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Potential probiotic *Pichia kudriavzevii* strains and their ability to enhance folate content of traditional cereal-based African fermented food

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ABSTRACT

With the aim of selecting starter cultures with interesting probiotic potential and with the ability to produce folate in a food matrix, yeast strains isolated from fermented cereal-based African foods were investigated. A total of 93 yeast strains were screened for their tolerance to pH 2 and 0.3 % of bile salts. *Pichia kudriavzevii* isolates gave the best results. Selected *P. kudriavzevii* strains were tested for survival to the simulated human digestion and for adhesion to Caco-2 cells. Moreover, presence of folate biosynthesis genes was verified and production of extra and intra-cellular folate determined during growth in culture medium. 31 % of yeast strains could tolerate pH 2, while 99% bile salts. Survival rate after simulated digestion ranged between 11 and 45 %, while adhesion rate between 12 and 40 %. Folate production was mainly intracellular, maximum after 24 hours of growth. To be closer to traditional cereal-based fermentations, a *P. kudriavzevii* strain with good probiotic potential was co-inoculated with *Lactobacillus fermentum* strains in a pearl millet gruel. This resulted in *in situ* folate production that peaked after 4 hours. The use of strains with both probiotic and nutritional enrichment properties may have a greater impact for the consumers.

Keywords: yeasts; *Pichia kudriavzevii*; probiotics; food enrichment; folate, LAB.

1. INTRODUCTION

Cereal-based fermented foods in Africa are major contributors to the diet of populations. Fermented gruels are frequently used as complementary foods to breast-feeding for young children (Tou et al., 2007b). Nevertheless, their nutritional density is weak and do not fulfill the requirement of complementary foods for young children (Mouquet-Rivier et al., 2008). Besides being important in those fermentation processes, yeasts have shown numerous probiotics effects on human health (Łukaszewicz, 2012; Moslehi-Jenabian et al., 2010). Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (WHO, 2001). To evaluate the properties of putative probiotic strains it is recommended to perform preliminary *in vitro* assessment (FAO/WHO, 2001, 2002). This assessment has traditionally paid special attention to the ecological origin of the strains, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to intestinal surfaces (Morelli, 2007). However, the ultimate criterion to select a successful probiotic strain is the ability to confer to consumers a health benefit. Among the potential benefits, yeasts can be used to produce vitamins or to increase the bioavailability of minerals (Łukaszewicz, 2012).

Folate, also known as vitamin B9, has an essential role in cellular metabolism and is relevant to the etiologies of chronic diseases and birth defects (Combs, 2012). In particular, folate is active as coenzyme in single-carbon metabolism and it is involved in the methylations and formylations that occur as part of nucleotide biosynthesis (Combs, 2012). Mammals do not synthesize their own folate and it needs to be obtained from the diet. In some diets, cereals, especially fermented whole grain products, are major contributors to folate intakes (Kariluoto et al., 2006). Increase of folate content of cereal products during fermentation has been mainly associated with the growth of yeasts (Osseyi et al., 2001; Kariluoto et al., 2004). They are naturally able to produce high quantities of folate and have already been used successfully for biological enrichment of foods in folate via fermentation (Hjortmo et al., 2008b, 2008c, 2005; Kariluoto et al. 2006, 2004; Korhola et al., 2014; LeBlanc et al., 2007; Patring et al., 2006; Wittholf et al., 1999). However, in natural fermentation of cereals, there is a coexistence of yeasts and bacteria, mainly lactic acid bacteria (LAB). Even if many LAB are folate consumers, several authors showed that some of them are able to produce folates in culture medium as well as in food (Le Blanc et al., 2007).

Together with their ability to produce folate, yeasts have also been used in food to increase mineral bioaccessibility, through hydrolysis of phytates by phytases (Kaur et al., 2007). We have previously showed that *Pichia kudrivazevii* strains possess a high ability to hydrolyze phytate (Greppi et al., 2015). Biofortification of foods via controlled fermentations by the use of culture starters would allow producing food products with improved vitamin and mineral contents at low cost, which is of particular importance in African countries where the resources of households are

often limited. Moreover, if the starter cultures have also the ability to survive the GIT passage and adhere to intestinal cell, all its potential beneficial effects may also be achieved directly inside the human host, increasing its impact on the host health.

In the present study, the probiotic potential of a collection of yeast strains isolated from traditional African cereal-based fermented foods was investigated. Survival to human gastrointestinal tract (GIT) conditions and adhesion to human intestinal cells was assessed *in vitro*. In addition, the ability to synthesize folates was checked in a cultivation medium on a selection of the most interesting *P. kudriavzevii* strains using genetic and biochemical screening. Finally, the most efficient folate producer was inoculated in a pearl millet-based gruel, together with LAB strains previously selected for their folate-producing ability at genetic level and by measurement of folate production in culture medium, but not yet *in situ* (Turpin et al., 2011; Saubade et al., 2014). This fermentation model was used to mimic the natural conditions where yeasts and LAB co-exist in the same food matrix and was used to investigate *in situ* folate production.

2. MATERIAL AND METHODS

2.1 Strains

A total of ninety-three yeast strains, previously isolated from spontaneously fermented cereal-based traditional food of West Africa and identified as described by Greppi et al. (2013a, 2013b) were used. They were maintained in glycerol (30%) and YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose (Biogenetics, Milan, Italy)).

Two LAB strains, namely *Lactobacillus fermentum* 8.2 and *L. fermentum* 6.9 isolated from an African pearl millet fermented porridge (*ben-saalga*) were also used for co-inoculation trials (Saubade et al., 2014; Turpin et al., 2011). They were maintained in glycerol (40 %) and de Man Rogosa and Sharp medium (MRS, Difco, France).

