Original Research Article

Microbiological and probiotic assessment of yeast isolated from wholegrain millet sourdoughs

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ABSTRACT

Introduction

The term probiotics have been described as live microorganisms associated with fermented foods that confer health benefit to the host. For a long time, researches into the world of probiotics have extensively and predominantly centred upon species of lactic acid bacteria and until recently *Saccharomyces cerevisiae*, as the only well-defined and proven probiotic yeast strain.

Aim

The purpose of this study was to isolate and characterise the yeast species associated with the fermentation of wholegrain millet sourdoughs and investigate *in vitro* the possible probiotic potential of the isolates.

Methodology

Wholegrain millet sourdoughs were prepared by spontaneous fermentation of the flours with tap water in the ratio 1:1 (w/v) for 48 h at 28 ± 2 ^{o}C through backslopping. A total of twenty five yeasts from the individual sourdoughs were identified based on their cultural, morphological and biochemical characteristics. The selected isolates were characterized to species level using API 20 C AUX test identification kit. Probiotic properties examined included bile salt and acid tolerance under conditions simulating the human gastrointestinal tract (GIT) and positive antagonistic activity against selected pathogens following well established procedures.

Results

The selected isolates investigated were characterized to belong to species of *Saccharomyces* and *Kluveromyces*. All of the isolates were discovered to exhibit sufficient survival under acidic pH of 2.0 with values ranging from 1.0log cfu ml^{-1} to 7.8log cfu ml^{-1} and showed high resistance to bile salt with values ranging from 63-99%. They also exhibited good antimicrobial activity against enteric pathogens of *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Pseudomonas sp*

Conclusion

Millet sourdoughs can serve as an affordable nutritionally healthy substrate for delivery of probiotics to the gastro-intestinal tract, thereby proffering basic health functionality. This study allowed to isolate and to identify yeast species present in millet sourdoughs with technological potential for sourdough applications.

Keywords Fermentation, Probiotics, Gastro-intestinal tract, Inhibition, Pathogens, sub- Saharan

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1.0 INTRODUCTION

Fermentation of foods has been able to offer a microflora of organisms that are beneficial to the host. Of these organisms, yeast plays a very important role, with species of Saccharomyces being the only yeast proven to provide probiotic benefit. Utilizing the sourdough technology to indigenous nutritionally promising healthy grains such as millets especially in sub- Saharan Africa will help proffer health benefit while increasing the quality of life of the rural populace. Millets are widely cultivated by local farmers in sub-Saharan Africa. However, despite their excellent nutritional profile they are still under-utilized, and bringing the potentials of these grains to limelight can be tapped into to sustain food security. It is worthy of note that some of these non-conventional indigenous cereal grains such as pearl millet (*Pennisetum glaucum*), white fonio (*Digitaria exilis* Staph), black fonio (*Digitaria iburua*) and finger millet (*Eleusine coracana*), have continued to increasingly receive research attention [1, 2]

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The fermentation of these millets into sourdough is both homo-lactic and hetero lactic acid fermentation. As reported by several authors it involves a complex microbial succession between lactic acid bacteria (LAB) and yeast with yeast cells enhancing the growth of LAB and also capable of producing some desirable metabolites such as amino acids, ethanol, vitamins and purines, or degrade complex carbohydrates, and synthesize volatile compounds which are responsible for the organoleptic characteristics of the final product [1, 3, 4, 5, 6]. Sourdough is made from flour and water, which starts to ferment spontaneously and which is allowed to ferment for a certain temperature and time.

To be considered probiotic, an organism must be viable and considerably reach the action site alive, overcome the harsh conditions of gastric acid characterized by low pH, enzyme activity, bile salt and competitively outwit and express dominance over non-beneficial pathogenic organisms thus beneficially affecting the health of the host [6]. Practically, yeasts demonstrate antagonistic activity against spoilage microorganisms, resist low pH and high salt concentrations, produce desirable aromas and improve lactic acid bacteria growth. These distinct attributes enable yeast to be considered as agents of probiotic because quite a number of species are able to survive passage through the gastrointestinal tract and show favourable effects on the host. Going by this, yeasts may contribute to the improvement of the health of consumers by means of the production of vitamins and antioxidants, degradation of non-assimilated compounds (such as phytate complexes), inhibition of pathogens, decrease in cholesterol levels, adhesion to intestinal cell line Caco-2 and the maintenance of epithelial barrier integrity [7, 8].

