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In Vitro Probiotic Evaluation of Yeasts from Coconut and Raffia Juices

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Abstract.
Eukaryotic probiotics currently attract a lot of scientific attention, with *Saccharomyces cerevisiae* and *Saccharomyces boulardii* being the most widely investigated probiotic yeasts. The range of yeast species with probiotic potential needs to be broadened. In this respect, juice-providing plants may diversify eukaryotic probiotic sources for organism preference.

This study tested the probiotic potential of *Pichia kudriavzevii* and *Kluyveromyces marxianus* isolated from coconut juice and *Schizosaccharomyces pombe* and *Wickherhamomyces anomalus* isolated from raffia palm juice in Nigeria. The *in vitro* tests used the optical density method to assay the tolerance to acid (pH 2, 3, 5), alkaline (pH 7.5, 8.0), gastric juice (30%), bile (1, 2, and 3%), and osmotic pressure (5, 10, 15, 20, 25, and 30% glucose solution).

All four yeasts survived in the test environments, exhibiting varying degrees of probiotic potential. After 96 h in simulated gastric juice, *S. pombe* outperformed *K. marxianus* and *W. anomalus* by 13 and 97.7% ($p < 0.05$), respectively. *W. anomalus* appeared to be the least viable in 30% gastric juice. After 96 h in the acid media, all yeasts performed better at pH 3.0 than at pH 2.0, with roughly 89% (1.695/0.185 mean absorbance values) greater growth in pH 3.0 than in pH 2.0. The alkaline media had a better effect on the growth rate. *P. kudriavzevii* fared best at pH 2.0 and 3.0 for up to 96 h. All yeasts maintained viability in 1, 2, and 3% bile solutions, although the growth rate did not improve significantly in any of the assay periods. Only minimal growth increase was registered in increased bile concentrations. All samples demonstrated sustained viability in 5–30% glucose between 24 and 48 h of incubation. After 48 h of incubation, the yeast concentrations began to fall as the glucose concentration rose from 5 to 30%. *P. kudriavzevii* was the least affected after 96 h (41.8%) and demonstrated the best survival results by the four criteria tested in this study.

If this species meets all other non-assayed parameters which qualify a microorganism as a probiotic, *P. kudriavzevii* obtained from Nigerian coconut juice can be recommended as a potential source of commercial probiotics.

Keywords. Probiotics, yeasts, *in vitro*, absorbance, coconut juice, raffia juice, viability

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Дрожжи из соков кокоса и рафии: пробиотическая оценка *in vitro*

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Аннотация.
Эукариотические пробиотики привлекают внимание ученых. Наиболее изученными видами дрожжей – источниками пробиотиков – являются *Saccharomyces cerevisiae* и *Saccharomyces boulardii*. Спектр видов дрожжей с пробиотическим потенциалом можно расширить за счет растений, использующихся в качестве сырья при производстве напитков.
Объект исследования – пробиотический потенциал дрожжей *Pichia kudriavzevii* и *Kluyveromyces marxianus*, выделенных из кокосового сока, а также *Schizosaccharomyces pombe* и *Wickherhamomyces anomalus*, выделенных из пальмового сока рафии (Нигерия). Методом оптической плотности *in vitro* определили толерантность к кислоте (pH 2, 3 и 5), щелочи (pH 7,5 и 8,0), желудочному соку (30 %), желчи (1, 2 и 3 %) и осмотическому давлению (раствор глюкозы 5, 10, 15, 20, 25 и 30 %).
Все четыре вида дрожжей выжили, продемонстрировав разную степень пробиотического потенциала. После 96 ч пребывания в искусственном желудочном соке *S. pombe* превзошли по численности *K. marxianus* и *W. anomalus* на 13 и 97,7 % ($p < 0{,}05$) соответственно. В 30 % растворе желудочного сока наименее жизнеспособными оказались дрожжи вида *W. anomalus*. После 96 ч в кислой среде все образцы оказались более жизнеспособными при pH 3,0, чем при pH 2,0 – приблизительно на 89 %. Щелочная среда оказала благоприятное воздействие на скорость роста. Дрожжи *P. kudriavzevii* продемонстрировали лучшие показатели выживаемости при pH 2,0 и 3,0 в течение 96 ч. Все дрожжи сохраняли жизнеспособность в 1, 2 и 3 % растворах желчи, хотя скорость роста существенно не увеличилась. Повышение концентрации желчи вызвало минимальное увеличение роста. После 24–48 ч инкубации в 5–30 % растворах глюкозы все образцы продемонстрировали устойчивую жизнеспособность. После 48 ч инкубации концентрация дрожжей начала падать, а концентрация глюкозы выросла с 5 до 30 %. Вид *P. kudriavzevii* оказался наиболее жизнеспособным через 96 ч (41,8 %) по всем четырем критериям.
Если дальнейшие исследования подтвердят, что этот вид соответствует остальным, не охваченным в рамках данной работы параметрам, которые позволяют квалифицировать микроорганизм как пробиотик, то дрожжи *P. kudriavzevii*, полученные из сока нигерийского кокоса, могут быть рекомендованы в качестве потенциального источника коммерческих пробиотиков.