2.2 Tolerance of yeasts to simulated human gastrointestinal tract (GIT)

Assay on tolerance to low pH and bile salts

Tolerance to GIT conditions was investigated for all the 93 strains as described by Pedersen et al. (2012), with some modifications. Yeast colonies were pre-grown for 24 h at 30 °C in YPD. Yeast culture, set at A_{600} = 1, were inoculated in YNB (6.7 g L^{-1} yeast nitrogen base (BD, Milan, Italy) without aminoacids and 20 g L^{-1} glucose, pH 5.4), YNB pH 2.0 and YNB containing 0.3 % (w/v) of bile salts (Oxoid, Milan, Italy), pH 8. The experiment was performed on 200 µl 96-wells microtiter plates (Steroglass, Milan, Italy). The wells were inoculated in triplicates. YNB without inoculation was used as negative control. Absorbance at 600 nm was measured after 24 h of incubation at 37°C, to simulate a prolonged stress condition. The tolerance of the strains to pH 2.0

and 0.3 % of bile salts was measured relative to the growth in YNB. It was calculated according to the following equation:

$$\text{Tolerance rate (\%)} = A_{600}\ YNB_x / A_{600}\ YNB \times 100$$

where YNB_x = YNB at pH 2 or YNB with 0.3 % bile salts and YNB = normal growth medium, used as positive control. Three independent experiments were performed; mean and standard error were calculated.

Assay on survival in simulated in vitro *human digestion*

Eight *P. kudriavzevii* strains with the highest phytase activity (Greppi et al., 2015) and with the highest tolerance to low pH and bile salts were chosen, and subjected to a simulated human GIT digestion using the protocol described by Bautista-Gallego et al. (2013), with slight modifications. Briefly, the simulated gastric juice was prepared daily by suspending pepsin (0.0133 g L^{-1}) and lysozyme (0.01 g L^{-1}) in a sterile buffer solution at pH 2.0 containing NaCl (2.05 g L^{-1}), KH_2PO_4 (0.60 g L^{-1}), $CaCl_2$ (0.11 g L^{-1}) and KCl (0.37 g L^{-1}). Pepsin and lysozyme were sterilized by filtration (0.22 μm). Simulated pancreatic juice was daily prepared by suspending bile salts (3 g L^{-1}, Oxoid) and pancreatin (0.1 g L^{-1}, Sigma Aldrich, Milan, Italy) in a sterile buffer at pH 8.0 consisting of Na_2HPO_4 $7H_2O$ (50.81 g L− 1) and of NaCl (8.5 g L^{-1}). The pH was adjusted with a solution of KH_2PO_4. Yeasts were inoculated in 2 mL of sterile YPD medium and incubated at 37 °C for 12 h. Serial dilutions were prepared and plated on YPD agar plates to enumerate the yeasts before the treatment (N0). The remaining overnight cultures were then centrifuged (10,000 × g, 10 min), the pellet washed with isotonic solution to remove the medium and the cells were re-suspended in the synthetic gastric juice. After incubation for 3 h at 37 °C in an orbital shaker (~200 rpm) to simulate peristaltic movements, harvested cells were re-suspended in isotonic solution and serial dilutions prepared and plated for yeast enumeration (N1). Harvested cells from the gastric digestion step were washed with isotonic solution and re-suspended in the same volume of the simulated pancreatic juice. After shaking at 200 rpm overnight at 37 °C, the pellet was washed and re-suspended in isotonic solution. Serial dilutions were prepared, and plated onto YPD agar (N2). Plates were incubated at 30 °C for 48 h and thereafter counted. Percentages of survival were obtained comparing the initial yeast counts with those remaining viable at the end of the gastric and pancreatic step of simulated digestion. They were calculated according to the following equation:

$$\text{Survival rate (\%)} = CFU_{N1\ or\ 2} / CFU_{N0} \times 100$$

where N1 or 2 = the total viable count of yeast strains after treatment by simulated gastric and pancreatic juices respectively; and N0 = the total viable count of yeast strains before treatment. Three independent experiments were performed, mean and standard error of the counts calculated.

2.3 Adhesion of yeasts to Caco-2 cell lines

Growth and maintenance of mammalian cell lines

The human colon adenocarcinoma cell line Caco-2 was purchased from ATCC (HTB-37^{TM}) and cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum (FBS), 10 U m^{-1} of penicillin, 0.1 mg m^{-1} of streptomycin and 2 mM of L-glutamine. All reagents were purchased from Sigma Aldrich. The cells were routinely grown in 75 cm^2 culture flasks (BD biomedical, Franklin Lakes, New Jersey, U.S.) in an incubator (Galaxy 170 s, Eppendorf, Italy) at 37 °C with humidified atmosphere of 5 % CO_2 and 95 % air. The culture media were changed routinely and once the cells reached sub-confluence (80-90 %) they were subpassaged.

Adhesion assay on Caco-2 cells

Selected yeast strains were grown overnight in 5 mL of YPD at 30 °C. Afterwards, cells were harvested in sterile phosphate buffered saline (PBS) and suspensions at A_{600}= 1 were prepared in DMEM without antibiotics. This absorbance corresponds roughly to 10^7 yeast cells mL^{-1}, as determined by plating for the different strains studied. Anyhow, the concentration of each yeast suspension was quantified in each experiment by serial dilution and enumerated on YPD agar. The adhesion assay was performed as described by Botta et al. (2014) with minor modifications. Briefly, Caco-2 cells were seeded at 20 000 c/w in 96-well microtiter plates (BD biomedical) and grown in the conditions described above for 2-4 days until they formed a confluent monolayer. Before the experiment the confluent monolayer of cells was washed twice with PBS in order to remove traces of antibiotics. After which, 100 µl of the yeast suspension was transferred into the wells and incubated for 90 minutes at 37 °C. Unattached yeasts were removed from the epithelial cells by three rinses with PBS. Afterwards, Caco-2 cells and attached yeasts were homogenized in each well with 100 µL of Triton-X solution (0.25 % in PBS). After 30 minutes of incubation at 37 °C the solution with released yeasts was serially diluted and enumerated on YPD agar. In parallel, yeast cells from overnight culture were also treated with Triton-X and incubated in the same conditions as used in the experiments, to evaluate a possible effect of Triton-X on yeast cells vitality. The plates were incubated at 30 °C for 48 h. Adhesion ability was expressed as the percentage ratio between the yeast counts initially seeded and the counts after the washing steps (CFU mL^{-1}). All assays were repeated three independent times and mean and standard error were calculated.