Several published studies have shown that some yeast strains isolated from dairy products or human faeces have been considered as potential probiotics, as they can survive in the low pH and bile environment of the gastrointestinal tract which are a prerequisite for strain survival through the gastrointestinal tract where they would have to resist internal stress against gastrointestinal enzymes, organic acids and adverse temperatures [3, 4, 5]. In their study Suzuki et al. [10] and Golubev et al. [11] attributed the antagonistic activity of yeasts basically to (1) ability to successfully compete for nutrient, (2) changes in the pH of the medium due to the production of organic acid, (3) production of a high concentration of ethanol, (4) secretion of antimicrobial metabolites such as mycocins. Mycocins are defined as glycoproteins or extracellular proteins which disrupt the cell membrane function present in susceptible yeast bearing receptors for the compound especially against species closely

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related to the producer strain [12]. The antagonistic activities of yeast have been documented to have significant impact in food and agriculture, bio-control whereby they serve as yeast starter culture possessing antagonistic activity which contribute positively to product safety predominantly by inhibiting growth of pathogenic organisms during fermentation and prolonging the shelf life and sensorial quality of finished product by inhibiting the proliferation and activity of spoilage microorganisms [13].

Arroyo lópez et al. [14] also revealed that yeasts play a very vital role in fermented food and beverage industry, particularly in products such as wine, beer, bread and a host of others.

Microbiota associated with traditionally fermented foods and beverages include filamentous fungi, yeast and bacteria. Most of these are non-pathogenic strains that have received the status of Generally Recognized As Safe (GRAS) and have found application as probiotics because of the benefits they proffer on the host [15, 16]. Among the features that are responsible for the success of yeasts as probiotics include their massive size, cultural diversity, nutritional flexibility (ability to utilize a wide range of nitrogen, carbon, and phosphorous sources), stress tolerance ability (to low pH/oxygen/water activity, high osmotic pressure), antimicrobial/antioxidative/antitumor activity, ability to secrete a broad range of enzymes such as lipase, peptidase, amylase, invertase, phytase, etc. and capacity to produce several other useful metabolites [16].

The present study was carried out with the aim of identifying yeast species isolated from whole grain millet sourdoughs and exploring their possible probiotic diversity by assessing their antimicrobial activity against some selected pathogens of clinical significance, pH and bile tolerance of the isolates. For a long time, researches into the world of probiotics have extensively and predominantly centred upon species of lactic acid bacteria and until recently Saccharomyces cerevisiae. This study aimed at characterization of yeast isolates from whole grains millet sourdoughs with a view of investigating *in vitro* the probiotic characteristics of the isolates.

2.0. MATERIALS AND METHODS

2.1. Sample collection and processing

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All the chemicals used were of analytical grade and the test isolates were obtained from the Department of Food Science and Technology, Joseph Ayo Babalola University, Ikeji- Arakeji, Osun State, Nigeria. The samples (finger millet, pearl millet, black and white fonio) were all procured from Kaduna State, North-central, Nigeria.

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The millet grains were pulverized using a marlex grinder (Excella-3962110**,** Mumbai, India) and sieved to pass through a 300 µm mesh size, packed in air tight containers and kept in the refrigerator at 4 °C for further analyses. The millet sourdoughs were prepared by mixing individual flour with water in the ratio 1:1 (w/v) in a glass bowl and stirred manually using a glass stirring rod and allowed to stand at a temperature of 28 ± 2 °C for 48 h in order for fermentation to occur.

2.2. Isolation and enumeration of yeast in the sample

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A 10 g sample of each fermenting sourdough was homogenized in 90 ml of sterile phosphate buffered saline PBS (0.8% (w/v) sodium chloride, 0.02% (w/v) potassium chloride, 0.144% (w/v) disodium phosphate, 0.024% (w/v) potassium phosphate, at pH 7) to obtain a 10-fold serial dilution of the sourdoughs. One millilitre aliquot of each dilution was pour plated with yeast peptone-dextrose (YPD, Merck) Agar containing 0.5% (w/v) yeast extract, 1% (w/v) peptone and 0.5% (w/v) glucose, 1.8% (w/v) agar and incubated at 35 ± 2 °C for 3-5 days. All colonies were counted and recorded as cfu per gram. After incubation, representative yeast colonies on YPD agar plates were examined by phase contrast microscopy and pure cultures were obtained by successive streaking on malt extract agar (MEA, Merck). The pure isolates were kept on MEA agar slants and stored at 4 °C [16, 17].