Ключевые слова. Пробиотики, дрожжи, *in vitro*, абсорбция, кокосовый сок, сок рафии, жизнеспособность

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Introduction

Probiotics have a long history, dating back over 10 000 years. Fermented foods, such as yogurt, are high in probiotics and are widely consumed worldwide. These days, probiotics are part of various healthy diets, and supplements with probiotic microbes have long established themselves in commercial production [1, 2].

Criteria for *in vitro* assessment of potential probiotics. In 2002, the Food and Agriculture Organization of the United Nations published guidelines for evaluating probiotics in a variety of foods. Currently, the criteria for evaluating probiotic candidates include: low pH tolerance, bile salt tolerance, osmotolerance, phenotypic and genotypic stability, carbohydrate tolerance, etc.

In addition, probiotics possess antimicrobial activity against *Listeria monocytogenes*, *Shigella flexneri*, *Staphylococcus aureus*, *Salmonella enteritidis*, enteropathogenic *Escherichia coli*, *Escherichia coli* O157 H7, and *Bacillus cereus* [3, 4].

Although the exact mechanism through which probiotics exert their positive benefits remains uncertain, a range of beneficial outcomes derived from probiotics is well documented [5].

Sources of probiotics. Previously, most probiotics ingested by humans came from fermented foods, e.g., dairy products. Eventually, the human body itself became the predominant source, with faces and breast milk serving as the primary providers. Probiotics isolated from human breast milk are mainly of the *Lactobacillus* genus, while those isolated from feces of healthy human adults and breastfed infants belong to *Lactobacilli* and *Bifidobacterium* [6].

Fermented foods with potential probiotics can be of plant or animal origin. All other probiotic organisms are bacterial species, except for *Saccharomyces cerevisiae* (bakery and brewing), *Saccharomyces bayanus* (winemaking), and *Saccharomyces boulardii*. These yeasts have been isolated from a variety of sources, including soy paste, and used as a probiotic in medicine [7, 8].

In Middle Eastern countries, fermented foods are abundant sources of lactic acid bacteria. The list includes parboiled dried wheat, garlic, parsley and olives among many others. Non-germinated cereals, such as sorghum and millet grains, are known for their functional properties [9]. Traditional non-dairy fermented beverages are also high in probiotics. They are made from millets, legumes, fruits, and vegetables [10, 11]. Probiotic features of lactic acid bacteria include resistance to pH 3 and 3% bile, as well as antibacterial activity against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. Such new developments as paraprobiotics and postbiotics go beyond the current trend of consuming live bacteria in food or as supplements: they imply that bacterial viability alone may not be required for health benefits. This discovery presents a potential opportunity for functional food producers [12].

