

Full Length Research Paper

Antimicrobial activities of a multi-species probiotic ingredient derived from opaque sorghum beer during its propagation in a starchy career model

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This study evaluates the antimicrobial activities of a multi-species probiotic ingredient derived from the African opaque sorghum beer during its propagation in a starchy career model. The aim was to establish the optimum growth conditions that warrant the optimum antimicrobial activities in the product. The antimicrobial activities were tested against Gram-positive bacteria (*Staphylococcus aureus* ATCC 27844, *S. aureus* MR 825), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728, *Salmonella typhi* R 30951401, *Klebsiella pneumoniae* ATCC 35657), as well as against yeast (*Candida albicans* MHMR), using agar disc diffusion method. Also, the growth of viable cells and physicochemical parameters during the propagation were monitored. The results showed that the pH and dry matter content of the probiotic ingredient decrease significantly ($p < 0.05$) during the propagation whereas the lactic acid, the titratable acidity, lactic acid bacteria (LAB), yeasts and moulds counts increase significantly ($p < 0.05$). From 0 to 12 h, the product failed to inhibit the growth of all indicator strains. From 24 h and onward, the probiotic career inhibited all indicator strains except for *K. pneumoniae* (ATCC 35657) which could not be inhibited. Clearly, our study showed that 36 h of the propagation were sufficient to generate a probiotic ingredient with optimum antimicrobial activities.

Key words: Probiotic, antibiotic, opaque beer, antimicrobial activity.

INTRODUCTION

The use of antibiotics for diseases prevention or healing and as growth factors in animals feeding contributed to

the development of breeding through health improvement and zootechnic performances of animals. The estimated

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antibiotic use in animal agriculture is 7.36 to 11.18 million kilogram per year (Khachatourians, 1998; Doyle, 2001). Globally, tonnes of antibiotics have been distributed in the biosphere during an antibiotic era of only about 60 years duration (Balcázar et al., 2006). However, these amounts of antibiotics have favored a strong selection among microorganisms, which developed resistance to these antibiotic chemicals, mainly by a horizontal and promiscuous flow of resistance genes (Aarestrup et al., 2000; Randall et al., 2003; SCAN, 2003). Currently, the potential for agricultural antibiotics to contribute to the development of antibiotic-resistant bacteria of human concern is the subject of intense debate and research (Wegener et al., 1999). Resistance mechanisms can arise by two ways: Chromosomal mutation or acquisition of plasmids. Chromosomal mutations cannot be transferred to other bacteria but plasmids can transfer resistance rapidly (Lewin, 1992). There is an increasing interest in finding alternatives to the use of antibiotics in animals feeding due to the ban of subtherapeutic antibiotic usage in Europe (Fajardo et al., 2012). Thus, several alternative methods are explored. Among them, probiotics are a subject of particular attention.

The probiotics with antimicrobial properties act by producing bacteriocins such as nisin (Yateem, et al., 2008) or by lowering the pH as a result of acidic compounds production such as lactic acid (Psomas et al., 2001). The progressive reduction of the use of antibiotics in animals feeding, as growth promoters, has raised renewed interest in the incorporation of microbial strains in animals feeding, in order to maintain the beneficial effect obtained with antibiotics (Guillot, 2001). Kpete-kpete is the starter culture used to ferment tchoukoutou, the most produced and consumed opaque sorghum beer in Benin.

Lactic acid bacteria and yeasts have been reported (Kayodé et al., 2007) to be the major microorganisms involved in the fermentation of tchoukoutou. These fermentation microorganisms have been reported to possess probiotic effects, to reduce the level of pathogenic bacteria occurring in beverages and to reduce the severity duration and morbidity of diarrhea (Mensah et al., 1991; Kimmons et al., 1999). Recent researches in Ivory Coast showed net body weight increase and increased feeding efficiency in broilers when settling of palm wine or yogurt probiotic were used as feeding supplementations (Bohoua, 2008). In Benin, an investigation by Houndonougbo et al. (2011) showed low mortality and increased body weight gain when chicken's feed was supplemented with starter culture harvested from opaque sorghum beer. In addition, a recent survey conducted by N'tcha et al. (2015) showed that Kpete-kpete is used to cure humans and animals diseases such as diarrhoea, dysentery and wounds. The aim of the present study is to optimize the antimicrobial activity of Kpete-kpete during its propagation in a sorghum-based starch used as career.