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2.2.1. Characterisation and identification of isolated yeast to species level

Characterization of the isolates was performed phenotypically on the basis of their cultural, morphological and biochemical characteristics. These were carried out by observing the colonies directly on the plates for size, elevation, consistency, shape, colour and Gram's staining, catalase test, endospore test, sugar fermentation test, growth at different temperatures and pH, citrate test, oxidase test, and urease test. Final identification was investigated using API 20 C AUX strips and medium (Bio-Merieux, Marcy1'Etoile, France) according to manufacturer's instruction.

2.3. Survival under acidic conditions

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To evaluate the survival of yeast isolates under acidic conditions, young cultures of 18-24 h were sub-cultured in MEA broth containing 0.5% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose and incubated at 37 °C. The cultures were then centrifuged at 7000 rpm for 10 min at 4 ºC. The pellets were washed and re-suspended in sterile PBS (0.8% (w/v) sodium chloride, 0.02% (w/v) potassium chloride, 0.144% (w/v) disodium phosphate, 0.024% (w/v) potassium phosphate, at pH 7). The effect of exposure to low pH was determined by inoculating 1% of activated yeast cultures into PBS pH 7 maintained at pH 2, using 1N HCl. It was then incubated at 37 °C for 3 h to simulate conditions of the human GIT. Samples were taken every hour for 3 h and the viable numbers of the yeast were enumerated by pour plate counts of all samples using 10-fold serial dilution prepared in 0.1% peptone water [16].

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2.4. Tolerance to bile salts

A modified method of Pederson et al. [17] was used to evaluate the viability of the yeast in bile salt. One millilitre of freshly prepared broth culture of yeast was added to 9 ml of MEA broth. The cultures were then centrifuged at 7000 rpm for 10 min at 4 ºC and pellets washed and re-suspended in sterile PBS. It was then supplemented with 0.1, 0.3, 0.5, 1.0 and 2.0 (w/v) of bile (Oxoid, Basingstoke, Hants, UK). A control was set up by inoculating cells in MEA broth without bile. The isolates were incubated at 37 °C for 24 h. Absorbance readings at 600 nm were recorded and the growth survival calculated thus

Percentage survival of isolates = $(MEAc - MEAt/MEAc) \times 100$

Where MEA_C = control

MEA_t =yeast isolates

2.5. Growth at different temperatures

This was determined by inoculating an approximate cell density corresponding to 2 McFarland (10^8 cfu/ml) freshly grown cultures into MEA broth medium and incubating at 30 °C, 37 °C and 45 °C for 24 h respectively. The uninoculated MEA broth served as blank. Growth viability was estimated by measuring the optical density at 600 nm [18].

2.6. Antagonistic activity against enteric pathogens

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Resistance of the yeast isolates to pathogens was done using the agar-well diffusion method. Bacteria cultures of *Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pyogenes*, *Staphylococcus aureus,* and *Salmonella typhimurium* were obtained from stock culture collection of the Microbiology Department of Ladoke Akintola University of Technology, Osun State, Nigeria. The cultures were grown in nutrient broth at 37 ^{o}C for 24 h. Also all isolates were grown over night in MEA broth, centrifuged at 7,000 revolutions per min for 10 min and washed with sterile peptone water**.** The bacteria cultures were spread on Muellar-Hinton agar plates using sterile effusion. Wells were made and filled with 100 µl of yeast isolate supernatant. The inoculated plates were incubated at 37 ^{o}C for 24 h and the diameter of the zone of inhibition was measured in millimetre using vernier caliper [19, 20].

2.7. Statistical analysis

All the experiments were carried out in triplicate and data obtained were analyzed using analysis of variance (ANOVA) and Duncan's new multiple range tests using a 5% significance level (SPSS version 19 computer software).

2.8. RESULTS AND DISCUSSION

2.8.1. Morphological and phenotypic characteristics of isolates

The morphological features of the colonies on YPD agar plates are presented in Table 1. Colonies obtained were seen to be big, smooth and whitish. When touched with a loop, the colonies were mucoid like in nature, the tests also revealed the isolates to be oxidase negative and non-spore forming.