Coconut and raffia palm juice are widely consumed around the world. In tropical countries such as Nigeria, they are natural refreshing beverages used to quench thirst. In addition to minerals, these fruit drinks contain several local microorganisms. For instance, coconut juice contains *Pichia kudriavzevii* and *Kluyveromyces marxianus* while raffia palm juice contains *Schizosaccharomyces pombe* and *Wickherhamomyces anomalus*. *Raphia rinfera*, *Raphia hookeri*, and *Elaeis guineensis* are the most common sources of palm wine in Nigeria. Fresh palm wine is widely regarded as a healthy beverage that aids lactation, heals conjunctivitis, and even improves vision [13, 14]. This delicious drink is popular in southeastern Nigeria, as well as in many tropical countries all over the world, including Asia and South America.

This study featured four yeasts. *P. kudriavzevii* and *K. marxianus* were isolated from coconut juice; *S. pombe* and *W. anomalus* came from raffia palm juice. They were tested for potential use as probiotics.

Study objects and methods

Yeast strains. *Pichia kudriavzevii* and *Kluyveromyces marxianus* (coconut juice) and *Schizosaccharomyces pombe* and *Wickherhamomyces anomalus* (raffia palm juice) were obtained from the Microbiology Department of Landmark University, Omu-Aran, Nigeria. They were subjected to four *in vitro* tests: survival in low and alkaline pH, survival in 30% simulated gastric juice, survival in 1, 2, and 3% bile, and survival in 5, 10, 15, 20, 25, and 30% sugar (glucose) to check osmotolerance.

Culture conditions. This study employed yeast extract peptone dextrose broth and agar as culture media. After purification, the yeasts were counted to obtain 10^9 CFU/mL in sterile phosphate buffered saline, as proposed by Moradi *et al.* [15]. The tests took place within 60 min after the count.

Gastric juice tolerance test. To test the capacity of the yeasts to survive in simulated gastric juice, we modified the procedure described by Lohith & Anu Appaiah and Ragavan & Das [16, 17]. In brief, the simulated gastric juice was prepared by dispensing 10 mL of phosphate buffered saline (0.9% w/v) into sterile universal tubes (n = 4) and adjusting pH to 2.0 with HCl. After that, we added 0.03 g pepsin into the solution to achieve a concentration of 3 mg/mL. Subsequently, we put 20 μL of overnight cultures (~ 10^9 CFU/mL) of each yeast into the simulated gastric juice to inoculate and incubate them at 37°C for 90 min. Following the incubation, 10 μL simulated gastric juice with yeast cultures was added to 10 mL yeast extract peptone dextrose broth. Each test was performed in triplicates. The optical density (absorbance) values made it possible to determine the viability of the yeasts spectrophotometrically. The test involved the use of a UV/VIS Spectrophotometer, Model AE S80-2S (A&E Lab, UK). After 0, 24, 48, 72, and 96 h of incubation, we measured the absorbance at 660 nm. All the tests were carried out in triplicates, and each value was a mean calculated from all three.

Survival in acid and alkaline environments. The study used the methods developed by Lohith & Anu Appaiah and Ragavan & Das with minimal modifications [16, 17]. The pH of the yeast extract peptone dextrose broth was adjusted with 1N HCL to 2.0, 3.0, and 5.0 for acidic conditions. For alkaline conditions, the adjustment was carried out using 1N NaOH to bring pH up to 7.5 and 8.0. The samples of pH-adjusted yeast extract peptone dextrose broth (9.9 mL) were dispensed into clean universal bottles. After that, we inoculated 0.1 mL (~ 10^9 CFU/mL) yeasts purified in phosphate buffered saline into 9.9 mL of pH-adjusted broths. The obtained mixes were swirled to homogenate. The absorbance of each inoculated broth at 660 nm was measured before incubation and repeated the procedure every 24 h for a total of 96 h. All the tests were carried out in triplicates, and the growth and survival of the yeasts were measured from the mean absorbance values recorded for each yeast organism.

Bile tolerance test. We prepared 1, 2, and 3% bile in the yeast extract peptone dextrose broth. Then, we dispensed 0.2 mL (~ 10^9 CFU/mL) of overnight culture in phosphate buffered saline into the broth and mixed. The absorbance of the broth cultures was measured at 660 nm before the incubation and 24, 48, 72, and 96 h after the incubation. All the tests were carried out in triplicates, and each value was a mean calculated from all three.