2.4 Folate-producing capacity of yeasts

Identification of genes

Nineteen *P. kudriavzevii* genes involved in folate biosynthesis were identified in a draft genome of *P. kudriavzevii* (GeneBank #ALNQ00000000.1, [Chan et al., 2012]). The genes were identified on different contigs by aligning with Blast Program (Altschul et al., 1997) the protein sequences retrieved from both SGD and CGD databases, against the whole genome shotgun contigs (wgs) of *P. kudriavzevii*. For *FOL3*, *FOL2*, *FOL1*, *ABZ1*, *ABZ2*, *DRF1* and *CDC21* genes, responsible for the conversion of folate precursor (GTP) into tetrahydrofolate (THF), specific primers were designed by Primer 3 (version 4.0.0) (http://primer3.ut.ee/). Primers sequences are shown in Table 1.

DNA extraction and PCR conditions

DNA of each strain was extracted from the pellet obtained from 1 mL of 24 h YPD pure culture and centrifuged at 14 000 x g for 10 min, 4 °C. The pellet of cells was subjected to DNA extraction according to the procedure described by Cocolin et al. (2000). DNA was quantified using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and diluted to a concentration of 100 ng mL^{-1}. The PCR temperature profile was as follow: 5 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C. At the end, 10 min at 72 °C were performed. Amplicons were analyzed by visualization on 1.2 % agarose gel electrophoresis in 1X TAE (80 V, 25 min) after staining with ethidium bormide (10 mg mL^{-1}). Amplified sequences were sent for sequencing (MWG Biotech, Ebersberg, Germany) and results aligned in GenBank using the Blast Program (Altschul et al., 1997), for identification purposes.

Growth conditions for the folate assay

Yeast pre-cultures were grown overnight at 30 °C in tubes containing 5 mL of YNB without aminoacids (folic acid content declared by the producer: 2 g L^{-1}). They were then inoculated to 50 mL of the same medium, in amounts to obtain an initial absorbance of 0.02 at A_{600} (UV-1800 Shimadzu UV Spectrophotometer, Kyoto, Japan). The cells were grown on a shaker (Julabo SW22, Seelbach, Germany) at 30 °C, 180 rpm, for 24 h. After 8 h, 16 h and 24 h, 4.5 mL of yeast culture were transferred into a falcon tube and kept at -20 °C until folate extraction. For each condition, three independent experiments were performed.

Folate extraction

Folate content was determined for the supernatant (i.e. extracellular folate) and for the cell biomass together with the supernatant (i.e. total folate). Intracellular folate was calculated by subtraction of the two values. Folate extraction protocol was adapted from the method described by Sybesma et al. (2003). The 4.5 mL of yeast-inoculated YNB without aminoacids broth were diluted twice with the extraction buffer (50 mM Ches/50mM Hepes buffer, pH 7.85) for total

folate determination. Then, to release folate from the cells, the tubes were flushed with nitrogen and heated at 100 °C for 10 min. They were vortexed twice during heating, and cooled on ice. After adjustment to pH 4.7 with acetic acid, 2 mL of conjugase prepared from desiccated hog kidney (5 mg mL^{-1} in water) was added. The tubes were incubated under shaking for 3 h at 37 °C. After adjustment of the pH to 6.1, the volume was filled to 50 mL with 0.5 % sodium ascorbate, and the tubes were centrifuged (13 000 rpm, 10 min, 4°C). For determination of extracellular folate, the same process was used on supernatant collected from yeasts inoculated to YNB without aminoacids. Collected supernatant and falcon tubes for determination of total folate were immediately stocked at -20 °C and folate quantification performed within 48 h.

Folate quantification

The determination of folate concentration was done following the method described by Kariluoto and Piironen (2009) using 96-well microtiter plates. The growth indicator organism was *Lactobacillus rhamnosus* ATCC 7469, the calibrant was folic acid (Sigma-Aldrich) and the assay medium was Folic Acid Casei Medium (Difco, Sparks, MD, USA). In each plate, a blank sample consisting of sodium ascorbate, a blank reactive consisting of extraction buffer and a positive sample consisting of YPD medium were analyzed. After 10 s shake at 240 rpm by a microtiter plate reader (LABSYSTEM Multiskan Ascent, Helsinki Finland), absorbance was read at 590 nm. Folate production by yeasts was calculated by subtracting the folate content of the medium (YNB without aminoacids). Values were calculated as averages of three individual incubations, and each determination was the results of four technical replicates.

2.5 Inoculation of selected strains in pearl millet gruel

Microbial strains and materials

Based on all the tests performed, one *P. kudriavzevii* strain was chosen to inoculate a pearl millet gruel. In addition, to mimic conditions of some African cereal slurry fermentations (i.e. *ben-saalga, koko*) in which LAB are dominant, two *L. fermentum* strains, previously selected for folate-producing capacity *in vitro* (Saubade et al., 2014; Turpin et al., 2011), were also used. Pearl millet (*Pennisetum glaucum*) used in the preparation of the gruels was purchased on a market in Ouagadougou (Burkina-Faso).

Preparation of gruels

The gruels were prepared from pearl millet as described by Tou et al. (2007b). Briefly, the protocol comprises as main steps: soaking (16 h), washing, milling, sieving, pre-cooking and inoculation. The pre-cooking step consisted in heating for 10 min at 80 °C the unfermented slurry resulting from sieving. After the pre- cooking step, the gruel (10 % dry matter content) was

divided into four sterile beakers, named gruel A, B, C and D, depending on the combination of LAB and yeast used. Each model was inoculated at a rate of 10 % (w/w) to the unfermented paste, when it had cooled down to 35 °C. Start concentration of 10^6 g^{-1} yeast cells and 10^7 g^{-1} bacteria cells was chosen to be close to field conditions (Tou et al., 2007a, 2007b).

For each combination, samples were taken at different times during the fermentation (from 0 to 24 h) for folate and pH measurements, together with microbiological enumeration. The dry matter content of gruels was determined at the end of the fermentation by oven drying at 105 °C to constant weight. Results are means of triplicate experiments.

