic activity of the selected isolate. Fresh isolates grown for a night were spot inoculated on Blood Agar plates (HiMedia) and incubated for 48 h at 37°C.

Production of DNase enzyme by isolates

Gupta and Malik (2007) method was followed in this study. A clear pinkish zone around the colonies was considered to mean the isolates produced the enzyme DNase.

Gelatinase enzyme production

Evaluation of gelatinase enzyme production was done according to Harrigan and McCance (1990). MRS agar plates containing 3% gelatin were prepared then inoculated with an overnight culture of isolates by streaking. Clear zones around the colony against a dark base indicated a positive effect.

Probiotic potential attributes of the isolates

Tolerance to low acid conditions

Liong and Shah (2004) method was followed with slight changes. MRS broth with pH adjusted to 1, 2, 3 and 6.5 were used to study how the LAB isolates can tolerate low pH for 1, 2 and 3 h. The cells were enumerated, growth expressed as colony forming units per milliliter (log CFU/ml) and percentage survival calculated.

Bile salts tolerance of the Isolates

Gilliland and and Walker (1990) and Aswathy et al. (2008) methods were considered to assess the effect of bile salt concentration on isolates growth rate. MRS broth was supplemented with bile salt (0.3, 1 and 2%) for 8 h. An inoculum of 100 µl was drawn and plated onto MRS agar. Incubation was followed at 37°C for 24 h and survival rate calculated.

RESULTS

The results of this research indicate that probiotic potential LAB could be isolated from spontaneously fermented cereal products. From all samples, 10 probiotic potential LABs (3, 4 and 3 from maize, finger millet and sorghum, respectively) were isolated 7 of which were presumptive *Lactococcus* and 3 *Lactobacillus* species (Table 1) based on biochemical characterization; a total of 3, 4 and 3. Out of the ten LAB, three isolates were found to be hetero-fermentative. Homo-fermentative LAB ferments carbohydrates with the production of only lactic acid which can lower the pH of medium close to 4.0-4.5, hetero-fermentative LAB on the other hand produces carbon-dioxide and other organic compounds (acetic acid, alcohol, acetaldehyde, diacetyl) which can further lower the pH to about 3.5.

Table 1 shows the tolerance of the 10 LABS to different conditions of temperature, salt concentration and pH. The temperature of 37°C and salt concentration of 2% favored the growth of all the bacteria. The isolates surviving at 45°C are comparable to yoghurt starter culture organisms that have an optimum between 38 and 42°C. Isolates MPU1, FPU1, FPU2 and SPU2 survived a temperature of 45°C. Five (5) isolates were able to withstand salt concentration of 6.5% while only one at 10%. The isolates that grew at 45°C, salt concentration of 6.5 and 10% and low pH are special species that could be of use in systems where either temperature of growth medium is elevated or pH fall extremely. Two isolates SPU2 and FPU1 can survive a low pH which is a desirable attribute for the survival of probiotic bacteria in the gut. Still those surviving at pH 2 are potential probiotics. MPU1 (*Lactobacillus* spp.), FPU1 (*Lactococcus* spp.) and SPU2 (*Lactococcus* spp.) are possible thermophiles and could thrive at reduced pH and moderate high salt concentration.

Safety assessment of LAB

The LAB isolates in this study were found to be sensitive to nearly all antibiotic used (Table 2). Any resistance shown was not biased towards a particular antibiotic, apart from Cefuroxime where two isolates showed resistance. The isolates' safety is guaranteed from the high percentage sensitivity, meaning they may not present resistance towards antibiotic use.

The isolates did not hemolyze the blood since clear zones were not seen around colonies on blood agar. Pathogenicity factors demonstrated by the production of gelatinase and DNase enzymes were not observed in this study among all isolates. The clear pinkish color around the colonies to demonstrate DNase enzyme production was not observed.

Table 1. Phenotypic characteristics of probiotic potential LAB isolated from traditionally fermented Cereals from Vihiga.