MATERIALS AND METHODS

Starchy career model

Starch extracted from a red variety of sorghum (*Sorghum bicolor* (L) Moench) was used as starchy career. The starch was extracted following the process described by Kayodé et al. (2012). Ten kilogrammes (10 kg) of cleaned sorghum grains were dehulled using a mini-PRL dehuller (Thiès, Sénégal) and then ground into flour.

Propagation of the probiotic ingredient and sampling

The sorghum flour is mixed with distilled water to obtain a dough (45% w/w), which is inoculated with 10% (w/w) of Kpete-kpete, kneaded into dough and allowed to ferment in a plastic bucket with lid for 72 h. Samples were withdrawn at 0, 6, 12, 24, 36, 48, 60 and 72 h of propagation for analysis. At each time point, 12.5 g of sample were aseptically taken from the flask for microbiological analysis and antimicrobial activity (10 and 2.5 g, respectively). Another sample of 50 g was kept at -10°C for pH, titrable acidity and dry matter measurements within 4 h approximately after sampling. The remaining sample was frozen for further analysis. In order to check the effect of drying temperature on the functional properties of the ingredient, we dried (42°C for 24 h) the product obtained at 72 h of propagation and derived samples for analysis of antimicrobial activities.

Microorganism materials used for antimicrobial test

The assayed pathogens included Gram-positive (*Staphylococcus aureus* ATCC 27844, *S. aureus* MR 825), Gram-negative (*Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728, *Salmonella typhi* R 30951401, *Klebsiella pneumoniae* ATCC 35657) and one yeast (*Candida albicans* MHMR). *E. coli* O157:H7 ATCC 700728, *S. aureus* ATCC 27844, *S. typhi* R 30951401, *K. pneumoniae* ATCC 35657 were supplied by the Laboratory of Food Safety and Water Quality of Ministry of Health, whereas *E. coli* ATCC 25922, *S. aureus* MR 825 and *C. albicans* MHMR were obtained from the Laboratory of Biology and Molecular Typage in Microbiology. Each stock culture was maintained in the respective growth media, containing 30% of glycerol, and stored at -80°C. Before the use in experiments, the strains were transferred into fresh growth media and incubated at suitable temperature for 18 to 24 h. This was followed by two consecutive transfers in the medium and incubated under the conditions indicated. The antimicrobial activities of ingredients were evaluated by means of disc diffusion assays.

Physicochemical analysis

Water content was determined as described (AACC, 44-15 A, 1984). Titratable acidity and pH were determined as described by Nout et al. (1989). The pH was measured using a digital pH-meter (JENWAY, Model 3505, UK) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany).

High pressure liquid chromatography (HPLC) analysis of sugars and organic acids

Lactic acids and soluble sugars were determined following the method developed by Mestres and Rouau (1997). 50 mg of samples were extracted with 5 mM sulphuric acid in 1.5 mL centrifuge tubes under continuous agitation for 30 min at room temperature. After extraction, samples were centrifuged at 3500 x g for 30 min and filtered through a 0.45 µm pore filter before quantifi-

cation by HPLC using an Aminex HPX-87H⁺cation-exchange column (BioRad Hercules, USA) thermostated at 37°C. Detection was done at 210 nm with an IR-detector. Elution was with sulphuric acid 5 mM at a flow rate of 0.6 mL min⁻¹. The injection volume of the sample was 20 µL. Organic acids and sugars were expressed as g/kg dry matter. Analyses were performed in duplicate.

Counts of viable microorganisms

Total counts of lactic acid bacteria (LAB), yeasts and moulds were enumerated according to the method described by Nout et al. (1987). At each sampling time, duplicate samples (10 g) were diluted in 90 mL sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1000 mL distilled water, pH = 7.0) and homogenized with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Viable counts of LAB were determined on de Man, Rogosa and Sharpe Agar (MRSA, CM 361, Oxoid, Hampshire, England) containing 0.1% (w/v) natamycin (Delvolid, DSM, The Netherlands) with incubation in anaerobic jar (Anaerocult A, Merck KGaA, Germany) at 30°C for 72 h. Yeasts and moulds were enumerated using Malt Extract Agar (MEA, CM 59 Oxoid, Basingstoke, Hampshire, England). MEA plates were incubated at 25°C for 72 to 120 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (log₁₀ CFU/g) of the sample.