Enumeration of LAB and yeasts

Bacteria and yeasts were enumerated during fermentation in all process combinations after serial dilutions in 9 g L^{-1} sodium chloride. LAB were enumerated on MRS plates supplemented with Delvocid (200 mg mL^{-1}) to avoid the growth of yeasts. Yeasts were enumerated on yeast glucose chloramphenicol plates (50 mg mL^{-1}; YGC, Oxoid, France), to avoid the growth of LAB. All plates were incubated at 30 °C for 48 h. Results are means of triplicate enumerations in samples of each experiment.

Total folate assay

The folate content of the gruels at different fermentation times was quantified according to Kariluoto and Piironen (2009).

2.6 Statistics

To detect differences among strains in each tested conditions (tolerance rate, survival rate, adhesion rate, and total folate production *in vitro* and *in situ*), statistical analysis was performed by one-way ANOVA analysis of variance (SPSS 22.0). When significant differences were detected, Duncan's post hoc test was performed. Data on probiotic tests were elaborated by principal component analysis (PCA) in R environment.

3. RESULTS

3.1 Probiotic properties of yeast strains

The results obtained from the tolerance of the 93 yeast strains to pH 2 and to the presence of 0.3 % of bile salts are presented in Fig. 1. In general, the isolates were more sensitive to the pH treatment (31% tolerate) than to the presence of bile salts (99% tolerate). Isolates belonging to the species *P. kudriavzevii* showed the best tolerance in both conditions. Thirty-eight percent of *P. kudriavzevii* isolates could grow in the presence of bile salts to the same or even higher extent than in the

positive growth medium. On the contrary, *Saccharomyces cerevisiae* isolates were the less tolerant to both conditions, 90 % of the strains from this species did not tolerate pH 2. There were less yeasts in each of the other species, but in general, tolerance to both conditions were variable. The most interesting *P. kudriavzevii* strains in terms of tolerance to pH 2 and bile salts and of phytase-producing capacity (Greppi et al., 2015) were selected for further *in vitro* tests; namely, *P. kudriavzevii* M26, M28, M29, O9, G6, G5, M31, M30 (Table 2).

Concerning the survival rate in synthetic gastric and pancreatic juices among the selected strains, strain M28 survived significantly more after gastric juice incubation (40.3 ± 14.5 %), while strain M26 was significantly more tolerant to the passage after pancreatic juice indicating the overall digestion survival rate (45.1 ± 6.6 %). Results on adhesion rate capacities showed that only 12.7 ± 2.4 % of the cells of strain M30 adhered on Caco-2 monolayer, significantly less ($p < 0.05$) than all the other yeast strains. On the other hand, strains M28 and O9 have a significant ($p < 0.05$) higher adhesion capacity, being 38.9 ± 2.7 % and 40.9 ± 5.9 %, respectively (Table 2). PCA analysis, showed in Fig. 2, allowed the segregation of two strains (O9 and M28) characterized by high adhesion rate and another three (M29, G6 and M26) for their high survival rate after simulation of human overall digestion (passage after pancreatic juice).

3.2 Folate production in YNB medium by selected yeasts strains

All the genes involved in folate biosynthesis were identified on the draft genome of *P. kudriavzevii*: *FOL3*, *FOL2*, *FOL1*, *ABZ1*, *ABZ2*, *DRF1*, *MET7*, *CDC21*, *MET13*, *ADE3*, *MIS1*, *SHM1*, *SHM2*, *LPD1*, *GCV2*, *GCV1*, *GCV3*, *MTD1* and *MET12* (data not shown). However, selected *P. kudriavzevii* strains were only screened for the presence/absence of the 7 main genes responsible for the conversion of folate precursor (GTP) into tetrahydrofolate (THF) (Table 1). The genes were identified in the shotgun genome sequence of *P. kudriavzevii* on different contigs and primers were designed to amplify the selected genes (Table 1). All the strains possessed the genes for *de novo* biosynthesis of folate.

Concerning folate quantification, the folate content of YNB medium was 2.6 ± 0.3 ng mL^{-1}. This value was subtracted from the folate quantification of the strains in that medium. In general, all the tested strains showed a higher total folate production after 24 h (Fig. 3). The comparison of the eight strains at each incubation time showed that net folate production was higher after 8 h for the strain G6 (14.0 ± 1.5 ng mL^{-1}), after 16 h for the strain M31 (139.6 ± 43.3 ng mL^{-1}) and after 24 h for the strain M28 (173.7 ± 33.6 ng mL^{-1}). For all the strains, the folate produced appeared to be almost completely intracellular.

3.3 Inoculation of selected LAB and yeasts in a model of fermented pearl millet-based gruel

Based on the previous results, *P. kudriavzevii* strain M28 was chosen for inoculation in the gruels, together with *L. fermentum* 8.2 and 6.9 to mimic a real cereal-based African fermentation. In particular, Gruel A was inoculated with *L. fermentum* 8.2 + *P. kudriavzevii* M28, gruel B with *L. fermentum* 6.9 + *P. kudriavzevii* M28, gruel C with *L. fermentum* 8.2 alone and gruel D with *L. fermentum* 6.9 alone.

Results of microbial counts and pH after the inoculation of selected LAB and yeast strains in the gruels are presented in Fig. 4. During fermentation, pH decreased quickly until 8 h and remained low until the end of fermentation when LAB were inoculated alone (C and D). On the other hand, when yeasts were present together with LAB, there was a slight increase of the pH from 16 h of fermentation (A and B).

As expected, only slight yeasts development was measured after 24 h of fermentation in conditions C and D where no yeasts were inoculated. In gruels A and B, a significant ($p < 0.05$) increase of yeasts concentrations was detected after 4 h and remained stable until 24 h reaching 6.3 ± 0.3 and 6.3 ± 0.6 log CFU mL^{-1}. LAB concentration also increased in all conditions after 4 h of fermentation and reached a concentration of 8 log CFU mL^{-1} until 24 h in presence or absence of yeasts.

