International Journal of Agriculture and Biosciences 2025 xx(x): xx-xx.
https://doi.org/10.47278/journal.ijab/2025.068

RESEARCH ARTICLE

eISSN: 2306-3599; pISSN: 2305-6622

In Vitro Assessment of the Probiotic Potential of *Kluyveromyces marxianus* (Strain CLARA-E): Stress Tolerance and Metabolic Activity for Animal Feed Applications

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ABSTRACT

Probiotics, live microorganisms conferring health benefits when administered in adequate amounts, are increasingly explored as feed supplements to enhance intestinal health, productivity, and animal welfare. This study aimed to evaluate the probiotic potential of *Kluyveromyces marxianus* as a feed additive through *in vitro* assessments. The strain, preserved in YPD medium and molecularly identified via ITS gene sequencing using the BLAST algorithm, was confirmed as *Kluyveromyces marxianus* (strain CLARA-E). The yeast's resilience to environmental stressors, including temperature, pH, bile salts, and high sodium chloride concentrations, was tested, alongside its glucose fermentation capacity. Results indicated optimal survival at pH 5.6, 0.1% (w/v) bile salts, and 43°C, with notable tolerance to elevated NaCl levels. Glucose fermentation was confirmed by gas production in Durham tubes. These findings suggest that *K. marxianus* CLARA-E exhibits promising probiotic properties, such as stress tolerance and metabolic activity, positioning it as a potential feed additive to modulate intestinal microbiota and enhance immune responses. However, further in vivo studies are necessary to validate its efficacy and beneficial effects in animal models. This research underscores the potential of native microbial strains in developing sustainable alternatives for animal nutrition and health.

Keywords: Gut microbiota; Bile salt tolerance; Yeast fermentation; Microbial biotechnology; Animal health enhancement.

Article History
Article # 25-122
Received: 14-Mar-25
Revised: 15-Apr-25
Accepted: 17-Apr-25
Online First: 09-May-25

INTRODUCTION

Probiotics are defined as live microorganisms that, when administered in appropriate concentrations, confer health benefits to the host (Konieczka et al., 2023). In animal nutrition, probiotics play a crucial role in enhancing gut health, improving feed efficiency, and promoting overall animal well-being. They achieve these benefits through mechanisms such as competitive exclusion of pathogens, reinforcement of the intestinal epithelial barrier, modulation of immune responses, and production of bioactive compounds, including vitamins and antioxidants (Latif et al., 2023; Yarullina et al., 2024). As a result, probiotics have been widely studied as alternatives to antibiotics in livestock production, particularly in the context of growing concerns about antimicrobial resistance and sustainable animal husbandry practices (Thakur et al., 2016; Lee et al., 2022).

Among the microorganisms used as probiotics, bacteria—such as *Lactobacillus*, *Bifidobacterium*, and *Bacillus*—have been extensively investigated, while probiotic yeasts, including *Saccharomyces cerevisiae* and *K. marxianus*, have gained increasing attention due to their unique properties (Baralić et al., 2023; Coniglio, et al., 2023; Tullio, 2024). Yeasts offer several advantages over bacterial probiotics, including their ability to tolerate harsh gastrointestinal conditions, produce a diverse enzyme profile, detoxify mycotoxins, and resist antibiotic - induced disruptions of the gut microbiota (López Barreto et al., 2021; Alvarez et al., 2024). Additionally, yeasts serve as

Cite this Article as: Anaya MCV, Hurtado CAB, Rhenals DEL, Zumaqué LEO, Ruiz AJB and Sotelo DEP, 2025. *In Vitro* assessment of the probiotic potential of *Kluyveromyces marxianus* (Strain CLARA-E): stress tolerance and metabolic activity for animal feed applications. International Journal of Agriculture and Biosciences xx(x): xx-xx. https://doi.org/10.47278/journal.ijab/2025.068

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valuable protein sources in animal feed, contributing to improved digestive efficiency and enhanced animal performance (Lu et al., 2024; Reina-Posso & Gonzales, 2025).

K. marxianus is a thermotolerant yeast with rapid growth kinetics, a broad substrate utilization spectrum, and a well-established record of safety, being classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration and included in the Qualified Presumption of Safety (QPS) list by the European Food Safety Authority (Varela et al., 2017; Timmermans et al., 2023). Recent studies suggest that *K. marxianus* possesses probiotic potential, demonstrating antimicrobial activity against enteric pathogens, immunomodulatory effects, and the ability to enhance intestinal integrity in animal models (Marcišauskas et al., 2019; Cerutti Martellet et al., 2023). Furthermore, its fermentative capacity and production of short-chain fatty acids and bioactive peptides make it a promising candidate for improving rumen function and overall livestock productivity (Fadda et al., 2017). Despite these promising findings, the probiotic application of *K. marxianus* in animal nutrition remains underexplored, and key aspects of its physiological adaptability, including its tolerance to stressors such as temperature fluctuations, acidic environments, bile salts, and high NaCl concentrations, require further investigation (Liu et al., 2021).