Source	Gram's reaction & Cell shape	Cultural characteristics	Catalase test	CO_2 from Glucose	Growth at temperatures (°C)				Growth in NaCl concentration (%)				Growth at pH				Possible species
					15	37	45	50	2	3	6.5	10	2	3	4	6	
MPU1	+Cocci	Ppc	-	-(Homo)	-	+	+	-	+	-	-	-	-	+	+	+	*Lactococcus* spp.
MPU2	+Cocci	Ppc	-	+(Heter)	-	+	-	-	+	+	+	-	-	+	+	+	*Lactococcus* spp.
MPU3	+Rod	Ppc	-	-(Homo)	-	+	-	-	+	-	-	-	-	+	+	+	*Lactobacillus* spp.
FPU1	+Cocci	Ppc	-	+(Heter)	-	+	+	-	+	+	+	-	+	-	+	+	*Lactococcus* spp.
FPU2	+Cocci	Ppc	-	-(Homo)	-	+	+	-	-	-	+	+	_	+	+	+	*Lactococcus* spp.
FPU3	+Cocci	Ppc	-	-(Homo)	+	+	-	-	+	+	-	-	-	+	-	+	*Lactococcus* spp.
FPU4	+Rod	Ppc	-	-(Homo)	-	+	-	-	-	+	+	-	-	+	+	+	*Lactobacillus* spp.
SPU1	+Cocci	Ppc	-	+(Heter)	-	+	-	-	+	+	-	-	_	-	-	+	*Lactococcus* spp.
SPU2	+Cocci	Ppc		-(Homo)	-	+	+	-	+	+	+	-	+	+	+	+	*Lactococcus* spp.
SPU3	+Rod	Ppc	-	-(Homo)	-	+	-	-	+	+	-	-	_	-	+	+	*Lactobacillus* spp.

(+) Indicate growth, (-) no growth, (+/-) Gram positive/Negative, (Ppc) Pin Point Colony, Homo (homofermentative), Heter (Heterofermentative). (MPU1- MPU3): Maize isolates, (FPU1- FPU4): Finger millet isolates, (SPU1-SPU3), Sorghum isolate.

Table 2. Antibiotic sensitivity of the LAB isolates.

S/N	Antibiotic	Concentration (µg)	Sensitive/resistant									
			M_{PU1}	M_{PU2}	M_{PU3}	F_{PU1}	F_{PU2}	F_{PU3}	F_{PU4}	S_{PU1}	S_{PU2}	S_{PU3}
1	Gentamicin (GEN)	10	S	S	S	S	S	S	S	S	S	R
2	Ofloxacin (OFL)	30	S	S	S	S	S	S	S	S	S	S
3	Nitrofurantoin (NIT)	200	S	S	S	S	S	S	R	S	S	S
4	Cefaclor (CCL)	30	S	S	S	S	R	S	S	S	S	S
5	Nalidixic Acid (NAL)	30	S	S	S	S	S	S	S	S	S	S
6	Augmentin (AMC)	30	R	S	S	S	S	S	S	S	S	S
7	Minocycline (MIN)	30	S	S	S	S	S	S	S	S	S	S
8	Cefuroxime (CXM)	30	S	R	S	S	S	R	S	S	S	S
	% Sensitivity		87.5	87.5	100	100	87.5	87.5	87.5	100	100	87.5

S=Sensitive (Prevented isolates growth), R=Resistant (Growth noted).

Assessment of probiotic attributes

In this study (Table 3), the number of bacteria cells in the medium decreased below the pH of 3 due to loss of viability. At pH 1 in all the tested isolates no viable cells were seen after 2 h, implying most isolates were killed by severe low pH. A good probiotic potential LAB isolate must resist harsh conditions found in the gastrointestinal tract and also colonize intestinal epithelium. In this study SPU2 was able to grow at pH 1 (experienced on empty stomach) for 2 h while the

Table 3. Acid tolerance of probiotic potential LAB isolates from traditionally fermented cereals of Vihiga.