Osmotolerance test. Glucose concentrations of 5, 10, 15, 20, 25, and 30% (w/v) were prepared in the yeast extract peptone dextrose broth. From each of these stock solutions, we dispensed 19.8 mL into sterile universal tubes, to which was added 0.2 mL (~ 10^9 CFU/mL) of each yeast (in phosphate buffered saline) for subsequent testing. The test included three replicates per yeast. Before incubation, the absorbance of the broth cultures was read at 660 nm. During the incubation, measurement of the absorbance was repeated every 24 h for a total of 96 h and the mean values calculated.

Results and discussion

Gastric juice tolerance test. Three of the four yeasts showed remarkable ability to thrive in the simulated 30% gastric juice at pH 2.0 and 37°C for 96 h. *Schizosaccharomyces pombe* demonstrated the best results with absorbance value of 2.704 at 96 h. Its concentration exceeded that of *Kluyveromyces marxianus* by 13% and that of *Wickerhamomyces anomalus* by 97.7% ($p < 0.05$). *W. anomalus* appeared to be the least viable yeast in the 30% gastric juice environment. The samples showed no significant difference ($p > 0.05$) in viability within the first 24 h. *Pichia kudriavzevii*, with an absorbance value of 1.984 at 96 h, showed no significant difference ($p > 0.05$) from *S. pombe* (2.704) and *K. marxianus* (2.352) after 96 h. However, its difference from *W. anomalus* for the same period was significant ($p < 0.05$) (Fig. 1).

Acid and alkaline tolerance test. All four yeasts exhibited acidic tolerance, with *P. kudriavzevii* showing evidence of remarkable survival at pH 2.0 and 3.0 for up to 96 h when compared to *S. pombe*, *W. anomalus*, and *K. marxianus*. Notwithstanding, all the yeasts fared better at pH 3.0 than at pH 2.0 with an approximately 89% (1.695/0.185 mean OD values) higher growth in pH 3.0 and 2.0, respectively, after 96 h (Figs. 2a and b). Similarly, all four yeasts grew better at pH 5.0 than at pH 3.0 and 2.0. For pH 5.0, we recorded a mean increase of 14.05% for all four yeasts after 96 h. *K. marxianus* and *P. kudriavzevii* showed better survival results (Fig. 2c). At 96 h, *S. pombe* and *W. anomalus* survived best in the alkaline medium, with no discernible difference between growth and survival results in acidic pH 5.0 and alkaline pH 7.5 and 8.0. (Figs. 2c, d, e, and f).

Bile tolerance test. *S. pombe* maintained stable concentration in all three bile solutions for 96 h while other yeasts showed variable concentrations after 48 h (Fig. 3).

Each of the assay periods demonstrated minimal but not appreciable differences in yeast concentrations between 1 and 2% bile and between 2 and 3% bile.

P. kudriavzevii was consistently the most viable species in 1, 2, and 3% bile medium, especially at 48 h.

Osmotolerance test. All four yeasts demonstrated signs of survival (5–30%) in the glucose solutions (Figs. 4a–f). *P. kudriavzevii*, *K. marxianus*, and *W. anomalus* reduced in concentration as the glucose concentration increased from 5 to 30%. However, their concentrations increased as the incubation time proceeded from 0 to 96 h.

S. pombe followed the same pattern as the other three yeasts but had a slightly lower concentration as the incubation time increased from 0 to 96 h.

Overall, as the concentration of glucose increased from 5 to 30% after 48 h of incubation, we detected a variable degree of reduction in yeast concentrations, with *P. kudriavzevii* being the least affected (Fig. 4g).

Such yeast strains as *Saccharomyces boulardi* are popular in healthcare and food industry for their well documented therapeutic properties, e.g., alleviation of digestive issues. *K. marxianus* is another highly researched probiotic yeast with a set of established methods of screening and assessing probiotic potential [15].