Agar disc-diffusion assay

The capacity of the ingredients to inhibit a representative group of pathogens and other was determined by modifying the disc diffusion method of NCCLS (2003). Twenty milliliters (20 mL) of molten Mueller-Hinton Agar (MHA, CM 337, Oxoid, Basingstoke, Hampshire, England) were poured into sterile Petri dishes and allow to solidify. 100 µL of the overnight Mueller-Hinton broth (MHB, CM 405, Oxoid, Basingstoke, Hampshire, England) culture of each pathogen strain, which have been adjusted to 0.5 McFarland-turbidity, was spread on the plates. Once the plates were dried aseptically, five blank discs papers (6 mm in diameter) were placed onto the surface of the agar. The moist or dried sample of probiotic ingredient was reconstituted with sterile distilled water to obtain a solution of 500 mg mL⁻¹. This solution was stirred vigorously using a magnetic stirrer for 30 min and then centrifuged at 3 500 x g for 30 min. Forty microliters (40 µL) of each supernatant were placed into the discs. The plates were left at room temperature for 1 h so that the absorbed supernatant become diffused into the agar, and then incubated at 37°C for 24 h. The tests were carried out in duplicate.

Statistical analysis

The propagation trials were carried out in triplicate. Mean values and standard deviations were calculated from the experimental data. Statistical Package for Social Science (SPSS), version 16.0 (Chicago, IL, USA) was used. Data analyses involved one-way analysis of variance (ANOVA). Significant difference was established at 5%.

RESULTS AND DISCUSSION

Changes in pH, titratable acidity, lactic acid, maltose and glucose contents of the ingredient during the propagation

The pH value decreased significantly ($p < 0.05$) from 5.63

to 4.03 within 24 h of propagation (Table 1). After 24 h, the pH remains relatively stable around a value of pH = 3.84. The titratable acidity increased significantly ($p < 0.05$) from 8.95 g/kg at 0 h (calculated as lactic acid) to 40.21 g/kg at 72 h of propagation. The progressive fall in pH and increase in titratable acidity during the fermentation process is typically characteristic of lactic acid fermentation of cereal grains (Singh et al., 2003; Vieira-Dalodé et al., 2007).

According to Tharmaraj and Shah (2009), the production of organic acids such as acetic, citric and lactic acids are responsible for the decrease of pH in such product. Interestingly, Nout (1991) and Steinkraus (1996) reported that a pH of 3.5 to 4.0 is sufficient to inhibit Enterobacteriaceae and other Gram-negative bacteria. Concomitantly to these modifications, we observed a decrease in the dry matter content of the ingredient which shifted from 51.98% at 0 h to 48.89% at 72 h of propagation.

The changes in lactic acid, maltose and glucose concentrations of the ingredient during propagation are also reported in Table 1. The amount of lactic acid in the dough increased rapidly to reach a maximum at 36 h of propagation. Thereafter, a significant decrease was observed until the end of propagation. The maltose content increased from 2.13 g/kg at 0 h to 3.84 g/kg at 24 h of fermentation and then decrease to 1.85 at 36 h of propagation and no significant decrease was observed afterward. Similar trend was observed for the glucose content with the difference that the most significant change occurred at 36 h of propagation. Water and volatile compounds production during aerobic and anaerobic catabolism by yeasts and LAB might be responsible for these changes (Hounhouigan et al., 1993). Similar modifications were reported in other African fermented cereal products (Muyanja et al. 2002; Sefa-Dedeh et al., 2003; Vieira-Dalodé et al., 2007).

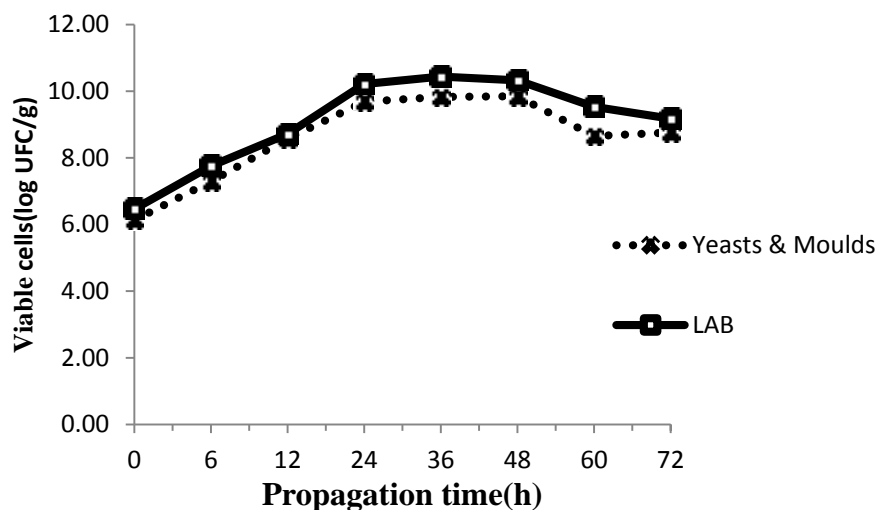
Changes in lactic acid bacteria, yeasts and moulds counts during propagation