Organisms	pH	Incubation time (min)								
		Cell survival (Cfu/ml)					% Cell survival			
		0	60	120	180	Mean	60	120	180	Mean
M_{PU1}	1	8.5	6.2	0	0	3.7	67.8	0	0	22.6
	2	8.6	6.7	6.1	5.8	6.8	73.3	66	62.7	67.3
	3	9.1	9.03	8.7	8.4	8.8	98.7	91	90.8	93.5
	6.5(Control)	9.12	9.14	9.23	9.25	9.2	100	100	100	
	Mean	8.83	7.77	6.0	5.86		84.95	64.25	63.38	
M_{PU2}	1	9.7	7.8	0	0	4.7	77.6	0	0	25.9
	2	9.5	8.1	6.5	5.8	7.3	78.6	62.8	55.4	65.6
	3	9.9	9.3	8.4	7.6	8.8	90.3	81.2	72	81.2
	6.5(Control)	10.05	10.3	10.35	10.46	10.29	100	100	100	
	Mean	9.79	8.88	6.31	5.97		86.6	61	56.85	
F_{PU1}	1	9.7	7.8	0	0	4.4	75.9	0	0	25.3
	2	9.7	7.02	6.4	5.6	7.1	68.2	61.8	54.1	61.4
	3	10.1	8.3	7.8	7.5	8.4	80.7	75.3	72.4	76.1
	6.5(Control)	10.15	10.28	10.36	10.38	10.29	100	100	100	
	Mean	9.9	8.37	6.14	5.87		81.2	59.28	56.63	
F_{PU2}	1	10.2	8.1	0	0	9.1	79	0	0	26
	2	10.12	8.3	6.6	5.7	7.68	80.9	63.8	54.8	66.5
	3	10.17	9.01	7.72	6.2	8.23	87.9	74.12	59.6	73.9
	6.5(Control)	10.21	10.25	10.34	10.41	10.3	100	100	100	
	Mean	10.18	8.92	6.17	5.58		86.95	59.48	53.6	
S_{PU2}	1	10.13	8.1	3.4	0	5.4	79.5	33	0	37.5
	2	10.13	8.4	6.3	5.4	7.6	82.4	61.5	52	65.5
	3	10.08	9.1	7.4	6.1	8.2	89.3	72.3	58.9	73.5
	6.5(Control)	10.15	10.19	10.24	10.35	10.2	100	100	100	
	Mean	10.12	8.95	6.84	5.46		87.8	66.7	52.73	

*Log CFU/ml = Average mean from 3 experimental results. *% cell survival = (log CFU/ml for pH 1, 2, 3/ log CFU/ml pH at 6.5) × 100. (MPU1-MPU2): Maize isolates, (FPU1- FPU2): Finger millet isolates, (SPU1), Sorghum isolate.

rest survived up to about 1 h. This study shows the tested isolates have the ability to grow well even at the lowest pH of 1.0 for 1 and 2 h of incubation, respectively used in this study.

In this study, MRS broth was supplemented with bile salt concentration of 0.3, 1.0 and 2.0% and its effect on growth rate of isolate monitored. In the small intestines the physiological concentration of bile salts is in the range of 0.2 to 2.0%. Bile salt causes an increase in permeability of bacterial cell membranes composed mainly of lipids and fatty acids. The cultures were grown in 0.3, 1.0 and 2.0% of bile salt concentration and survivability monitored for 4 and 8 h, respectively. Among the isolate tested, MPU1 showed the highest survival of 99, 88 and 83% on 8-h incubation followed by FPU1 with 92, 86 and 77% for the same period of time (Table 4). The viable cell count of the cells decreased with increase in the concentration of bile salt to 2.0%. Most isolates are resistant to 0.3% bile concentration with over 92% survivals. FPU1 is more resistant at bile concentration of 1% than all the rest while MPU1 is most resistant at 2% bile salt.

DISCUSSION

Lactic Acid Bacteria are generally Gram-positive rods or coccobacilli occurring in chain. They are non-spore former, usually non motile, non-acid fast, non-respiring, devoid of cytochrome and catalase negative (Mokoena, 2017). They grow well under anaerobic conditions but may grow in microaerophilic as well as aerobic conditions.

Table 4. Bile salt tolerance of probiotic potential LAB isolates.