Results on folate content of pearl millet fermented gruels after inoculation of selected strains are presented in Fig. 5. Folate quantification showed that all the fermentations allowed a significant higher production of folate, as compared with the non-fermented dough (time 0 h). In addition, whatever the combination of microorganisms, there was a significant high production of folate after 4 h of fermentation. At this time points, gruel A and B produced significantly higher folate compared to gruel C and D. Moreover, folate production was significantly higher in gruel A co-fermented with *L. fermentum* 8.2 and P. *kudriavzevii* M28 than in gruel C fermented with the bacteria alone. Folate concentration decreased until 16 h, with some significant differences among the gruels at 6, 8, 12, 14 and 16 h (Fig. 5, small letters). A second significantly higher production of folate was detected at 24 h. Co-fermentations with yeast (gruels A and B) produced significant higher folate, compared to those without yeast (gruels C and D) (Fig. 5).

4. DISCUSSION

Here, the probiotic potential of yeast strains isolated from fermented food has been studied as well as the potential of the same strains to nutritionally enrich the food product during fermentation.

Most of the published investigation on probiotics focused on bacteria, especially lactic acid bacteria. However, there is an increasing interest in finding new yeast strains with probiotic potentials (Hatoum et al., 2012; Kumura et al., 2004; Moslehi-Jenabian et al., 2010; Perricone et al., 2014). Presently, most probiotics are assessed by their ability to survive in, and subsequently colonize, the gastrointestinal environment. In fact, resistance to GIT conditions, as well as the

ability to adhere to intestinal mucosa, is important to reach the target site in a viable state and to provide beneficial effects on the hosts (Ouwehand et al., 2002).

To reach the intestine, strains must first pass through the stomach, which provides a powerful barrier to the entrance into the gut. Most of the yeast isolates tested in this study were to some extent able to tolerate pH 2, which is an extreme value that is commonly used to mimic the pH encountered in the stomach, in fact a pH of 4 would have been more realistic. In the intestine, yeasts would be exposed to the presence of bile salts. The strains tested here appeared to tolerate better bile salts, compared to the acidic pH of the stomach, as already proven for other yeasts (Perricone et al., 2014). As almost 20 % of the yeasts strains could tolerate and grow with 0.3 % of bile salts in the medium even more than in an unmodified growth medium, we can hypothesized that the survival potential of those strains to GIT conditions would be rather good.

From the first screening on the tolerance to GIT conditions, *P. kudriavzevii* appeared to be the most promising. This species previously demonstrated a high persistence in cereal-based fermentations (Greppi et al., 2013a, 2013b) and a high capacity to secrete phytases (Greppi et al., 2015; Hellstrom et al., 2012; Qvirist et al., 2015). For these reasons, the most interesting *P. kudraivzevii* strains were selected and subjected to an *in vitro* simulation of human digestion. This was carried out with the successive use of complete synthetic gastric and pancreatic juices. After the passage in gastric juice, the strains that survived were subjected to pancreatic juice. In general, all the selected strains could survive both conditions, maintaining a concentration compatible with their use as potential probiotics (between 10^6 and 10^5 CFU mL^{-1}). The most promising strains survived between 16.8 % and 45.1 % of the initial concentration (i.e. 10^7-10^8 CFU mL^{-1}). Thereafter, the ability of those strains to adhere to Caco-2 cells was evaluated. The adhesion rate of tested strains varied between 12 and 40 %, which corresponds to a concentration comprised between 10^5 and 10^7 CFU mL^{-1}. These results were in agreement with previous screening of yeasts isolated from kefir (Kumura et al., 2004).

Once survived the GIT and eventually adhere to cell lines, yeast strains ingested via fermented food could provide some beneficial effects to the hosts. Among others, degradation of phytates and biofortification of folates are known (Moslehi-Jenabian et al., 2010) and might be really important in Africa, where deficiencies in micronutrients may be high. In fact, it has been shown that microbial phytase activity introduced by the diet is responsible for the degradation of phytates in the stomach and small intestine (Sandberg and Andlid, 2002). Considering the high phytase activity of the selected *P. kudriavzevii* strains (Greppi et al., 2015), their survival to *in vitro* GIT digestion and their ability to adhere to the intestinal cells, their potential as folate producers was evaluated. Folate deficiency is an important health problem in many parts of the world, particularly where there is poverty and malnutrition (Mitchell et al., 2012). Humans are autotrophic for this vitamin and must therefore satisfy their needs by the diet. The biosynthetic

pathway of folate in yeasts is mostly characterized, and it is similar to that of other microorganisms (Walkey et al., 2014). The identification of genes involved in the biosynthesis of folate in a draft genome of *P. kudriavzevii* allowed the verification of the presence of the molecular requirements necessary to produce the vitamin. Moreover, since molecular tools for manipulation of yeasts genes expression are well developed these genes might be used as possible target to achieve the production of foods with increased level of folate as recently done for wine (Walkey et al., 2014). As expected, genes for *de novo* synthesis of folate were found in all isolates. All the tested strains were also able to produce folate *in vitro* during growth, but with significant differences among them, despite common genetic equipment. The higher production took place after 24 h, i.e. at the beginning of the stationary phases, and it appeared to be almost completely intracellular. This is in agreement with previous publications testing other yeast species (Kariluoto et al., 2006). Low folate content in the supernatant may indicate the folates to be highly conjugated and therefore remaining intracellular (Hjortmo et al., 2005). The growth medium influence the folate production, yeasts grown in YPD as complex media produced much lower folate content than in other synthetic growth media, and peptone seemed to reduce as much as 90 % of their production (Hjortmo et al., 2008a). For this reason, the production was hereby quantified in a minimal growth medium (YNB without aminoacids). In addition, the physiological state of the cells clearly affects the folate content and a high growth rate i.e. respiro-fermentative growth (0-10 hours) should be the most favorable to obtain high specific folate content for yeasts (Hjortmo et al., 2008a). This is the reason why the production was hereby quantified in different phases of the yeast growth.