The changes in microbial count of the ingredient during the propagation are shown in Figure 1. The LAB counts increased from 6.47 to 10.44 log cfu/g. The most significant ($p < 0.05$) increase in the numbers of LAB was noted during the first 36 h of propagation. No significant growth was noted between 36 and 48 h. Further incubation led to a significant decrease of LAB counts. Similar trend was observed for yeast and mould counts. The symbiotic relationship between LAB and yeasts during the fermentation process of starch-based product is well established (Nout, 1991). Indeed, the development of yeasts is favored by the acidic environment created by LAB, while the growth of bacteria is stimulated by the presence of growth factors such as vitamins and soluble nitrogen compounds provided by yeasts.

Table 1. Trends of pH, titratable acidity, lactic acid, maltose, glucose and dry matter during propagation.

Time (h)	pH	Titratable acidity	Lactic acid	Maltose	Glucose	Dry matter
0	5.63±0.12 ^a	8.95±1.06 ^a	0.69±0.22 ^a	2.13±0.44 ^a	3.73±1.02 ^a	51.98±0.62 ^a
12	4.55±0.32 ^b	22.76±5.58 ^b	7.43±0.90 ^b	2.48±1.15 ^{ab}	3.56±0.49 ^a	51.17±1.46 ^{abc}
24	4.03±0.07 ^c	28.56±2.82 ^{bc}	16.33±1.94 ^c	3.84±0.89 ^b	3.51±0.20 ^a	50.45±0.95 ^{abc}
36	3.89±0.02 ^c	35.26±3.04 ^c	24.88±2.20 ^d	1.85±0.21 ^a	1.34±0.69 ^{bc}	50.25±0.97 ^{abc}
48	3.87±0.10 ^c	37.36±4.91 ^d	20.21±0.20 ^e	1.63±0.57 ^a	1.68±0.84 ^b	49.62±1.61 ^{bc}
60	3.80±0.06 ^c	40.22±5.95 ^d	20.61±0.88 ^e	1.21±0.32 ^a	0.26±0.05 ^c	49.10±1.59 ^c
72	3.84±0.13 ^c	40.21±6.05 ^d	18.10±1.47 ^{ce}	1.16±0.16 ^a	0.25±0.11 ^c	48.89±1.71 ^c

*Values are means ± standard deviations. Values bearing different letters in a column are significantly different (p < 0.05).

**Figure 1.** Changes in viable cells counts during probiotic ingredient production.

Antimicrobial activity

The antimicrobial activity was assessed during the probiotic ingredient propagation against indicator strains by Agar disc diffusion method. Figure 2 showed the inhibition zone diameter resulting from the antimicrobial activity of the probiotic ingredient against Gram positive, Gram negative bacteria and one yeast. A superior diameter to 1 mm around the disc was considered as a positive result. Thus from 0 to 12 h, the probiotic ingredient failed to show any inhibitory activity against all indicator strains. It is most likely that enough antimicrobial compounds were not produced by the functional microorganisms during this propagation time. Ahmad et al. (2014) reported that the antimicrobial effect of *Lysinibacillus* jx416856 started after 15 h of incubation in MRS broth and growth dependent bacteriocin activity was observed at early log phase (18 h). However, at 24 h of propagation, our probiotic ingredient inhibited indicator strains broadly except *K. pneumoniae* (ATCC 35657). After 24 h of propagation, all indicator strains were inhibited by the probiotic ingredient. The inhibition zone diameters observed at 36 h of propagation were