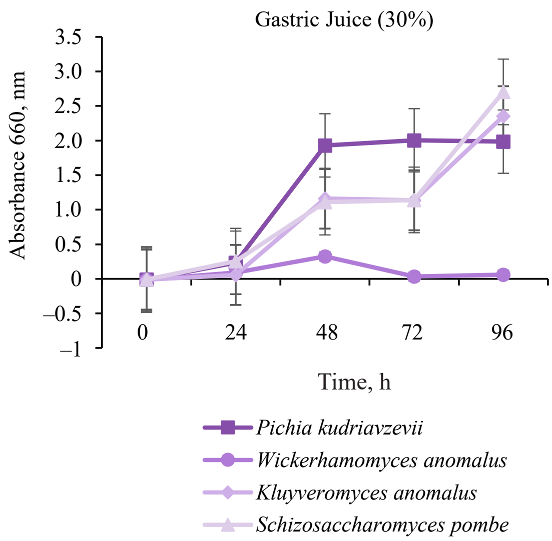

Figure 1. Viability of *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii* in 30% simulated gastric juice

Рисунок 1. Жизнеспособность дрожжей *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* и *Pichia kudriavzevii* в 30 % искусственном желудочном соке

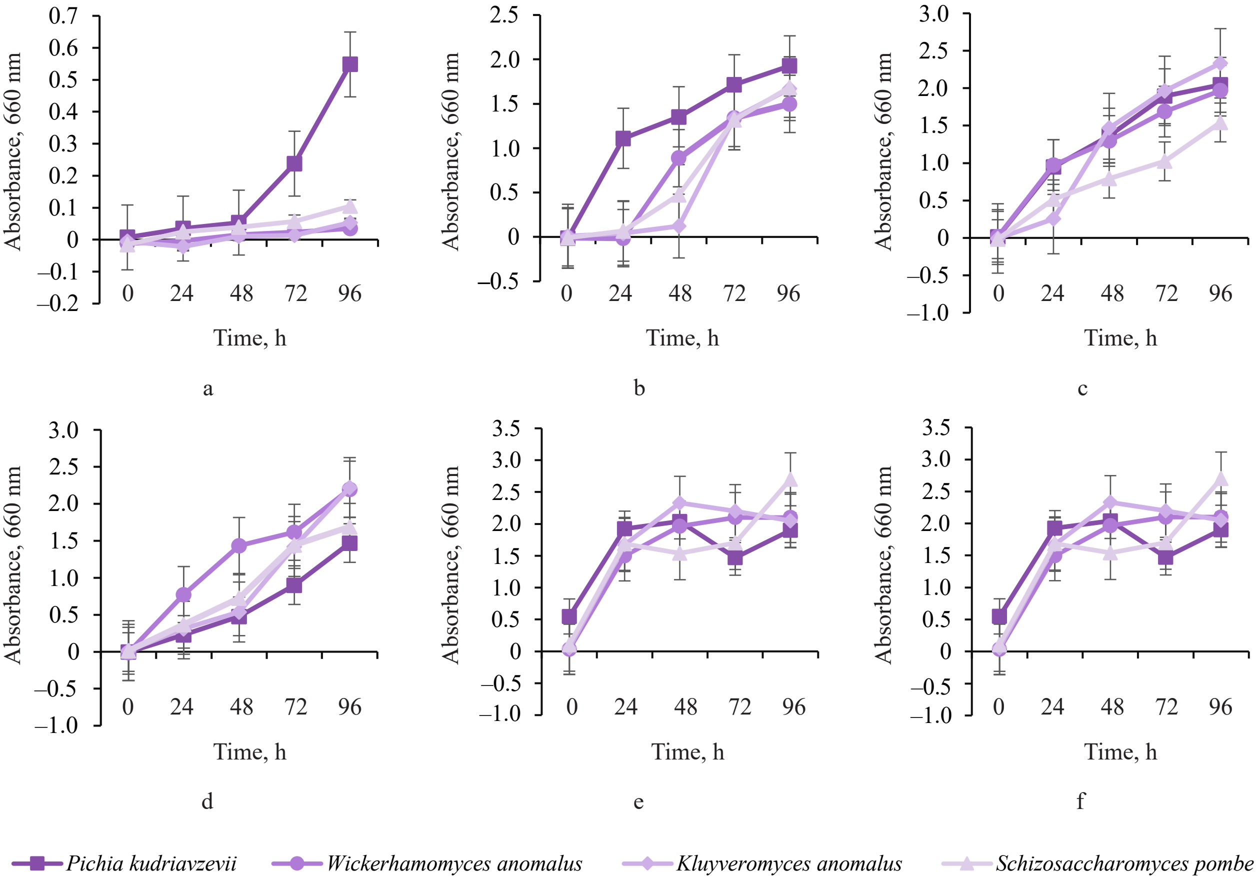

Figure 2. Viability of *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii* at pH 2.0 (a), 3.0 (b), 5.0 (c), 7.5 (d), and 8.0 (e); after 96 h of incubation (f)