It has been widely demonstrated how folate production in growth medium did not necessarily predict the production in a food matrix (Kariluoto et al., 2014). This is why it was mandatory to test the promising strains *in situ*. The most interesting yeast isolate (*P. kudriavzevii* M28) was then inoculated in a cereal-based gruel made from pearl millet as a model food. This is a model of fermented food highly consumed in West Africa (*ben-saalga*), which is nutritionally poor (Tou et al., 2006; Mouquet-Rivier et al., 2008). It is consumed after a minimum of 2 and a maximum of 20 hours of fermentation (Tou et al., 2006). As in traditional process there is a natural combination of yeasts and LAB, both microorganisms were inoculated for a fermentation lasting 24 hours to reflect the common production process. LAB strains inoculated belong to *L. fermentum* species and were previously isolated from pearl millet fermented slurry (Turpin et al., 2011). Those strains have genetic equipment required to synthesize folate and they are able to produce folate in culture medium (Saubade et al., 2014). Moreover, many *L. fermentum* strains present technological and probiotic traits (Owusu-kwarteng et al., 2015).

Co-inoculation of *P. kudriavzevii* M28 with each strain of *L. fermentum* allowed the growth of both types of microorganisms to levels comparable to natural microbiota (Tou et al., 2007a).

Moreover, no negative or positive effects of yeasts on LAB growth were observed. In fact, when single cultures of *L. fermentum* strains were used, they reached the same final concentrations as in yeast-LAB co-cultures.

In general, the concentration of folates in inoculated pearl millet fermentation peaked at 4 h of fermentation and decreased rapidly to stabilize at values between 2-4 µg/100 g fresh matter, probably due to an equilibrium between production and consumption by LAB strains. Increased production at 24 h might be due to cell autolysis in stationary phase. It may therefore be suggested that a fermentation of 4 hours could be the best solution to get the higher folate production in this model of fermented food. In addition to the strain selection, the duration of fermentation is indeed a lever to improve folate content of fermented foods. In addition, fermentation of pearl millet gruels with combinations of the yeast and LAB strains resulted in folate levels significantly higher than in gruels fermented by LAB alone. The interactions between LAB and yeasts in the fermentation of cereal based fermented foods had been demonstrated (Kedia et al., 2007; Vieira-Dalodé et. al, 2008). Although one of the LAB strain consumed folate in culture medium, its effect in co-cultivations was minimal, as already reported (Kariluoto et al., 2006).

5. CONCLUSIONS

Throughout Africa, food fermentations are still driven by indigenous microorganisms, which influence the nutritional, organoleptic and quality of final products. However, for improved safety, quality and beneficial health effects a trend has emerged which involves the isolation of indigenous strains from traditional fermented products to be used as functional starter cultures (Owusu-kwarteng et al., 2015).

The final result of this study was the selection of a promising yeast strain as suitable starter for fermented cereal-based products. This strain possessed desirable probiotic characteristics *in vitro.* It was able to survive the conditions of the human GIT, to adhere to intestinal cell lines, and to produce folate and phytases. Therefore it seems to have a great promising ability to increase the nutritional quality of foods via natural fermentation as well as to exert probiotic beneficial effect in the human host. It is therefore a good candidate for further *in vivo* and functional studies.

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TABLES

Table 1. Genes of *P. kudriavzevii* involved in folate biosynthesis identified in a draft genome (GeneBank #ALNQ00000000.1), sequences of primers used to detect them and results of PCR assays.

Folate Biosynthesis Genes of *Pichia kudriavzevii*						
Gene name	Enzymatic activity	Contig [a]	Primer name	Primer sequence	Amplicon size	PCR
FOL3	dihydrofolate synthase	624	FOL3-f	TTT-GGC-TCC-CTT-GGT-TAT-TG	241 bp	+
			FOL3-r	AGG-AAA-CGT-GGG-TTC-ACT-TG		
FOL2	GTP-cyclohydrolase I	148	FOL2-f	TCG-TGA-AGA-GGG-CAG-TTT-TT	200 bp	+
			FOL2-r	TTG-GAC-CTG-GAA-CCT-TCT-TG		
FOL1	dihydroneopterin aldolase	297	FOL1-f	TGC-TAA-AGT-TGC-CGA-GGA-GT	227 bp	+
			FOL1-r	CCT-CTT-GGT-TTC-TTC-GTT-CG		
ABZ1	aminodeoxychorismate synthase	298	ABZ1-f	CAG-GAC-CAG-GTT-CAC-CAA-CT	198 bp	+
			ABZ1-r	TGG-AAG-AAT-GGT-CAA-CCA-CA		
ABZ2	aminodeoxychorismate lyase	157	ABZ2-f	TCA-CTA-GCA-TTG-CCT-GTT-GG	188 bp	+
			ABZ2-r	CAT-GAG-AGC-GTT-CAT-GAG-GA		
DFR1	dihydrofolate reductase	112	DRF1-f	TGG-TGC-CAT-TGA-GGA-GAT-TT	201 bp	+
			DRF1-r	GCT-TCG-AGT-TCA-CAA-TGC-AA		
CDC 21	thymidylate synthase	524	CDC21-f	CAC-AGA-CGC-AAA-ACT-CCT-CA	182 bp	+
			CDC21-r	CAT-TGG-TGT-AAT-CGC-TGT-GG		

[a] contig number of the shotgun genome in which the gene was identified

+ indicates the detection of PCR product

bp, base pair

Table 2. Probiotic properties of 8 selected *P. kudriavzevii* strains, as tested *in vitro*: *i)* Survival rate of the strains after passage in synthetic gastric and pancreatic juices, measured by plate counting (CFU mL^{-1}). Survival rate was calculated by comparison to the initial yeast counts with those after gastric and pancreatic digestion; *ii)* Adhesion rate of the strains to Caco-2 cell lines. All the results are expressed as mean percentage ± standard error of three independent experiments.