significantly higher for all indicator strains. The antimicrobial effect increased until a stationary phase which occurs at 36 h and remained constant till 72 h. Ahmad et al. (2014) reported similar trend for *Lysinibacillus* jx416856 and even though indicated that its antimicrobial activity decreased eventually with a constant level. In another study, Djadouni and Kihal (2012) recorded optimal bacteriocin production in MRS after 24 h of incubation. These results supported our findings since the growth of all indicator strains tested were inhibited after 24 h of the propagation time. Interestingly, the lactic acid production, and probably bacteriocin, significantly increased after 24 h of the propagation. Between 24 and 36 h the inhibition zone diameters increase steadily and remains constant ($P > 0.05$) till 72 h of propagation. This suggested that 36 h of propagation is enough to inhibit all pathogen tested. Among the indicator strains, *C. albicans* MRMH (16±1.41 at 72 h), *S. aureus* RM 825 (15±1.41 at 48 h), *E. coli* O157: H 7 ATCC 700728 (14.75±0.35 at 36 h) were extensively inhibited by the probiotic ingredient while *S. typhi* R30951401 (12.5±2.12) and *K. pneumoniae* ATCC 35657 (11.5±2.12) were weakly inhibited. Clearly, the

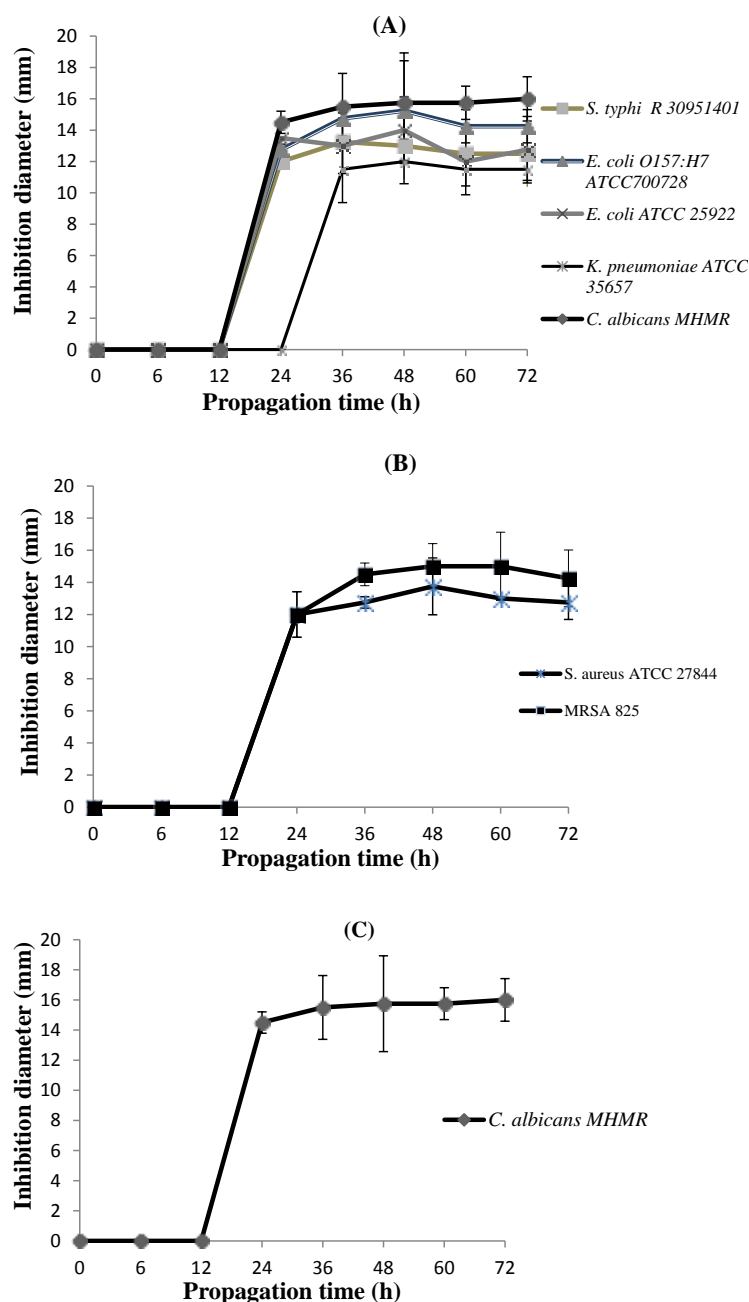


Figure 2. Changes in antimicrobial activity of probiotic ingredient against Gram negative (A) and Gram positive (B) bacteria and yeast (C) during propagation time. The inhibition diameter was measured after 24 h of incubated at 37°C by disc diffusion method.

inhibitory activity depends on the propagation time and on the type of microorganism considered. In other to check the effect of drying temperature on the functional properties of the ingredient, we dried the product at 42°C for 24 h. Thus, after drying, the probiotic ingredient at 72 h also exhibited antimicrobial activities against all pathogen strains (Figure 3). There was no significant difference between inhibition zone diameters recorded for the dried and undried probiotic ingredient.