Рисунок 2. Жизнеспособность дрожжей *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* и *Pichia kudriavzevii* при pH = 2,0 (a), 3,0 (b), 5,0 (c), 7,5 (d) и 8,0 (e) и после 96 часов инкубации (f)

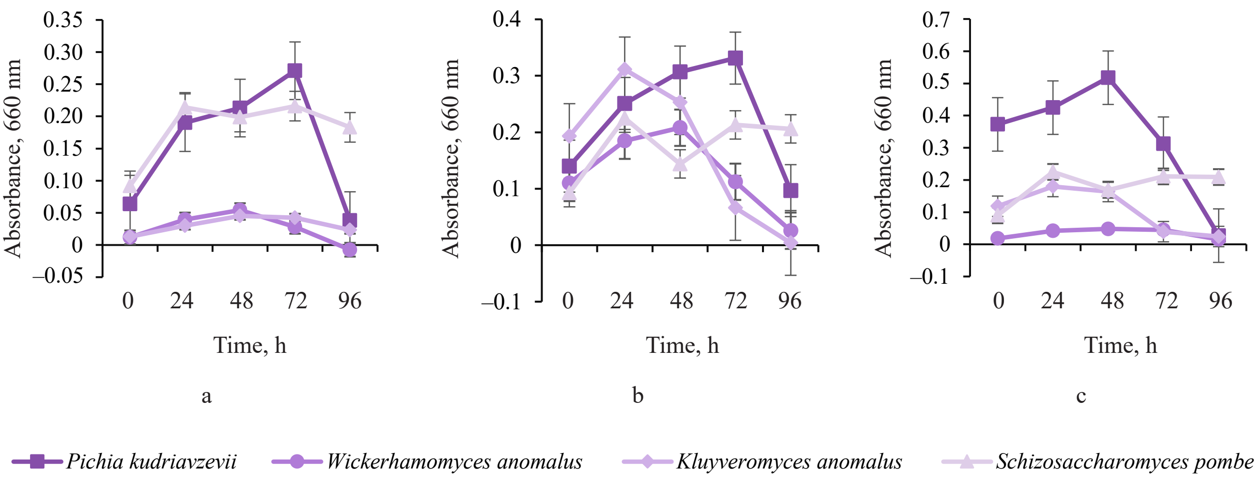

Figure 3. Viability of *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii* in 1 (a), 2 (b), and 3% (c) bile solution

Рисунок 3. Жизнеспособность *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* и *Pichia kudriavzevii* в 1 (a), 2 (b) и 3% (c) растворе искусственной желчи