Strains/Conditions	Survival Rate		Adhesion Rate
	Gastric juice	Pancreatic juice	Caco-2 cells
Pichia kudriavzevii			
M26	16.1 ± 2.8^{a}	45.1 ± 6.6^{d}	14.9 ± 2.7^{ab}
M28	40.1 ± 14.5^{c}	19.5 ± 2.8^{ab}	38.9 ± 4.0^{c}
M29	20.2 ± 2.3^{ab}	26.7 ± 2.8^{bc}	18.0 ± 3.3^{b}
O9	8.5 ± 1.4^{a}	18.6 ± 1.2^{ab}	40.9 ± 5.9^{c}
G6	35.3 ± 5.0^{bc}	33.6 ± 6.9^{c}	17.8 ± 4.1^{ab}
G5	8.0 ± 0.9^{a}	11.4 ± 1.4^{a}	15.6 ± 0.6^{ab}
M31	9.4 ± 0.9^{a}	16.8 ± 1.3^{ab}	13.7 ± 1.8^{ab}
M30	11.9 ± 2.8^{a}	23.3 ± 2.8^{abc}	12.7 ± 2.4^{a}

For each condition, different letters indicate significant differences ($p < 0.05$) among the strains (ANOVA and Duncan's test)

FIGURES

Figure 1. Tolerance rate of 93 yeast strains after 24 hours of incubation at 37 °C in YNB without aminoacids at different conditions (YNB_x): i) pH 2 (black series), ii) 0.3 % (w/v) bile salts (grey series). The tolerance rate was calculated relative to their growth in YNB, which was used as a positive control for growth: A_{600} YNB_x/ A_{600} YNB *100. Results are expressed as mean + standard error of three independent experiments.

***** Strains selected for further *in vitro* tests.

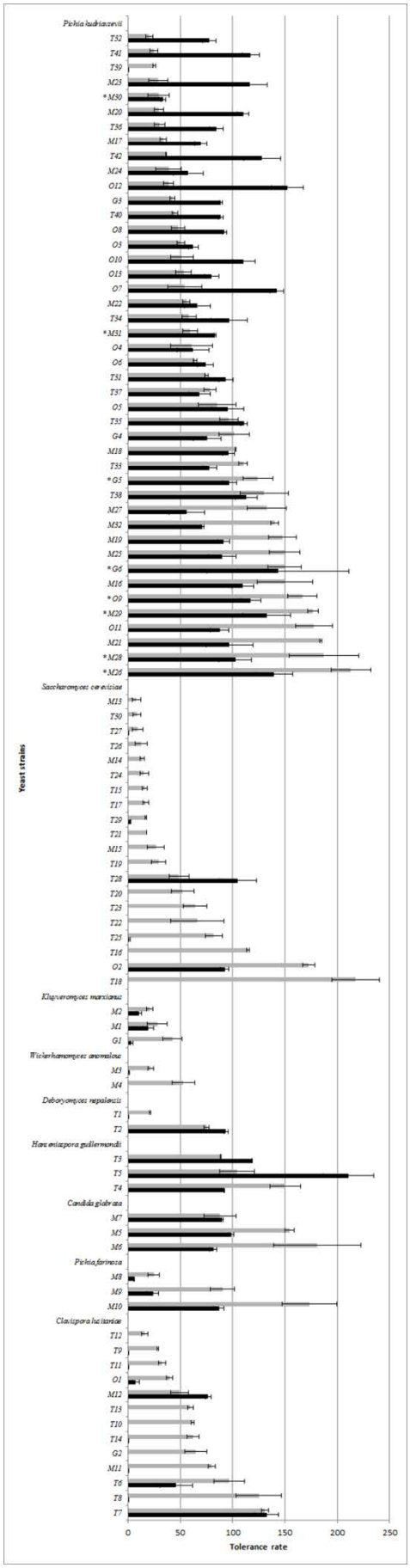
Pichia kudriavzevii
T52
T41
T39
M23
*M30
M20
T36
M17
T42
M24
O12
G3
T40
O8
O3
O10
O15
O7
M22
T34
*M31
O4
O6
T31
T37
O5
T35
G4
M18
T33
*G5
T38
M27
M32
M19
M25
*G6
M16
*O9
*M29
O11
M21
*M28
*M26
Saccharomyces cerevisiae
M13
T30
T27
T26
M14
T24
T15
T17
T29
T21
M15
T19
T28
T20
T23
T22
T25
T16
O2
T18
Kluyveromyces marxianus
M2
M1
G1
Wickerhamomyces anomalus
M3
M4
Deboryomyces nepalensis
T1
T2
Hanseniaspora guillermondi
T3
T5
T4
Candida glabrata
M7
M5
M6
Pichia farinosa
M8
M9
M10
Clavispora lusitaniae
T12
T9
T11
O1
M12
T13
T10
T14
G2
M11
T6
T8
T7
Yeast strains
0
50
100
150
200
250
Tolerance rate

Figure 2. Principal component analysis (PCA) of the data on the probiotic properties of eight selected *P. kudriavzevii* yeast strains. Projection of the variables (a.) and cases/isolates (b.) onto the plane formed by the first two factors deduced by PCA.

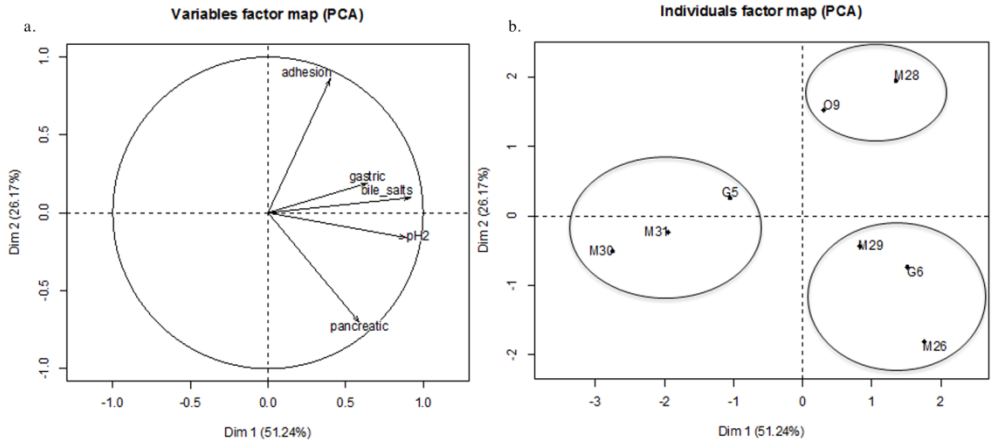

Figure 3. Total folate production (ng mL $^{-1}$) of eight *P. kudriavzevii* strains (M26, M28, M29, O9, G6, G5, M31, M30) after 8, 16 and 24 hours of growth in YNB without aminoacids. Initial folate content of the culture medium was subtracted. Extracellular folate (strike series) and intracellular folate (grey series) are expressed as mean and standard deviations of three independent experiments. For each series, different letters indicate significant differences ($p < 0.05$): 8 hours (small letters), 16 hours (capital letters), 24 hours (x and y).