We studied the relationship between some measured parameters (Table 2). High negative correlation exists between the lower pH and the antimicrobial activity ($r = -0.932$). In a similar study, Tejero-Sariñena et al. (2012) reported that the pH was inversely correlated with the diameter of inhibition against the pathogenic indicator strains. Level of lactic acid is in high positive correlation with the antimicrobial activity. The pH-lowering effect resulting from acid production is most likely responsible

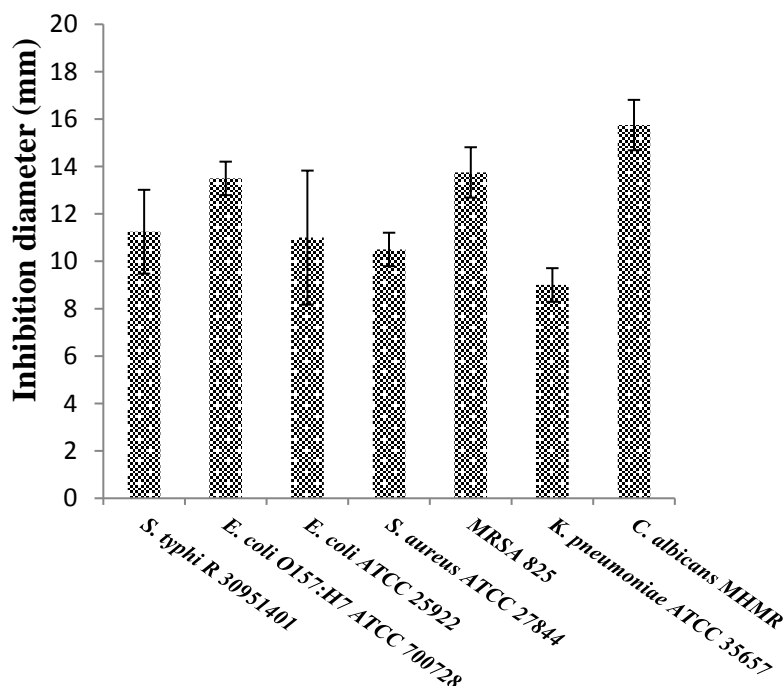


Figure 3. Antimicrobial activity of dried probiotic ingredients against indicator strains by agar disc diffusion method.

Table 2. Pearson correlation matrix between physicochemical, microbiological and antimicrobial activity.

Activity	pH	Dry matter	Lactic acid	Maltose	Glucose	Yeasts and moulds	LAB
Dry matter	0.617*						
Lactic acid	-0.944**	-0.439					
Maltose	0.228	0.222	-0.220				
Glucose	0.705**	0.522	-0.719**	0.602*			
Yeasts and moulds	-0.772**	-0.280	0.758**	0.064	-0.191		
LAB	-0.861**	-0.193	0.856**	0.008	-0.288	0.942**	
Antimicrobial activity	-0.932**	-0.639*	0.931**	-0.345	-0.770**	0.644**	0.779**

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; antimicrobial activity (expressed in mm of inhibition diameter).

for the inhibitory mechanism. Indeed, the inhibitory effects of lactic acid bacteria might be due to either individual or joint production of organic acids, hydrogen peroxide, or bacteriocins (Ennahar et al., 2000; Villamil et al., 2003; Vázquez et al., 2005). Moreover, it has been reported that LAB produce a large number of antimicrobial compounds such as organic acids, H_2O_2 , diacetyl, enzymes, bacteriocin, and biosurfactants which are effective against food spoilage and pathogenic bacteria (Sharma and Saharan, 2014).

Conclusion

The probiotic ingredient derived from the African opaque sorghum beer exhibited antimicrobial activity with a large

spectrum being effective against Gram negative, Gram positive and the yeast microorganisms. This antibacterial activity is preserved during the propagation process of the functional microorganisms in a cereal-based starchy career. 36 h of propagation is enough to inhibit all pathogens tested. The microorganisms involved in the starter of African sorghum beers could an alternative to antibiotic chemicals. More in-deep researches are ongoing on the molecular and functional characterization of these microorganisms.

Conflict of Interests

The authors have not declared any conflict of interests.

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