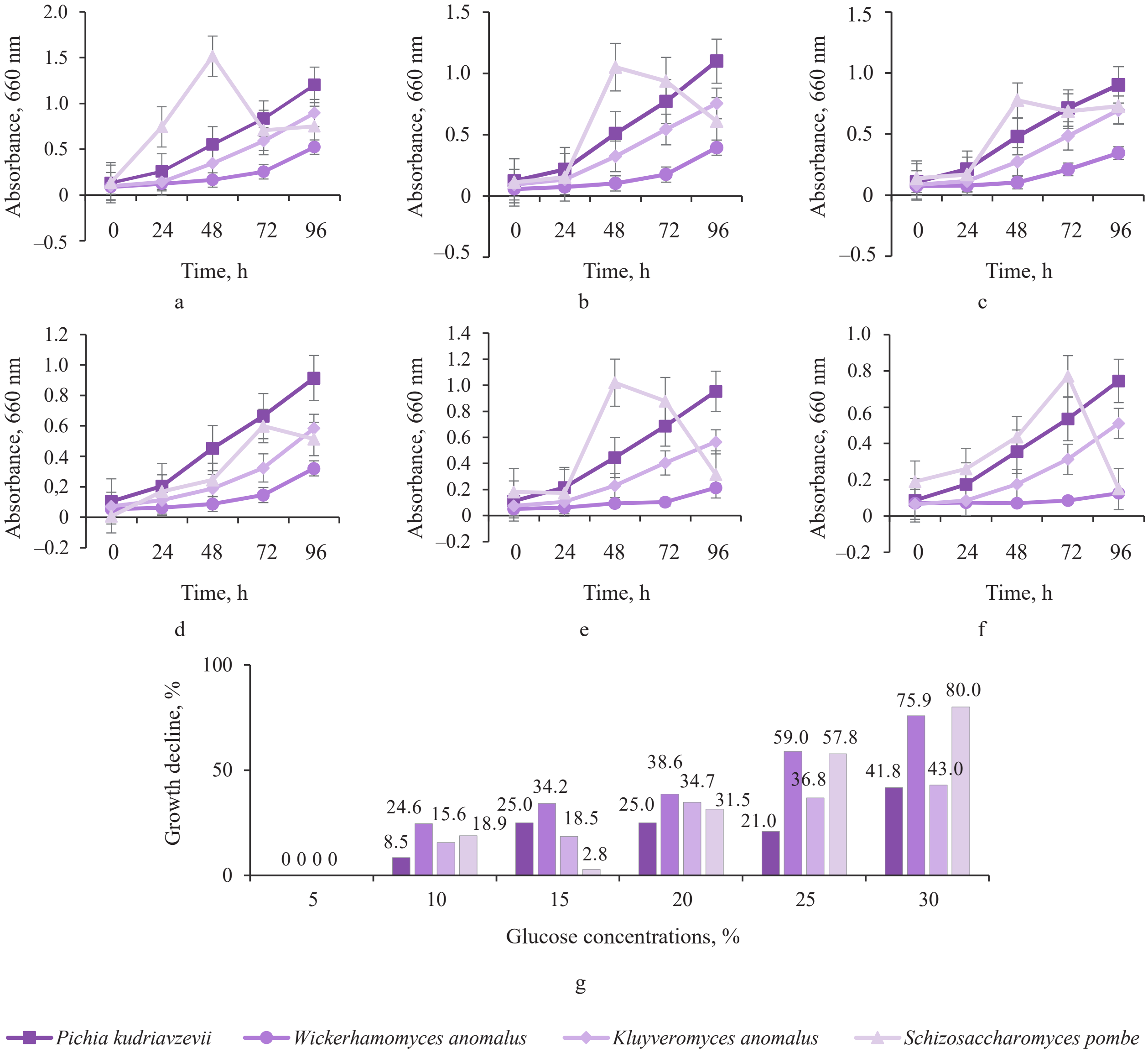

Figure 4. Viability of *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii* in increasing glucose concentration 5% (a), 10% (b), 15% (c), 20% (d), 25% (e), 30% (f); decline in viability yeast (g), 96 h after incubation

Рисунок 4. Жизнеспособность дрожжей *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* и *Pichia kudriavzevii* при повышении концентрации глюкозы 5 % (a), 10 % (b), 15 % (c), 20 % (d), 25 % (e), 30 % (f); снижение жизнеспособности дрожжей (g), 96 ч после инкубации

Because low pH is one of the most basic criteria, most *in vitro* studies recommend selecting probiotic yeast strains that can grow at extremely low pH. All the yeasts tested in this work showed resilience to low pH of 2, 3, and 5 for up to 96 h. *P. kudriavzevii* performed remarkably well: its ability to thrive in low pH exceeded that of the other three yeasts. In addition, its pH corresponded to the pH range of human stomach, which is 1.5–3.5. This fact qualified *P. kudriavzevii* as a protic candidate, provided the strain meets other, untested criteria. The pH of human intestine ranges between 6 and 7, and all the yeasts in this study were able to thrive in a comparable environment, which also indicates their probiotic potential.