Table 1: Morphological characteristics of isolates from sourdoughs on YPD agar plates

Morphology	Pearl millet	Finger millet	Black fonio	White fonio
Size	Big	Small	Small colonies in chain	Big
Surface	Moist and smooth	Dry and rough	Dry and rough	Moist and smooth
Shape	Round with filaments	Round	Round	Round
Colour	White	White	Creamy	White
Consistency	Butyrous	Butyrous	Mucoid	Butyrous
Elevation	Raised	Flat	Flat	Raised

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Shape	Oval	Oval	Oval	Oval
Budding	+	+	+	+
Pseudohypha	+	-	+	+

[+] positive, [-]negative

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A total of twenty five yeasts were isolated and following their morphological and biochemical profile and eight of the isolates were identified based on the carbohydrate fermentation pattern of API 20 C AUX test kits and API database, as belonging to the genus *Saccharomyces* and *Kluveromyces*. All the identified yeast species have been established to be present in sourdough [21]. Table 2 shows response of the isolates to carbohydrate fermentation using API 20 C AUX kit (BioMerieux, Marcy l'Etoile, France).

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Table 2: Characterization of Yeast using API 20 C AUX kit /sugar fermentation profile using API 20 C AUX kit

sugars	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
O	_	_	_	_	_	_	_	_
GLU	+	+	+	+	+	+	+	+
GLY	+	+	+	+	+	_	+	+
2KG	_	+	V	_	+	+	+	+
ARA	+	_	_	_	+	+	+	+
XYL	+	_	_	_	+	+	+	+
ADO	_	_	_	_	+	_	+	_
XLT	-	+	_	_	_	_	+	_
GAL	+	+	+	_	+	_	V	_
INO	+	_	_	_	+	_	_	_
SOR	-	+	_	_	+	_	+	+
MDG	_	+	+	_	+	+	+	V
NAG	+	+	_	+	+	+	+	+
CEL	+	+	_	_	+	+	_	+
LAC	+	+	_	_	_	+	+	_
MAL	+	+	_	_	_	+	+	+
SAC	+	+	+	_	V	+	+	+

TRE	+	_	_	_	V	+	+	_
MLZ	_	+	_	_	V	_	+	+
RAF	+	+	+	V	_	+	+	+
% reliability of identification	96.2	99.9	98.5	95.6	98.5	93.1	96.2	89.6
Isolate identity	*Saccharomyces cerevisiae1*	*Kluveromyces lactis 1*	*Saccharomyces cerevisiae2*	*Kluveromyces lactis 2*	*Saccharomyces boulardii 1*	*Saccharomyces cerevisiae3*	*Saccharomyces boulardii 2*	*Kluveromyceslactis 3*

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\+ = positive, - = negative, v = variable

O =Aucun, GLU= D-glucose, GLY=glycerol, 2KG=calcium 2-ceto- gluconate, ARA= L-arabinose, XYL= D-xyLose, ADO= adonitol, XLT, xylitol, GAL= D-galactose, INO=inositol, SOR=D-sorbitol, MDG=methyl-αd-glucopyranoside, NAG=N-Acetyl-glucosamine, CEL, D-celiobiose, LAC=D-lactose (origine bovine), MAL= D-maltose, SAC=D-saccharose, TRE=D-trehalose, MLZ= D-melezitose, RAF=D-rafinose

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2.8.2. **<u>Acid tolerance at pH 3.0 under acidic condition in HCl solution</u>**

All the yeasts were able to survive the high acidic condition up to 3 h incubation time with high survival rate.

Table 3: Survival of yeast isolates under acidic condition at pH 3.0 in HCl solution

pH 2.0	Log of counts (cfu/g)			
Isolates	**0 h**	**1 h**	**2 h**	**3 h**
S. cerevisiae 1	4.67^{b}	3.6^{ab}	1.3^{a}	–
K. lactis 1	6.12^{ab}	4.0^{ab}	1.9^{a}	–
S. cerevisiae 2	7.1^{a}	4.5^{a}	2.1^{a}	1.0^{a}
K. lactis 2	7.8^{a}	4.5^{a}	2.6^{a}	–
S. boulardii 1	6.9^{a}	2.5^{b}	1.63^{a}	1.0^{a}
S. cerevisiae 3	7.2^{a}	3.9^{ab}	2.33^{a}	–
S. boulardii 2	6.9^{a}	3.9^{ab}	2.43^{a}	–
K. lactis 3	6.3^{ab}	3.0^{ab}	2.0^{a}	1.0^{a}

– no growth

Means with different superscripts along the column are significantly different ($p<0.05$).

Values are means ± SD of triplicate measurement.