Organism	Time	Bile salt concentration (%)								
		Cell count (Cfu/Ml)					Cell survival rate			
		0	0.3	1	2	Mean	0.3	1	2	Mean
MPU1	4	10.3	10.06	9.2	8.8	9.6	99	88	85	91
	8	10.13	9.7	8.9	8.4	9.2	96	88	83	88
MPU2	4	10.04	9.3	8.85	7.7	8.97	92.6	88	76.7	85.8
	8	10.33	9.4	8.4	7.6	8.9	91	81.3	73.6	82
FPU1	4	10.3	10.1	9.5	8.8	9.7	98	92	85	92
	6	10.2	9.4	8.8	7.9	9.1	92	86	77	85
FPU2	4	10.32	10.04	9.2	8.6	9.5	97.3	89	83	89.8
	8	10.11	9.5	8.7	7.4	8.9	94	86	73.2	84.4
SPU2	4	10.2	9.7	8.8	8	9.5	95	86.3	78.4	86.6
	8	10.24	9.5	8.6	7.5	8.96	92.8	84	73	83.3

*Log CFU/ml: Average mean results of three experimental values. *% Survivability = (log CFU/ml Bile concentration (%). 3, 1, 2/log CFU/ml Bile concentration 0) × 100. (MPU1- MPU2): Maize isolates, (FPU1- FPU2): Finger millet isolates, (SPU1), Sorghum isolate.

They exhibit optimum growth at slightly lower acidic condition (pH 5.5 t- 6.0). They are strictly fermentative, with lactic acid as the major end product during sugar fermentation (Khalil and Nural, 2015). LAB can be classified on the basis of their morphology (cocci or rods, tetrad formation), mode of glucose fermentation, growth at different temperatures and salt concentrations, and configuration of the lactic acid production (D, L or both) (Markowiak and Śliżewska, 2017). Lactic acid bacteria have low acidification activity (Bintsis, 2018). The fast-acidifying LAB strains are found to be good for fermentation process as primary starter culture while poor acidification strains can be used as adjunct cultures depending on other properties. The proteolytic activity of probiotic culture is essential for the growth of the organisms and it is involved in the development of organoleptic properties of different fermented products (Amani et al., 2016). They have two different metabolic pathways for hexose fermentation. In homo fermentative pathway, lactic acid (more than 85%) is major end product whereas in heterofermentative pathway lactic acid, ethanol/acetone and CO_2 are the terminal products. These compounds impart characteristics flavour to the fermented foods. This study revealed that 70% of the LAB isolates are homofermentative, while 30% heterofermentative. The isolates growing at high salt concentration (6.5-10%) could be useful in the production of lactic acid where it is precipitated as lactate (Bonatsou et al., 2017). The antibiotic sensitivity property of LAB enables formulation of safe probiotic products for human consumption (Georgieva et al., 2015). In this study, the probiotic potential isolates were sensitive to the eight antibiotics. Probiotic LAB should be sensitive to antibiotics in order to avoid disseminating the resistance property to other pathogenic bacteria in the same niche, or the antibiotic resistance among them should be non-transferable (Jose et al., 2015). The lysis of red blood cells and the subsequent release of their contents into surrounding fluid is referred to as hemolysis (Deidda et al., 2020). Most pathogens are able to cause hemolysis *in vitro* and *in vivo*. Among the selection criterion for probiotic strains, is the absence of haemolytic and gelatinase activity an indicator that these bacteria are non-virulent (Rastogi et al., 2020). The isolates in these research findings are there not pathogenic. When selecting LAB for probiotic use, acid tolerance is one of the most important factors to consider. This will assist in determining whether they will be able to survive, grow and perform therapeutic activity in the gastrointestinal tract where pH is low (Terpou et al., 2019). The probiotic LAB strain must survive the acid conditions during gastrointestinal tract transit. The acidic pH in the gut normally range from 2 to 4 in normal conditions and during fasting it can reach up to pH 1. In this study, SPU2 was able to grow at pH 1 for 2 h while the rest survived up to about 1 h.

Conclusion

This research study focused on the potential of LAB isolated from fermented cereal-based products as probiotics for functional foods. The incorporation of such probiotic bacteria in foods have great potentials to improve the quality of life. The ability to survive low pH and resistance to bile concentration (0.3 and 1%) are

some of the desirable attributes of probiotic potential bacteria. The susceptibility to selected eight antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed the safety status of the isolates. The use of the tested isolates in production of functional foods requires further evaluation on the sensory aspect and acceptability to enhance the application of the strains in food industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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