In a previous study, Moradi *et al.* compared *Saccharomyces cerevisiae* with *K. marxianus* and different strains of *P. kudriavzevii* [15]. They reported that the other yeasts thrived better in acid environments than *S. cerevisiae*. Our findings imply that *P. kudriavzevii* and *K. marxianus* from coconut juice, as well as *S. pombe* and *W. anomalus* from raffia juice, may have probiotic properties.

If these yeasts meet all other probiotic criteria that were not investigated in this study, their ability to thrive in low pH environments, gastric juice, bile, and 5–30%

glucose qualifies them as prospective probiotic strains. The capacity of bacteria to thrive in the stomach environment with its hydrochloric acid depends on their survival in gastric juice. This requirement makes it critical to test bacteria and yeasts *in vitro* for their ability to survive in gastric juice as part of the probiotic approval assessment. In this study, three out of four yeasts, namely *P. kudriavzevii*, *K. marxianus*, and *S. pombe*, demonstrated substantial survival ability in simulated gastric juice. Earlier, Fadda *et al.* identified six *Kluyveromyces* strains from a variety of cheese and proved that all of them thrived in simulated gastric conditions at pH 3.0 [18]. In addition, Moradi *et al.* reported the survival of five strains that were very marginally affected by gastric juice exposure, with *K. marxianus* showing the most resistance [15]. In this respect, our findings are consistent with those mentioned above.

The small intestine and colon contain relatively large quantities of bile salts, which are poisonous to living cells. As a result, the ability of bacteria and yeasts to tolerate bile is now an important criterion for probiotic organisms [19]. In the human digestive environment, the optimal bile content ranges from 0.30 to 0.60%.

In this research, all four yeasts isolated from plant sources were able to grow in simulated bile salt concentrations of 1, 2, and 3%, all of which exceeded the optimal concentration in human intestine. Their ability to pass the bile tolerance test suggests that they could be effective as probiotics.

Yeast can use a wide range of carbohydrates, including glucose, to fuel its growth. *S. pombe*, *W. anomalus*, *K. marxianus*, and *P. kudriavzevii* survived well in all glucose concentrations (5–30%). Other studies identified *P. kudriavzevii* as an osmotolerant yeast species to be used in bioethanol production [20]. In our research, *P. kudriavzevii* and *K. marxianus* remained the most stable yeasts in the varied glucose concentrations, which makes them excellent candidates for probiotics.

Conclusion

Bacterial species, such as *Lactobacillus* and *Bifidobacterium*, are universally accepted probiotic organisms. Currently, the only probiotic yeast in use is *Saccharomyces boulardii*. However, yeasts with therapeutic benefits can be found in a variety of fruits and dairy products that people consume on a regular basis in fermented drinks and yogurts.

As observed in this study, *Pichia kudriavzevii* isolated from coconut juice survived in both acidic and alkaline environments, concentrated gastric juice, 30% pepsin, 1–3% bile, and 5–30% glucose medium. Its survival properties exceeded those demonstrated by *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, and *Kluyveromyces marxianus*. *P. kudriavzevii* showed acid and osmotolerance survival which corresponds with some earlier reports of its usefulness as an ethanologenic yeast strain [21]. However, the other three yeasts also exhibited reasonable probiotic potential, particularly *S. pombe*, which thrived in the bile medium.

Probiotics' microbial viability and metabolic activity must be maintained throughout the production process, i.e., fermentation, which demands further *in vitro* and *in vivo* studies [22]. In this study, *S. pombe*, *W. anomalus*, *K. marxianus*, and *P. kudriavzevii* all proved viable in each of the four conditions studied. Presumably, other plants used in national cuisines can offer new sources of eukaryotic probiotic organisms with potential commercial use as part of functional foods.

Contribution

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Критерии авторства

Авторы в равной степени участвовали в написании рукописи и несут равную ответственность за плагиат.

Конфликт интересов

Авторы заявляют об отсутствии потенциальных конфликтов интересов в отношении исследования, авторства и/или публикации данной статьи.

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