The period of 0 h, is the time the isolates were immediately inoculated

In order to be classed as probiotics, microorganisms need to prove their survival against biological hurdles of high acidity and bile salt in the gastrointestinal tract. In this study, all the isolates which grew best in acidic pH 2.0 after 3 h were also able to survive the bile salt tolerance test which is similar to the results obtained by Katarrzyna and Alina [22] whereby high survival rate were observed for all tested strains of *S. cerevisiae* of kefir and fecal isolates in pH 2.5 simulated gastric juice. The result can also be compared with the report of Chen et al. [23] which identified 17 yeast strains and found them to be capable of growing in bile salt solutions and most of them tolerated low pH, surviving in gastric juice. This can be related to the results of Sridevi et al. [24] where species of *Rhodotorula*

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and *Candida* tolerated low pH. The potential of these isolates to withstand stress of the GIT and colonise it, is very essential to their being considered as probiotic organisms.

2.8.3. Tolerance of yeast isolates to different concentrations of bile salt

Tolerance to bile salt is an important prerequisite for the colonization of the GIT. Regarding this, all the isolates exhibited excellent activity of tolerance to this condition of bile salt with varying degrees (63%-99%). From this study, at 0.3% concentration, *S. cerevisiae, S. boulardii and K. lactis* each showed high percentage yield ≥94%, which is similar to the report of Chen et al. [23] which observed high resistance to bile salt at 0.3%. At 2.0% concentration, only *Saccharomyces cerevisiae and Kluveromyces lactis* showed the highest percent survival, in line with a similar trend observed by Syal and Vohra [16] at 1% concentration of bile salt where all five isolates of yeast tested showed extreme tolerance to high bile salts concentration at survival rate of 95% with no decline. Physiologically, the concentration of bile salt in the small intestine is between 0.2 and 2%, and it is mandatory for potential microorganisms being considered as probiotics to be able to survive this condition during passage through the gastrointestinal tract [25, 26]. *Kluveromyces lactis* was the least tolerant to the harsh condition of bile salts at all concentration as shown in table 4.

Table 4. Percentage survival of yeast isolates in YPD broth supplemented with bile salts at different concentrations at OD_{600}

	Growth at different conc. (%) of bile salt				
Isolates	0.1	0.3	0.5	1.0	2.0
***S. cerevisiae* 1**	93	90	90	72.5	63
***K. lactis* 1**	95	95.6	91.2	89.6	81.2
***S. cerevisiae* 2**	99	91.3	87.5	91.3	88.6
***K. lactis* 2**	99	99	94	95	92.5
***S. boulardii* 1**	99	96.3	95.1	94	91.3
***S. cerevisiae* 3**	94.7	94	87.5	84	77.5
***S. boulardii* 2**	86	87.5	80.4	86	75

Values are expressed as percentage survival at 3 h incubation with that of control

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Table 5. Growth of isolates at different temperatures at 600 nm

Isolates	**30 °C**	**37 °C**	**45 °C**
S. cerevisiae **1**	1.89^{b}±0.005	1.93^{a}±0.003	1.94^{a}±0.006
K. lactis **1**	1.88^{b}±0.000	1.90^{c}±0.002	1.92^{c}±0.006
S. cerevisiae **2**	1.83^{c}±0.006	1.76^{d}±0.001	1.77^{d}±0.000
K. lactis **2**	1.96^{a}±0.006	1.93^{b}±0.003	1.93^{b}±0.00
S. boulardii **1**	1.44^{e}±0.000	1.29^{g}0±.006	1.29^{g}±0.005
S. cerevisiae **3**	1.38^{f}±0.15	1.30^{f}±0.000	1.30^{f}±0.000
S. boulardii **2**	1.78^{d}±0.27	1.74^{e}±0.003	1.75^{e}±0.000

In this study, as presented in table 5 all the isolates were able to grow at 30, 37 and 45 °C. Growth at a stricter temperature of 45 °C was comparable with that of 37 °C, although decrease in growth viability was observed for *S. boulardii* and *S. cerevisiae.* This finding is consistent with the results of other authors Rajkowska and Kunicka-Styczynska, [17] and Syal and Vohra, [16] which noted similar survival of yeast strains at 30 °C and 37 °C