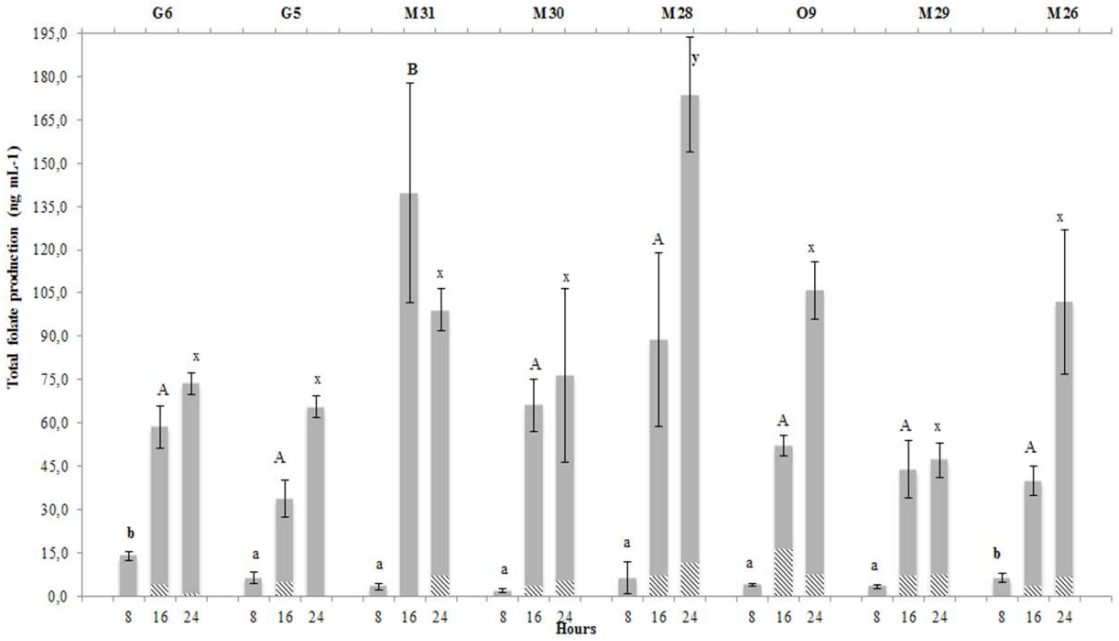

Figure 4. Changes in viable counts and pH during the fermentation of pearl millet fermented gruels after inoculation of cultures of selected yeasts and bacteria for 24 h, 30 °C. Four different gruels were inoculated with: A) *P. kudriavzevii* M28 + *Lb. fermentum* 8.2; B) *P. kudriavzevii* M28+ *Lb. fermentum* 6.9; C*) Lb. fermentum* 8.2; D) *Lb. fermentum* 6.9. a) pH measurements; b) Yeast counts; c) LAB counts are shown for each gruel during fermentation. Mean and standard deviation of three independent experiments are shown.

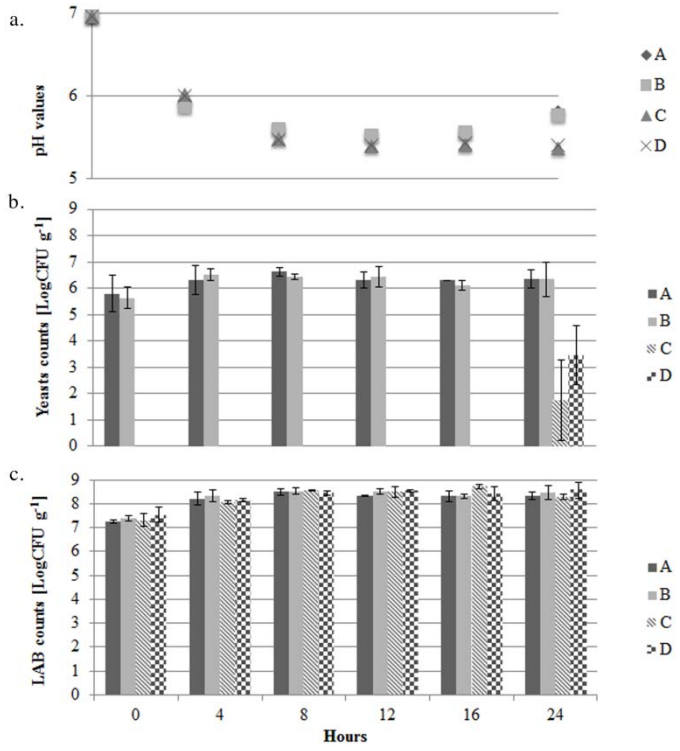

Figure 5. Folate content (μg/100 g fresh weight basis) of pearl-millet gruel inoculated with folate producing LAB and yeasts during fermentation (24 h, 30 °C). Four different gruels: A) *P. kudriavzevii* M28+ *Lb. fermentum* 8.2 (black series); B) *P. kudriavzevii* M28+ *Lb. fermentum* 6.9 (grey series); C) *Lb. fermentum* 8.2 (black squares series); D) *Lb. fermentum* 6.9 (grey dashed series). Results are the means and standard deviations of 3 independent experiments (n=3). Different letters show significant differences among the four gruels within the same time point (a, b, c); whilst K, V, W, X, Y, Z highlighted significant differences among the time points for each gruel (ANOVA and Duncan's post-hoc test: $p < 0.05$).