2.8.4. Antimicrobial activity of yeast isolates against selected pathogens

The result of the antagonistic activity against selected pathogens is presented in figure 1 and showed that *K. lactis* and *S. cerevisiae* isolates exhibited the strongest inhibitory action against the tested pathogens. *Saccharomyces cerevisiae* had the highest zone of inhibition against *Klebsiella sp.* with values ranging from 22.0-3.00 mm. *K. lactis* had high inhibition zone of 16.00 mm and 22.0 mm against *Pseudomonas sp.* and *Staphylococcus sp.* respectively. In a similar result, Velitchka et al. [28] also observed antagonistic activity of *Candida rugosa* (y28) and *Candida lambilla* (y30) towards most of the eight test pathogens and found inhibitory activity against the pathogens. It was observed that strains of *S. cerevisiae* had no antimicrobial activity against *Staphylococcus spp.* and weak inhibitions (2 mm) against *E. coli;* this is in collaboration with Venkatesan et al. [29] which showed that *S. cerevisiae* had no activity against *Staphylococcus aureus* and very poor inhibition against *E. coli.* Edema and Sanni [30] also recorded low inhibitory activity of *S. cerevisiae* against pathogenic bacterial tested.

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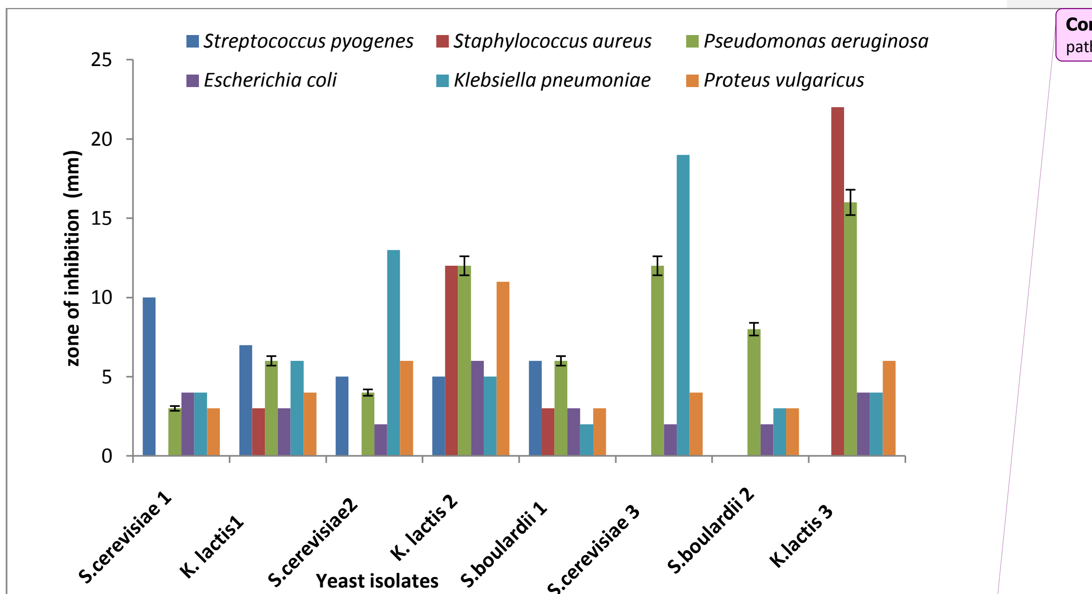

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Figure 1: Antimicrobial activity of yeast isolates against selected pathogenic organisms

The clear inhibition zones shown by the positive yeast isolates against the pathogens could be possible due to the fact that they may have competed for nutrient with the pathogens and simultaneously produced organic acids, hydrogen peroxide, diacetyl and bacteriocins which acted as antibiotic agents, and thus helped to eliminate the pathogens. The effectiveness of this positive elimination of pathogens in the small intestine is a plus for expression of probiotic effect for the host.

3.0. Conclusion

This research has been able to indicate that aside *Saccharomyces,* species of *Kluveromyces* have demonstrated health promoting effects by their ability to withstand the harsh condition of low pH and extreme tolerance to high bile salt concentration present in the gastrointestinal tract. Probiotic products from whole grain cereals taking advantage of the sourdough fermentation process can serve as a healthy and readily available substrate when our local grains are considered. With consumers becoming more aware of the importance of good nutrition and health, and therefore drifting to natural healthy foods, the positive effect of these isolates and or their metabolites can be harnessed as starter cultures for the improvement of traditionally fermented foods to improve the safety, shelf life and organoleptic properties of the resultant product. Selection of probiotics require that the organism should reach their action site alive and be able to overcome the primary gastric acid barrier in the stomach which is characterized by low pH, presence of enzymes and bile salt. Since all the tested

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isolates showed high tolerance to these conditions they could be subjected to further invivo assay in other to establish the possible probiotic prospect of other promising species such as Kluveromyces and also consolidate the phenotypic results with genotypic identification.

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Research involving human participants and/or animals

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Informed consent

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