Given the growing interest in alternative probiotics and the need for robust candidates capable of thriving in the gastrointestinal environment, this study aimed to evaluate the probiotic potential of *K. marxianus* for animal nutrition. Specifically, we assessed its tolerance to physiological stressors and its fermentative performance, key determinants of its viability as a feed additive. Our findings provide new insights into the resilience and functional properties of *K. marxianus*, contributing to the development of novel probiotic strategies for sustainable and efficient animal production.

MATERIALS & METHODS

Ethical Approval

All experiments involving the use of animals were approved by the Central Bioethics Committee of the University of Córdoba (Colombia), during the session of the Technical Knowledge Board for Veterinary Medicine and Animal Science, held on March 21, 2024, as recorded in Minute No. 002.

Study Area

Rumen content samples were collected at the Turipaná Research Center of AGROSAVIA, located at 8°50'79" N and 75°47'58" W in the municipality of Cereté, department of Córdoba, Colombia (Doria-Ramos et al., 2020). For this study, three Romo Sinuano breed cattle with rumen fistulas were selected. These animals were fed forage and corn silage, which are typical dietary components in Córdoba, Colombia. To perform the respective isolations, the samples were stored in a portable plastic cooler at 5°C and transported to the Biotechnology Laboratory (GRUBIODEQ) at the Department of Chemistry and the Department of Biology of the Universidad de Córdoba (8°47′037″ N; 75°50′51″ W, 15 masl) in Montería, Colombia (Fig. 1) (Pompelli et al., 2019).

Isolation and Preservation of the Strain

Rumen content samples were serially diluted using a saline solution (0.85% (w/v) NaCl). The resulting dilutions were plated on peptone dextrose agar (YPD), prepared with 20g of peptone, 10g of yeast extract, and 20g of glucose per liter of distilled water. The plates were incubated at 30°C for 24 to 48hours (Ullah et al., 2023). The isolated yeast strain was preserved in the GRUBIODEQ laboratory strain bank in YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone, 2% glucose, and 30% (w/v) glycerol) at 5°C until further use.

Yeast Growth under Stress Conditions

For all experiments, an initial culture of yeast grown in YPD medium for 24 hours at 30°C was used as the inoculum, adjusted to a final concentration of $1x10^8$CFU mL^{-1} (López Barreto et al., 2021). Growth was monitored by counting viable cells using the serial dilution technique. This method involved progressively diluting an aliquot of the initial culture in saline solution (0.85% (w/v) NaCl), preparing dilutions from 10^{-1} to 10^{-8}. Then, 100µL of each dilution was plated on YPD agar and incubated at 30°C for 24hours (Dong et al., 2025). Colony-forming units (CFU) were counted on plates with 30 to 300 colonies, ensuring the accuracy of the count. *In vitro* tests were performed in triplicate and are detailed below.

Growth at Different pH Levels

YPD liquid culture medium was prepared, and the pH was adjusted to 3.0, 4.0, 5.6, and 7.0 using a 5% hydrochloric acid (HCl) solution, followed by sterilization at 15PSI and 121°C for 15min. The cultures were inoculated and incubated at 30°C for 24hours (Wang et al., 2024). Growth was assessed visually by turbidity observation after shaking the tubes. Viable cell counts were performed as described in section 2.3. The survival percentage was calculated using Equation 1 (Cueto-Vigil et al., 2012):

% survival=(Log CFU treatment)/(Log CFU inoculum) x100 Ec.1

Growth at Different Bile Salt Concentrations

YPD broth was supplemented with bile salts (Sigma-Aldrich) at concentrations of 0.05, 0.1, 0.15, and 0.3% (w/v), followed by sterilization at 15PSI and 121°C for 15minutes. Cultures were inoculated and incubated at 30°C for 24hours (Wang et al., 2024). Growth was evaluated visually by the presence or absence of turbidity, and viable cell counts were performed as described in section 2.3. The survival percentage was calculated using Equation 1.

Growth at Different Temperatures

Cultures were inoculated into 15mL of YPD broth and incubated at 30, 37, and 43°C for 24hours (Wang et al., 2024). Growth was observed visually by turbidity after shaking the tubes. Viable cell counts were performed to determine survival rates, which were compared to the initial inoculum, and survival percentage was calculated using Equation 1.

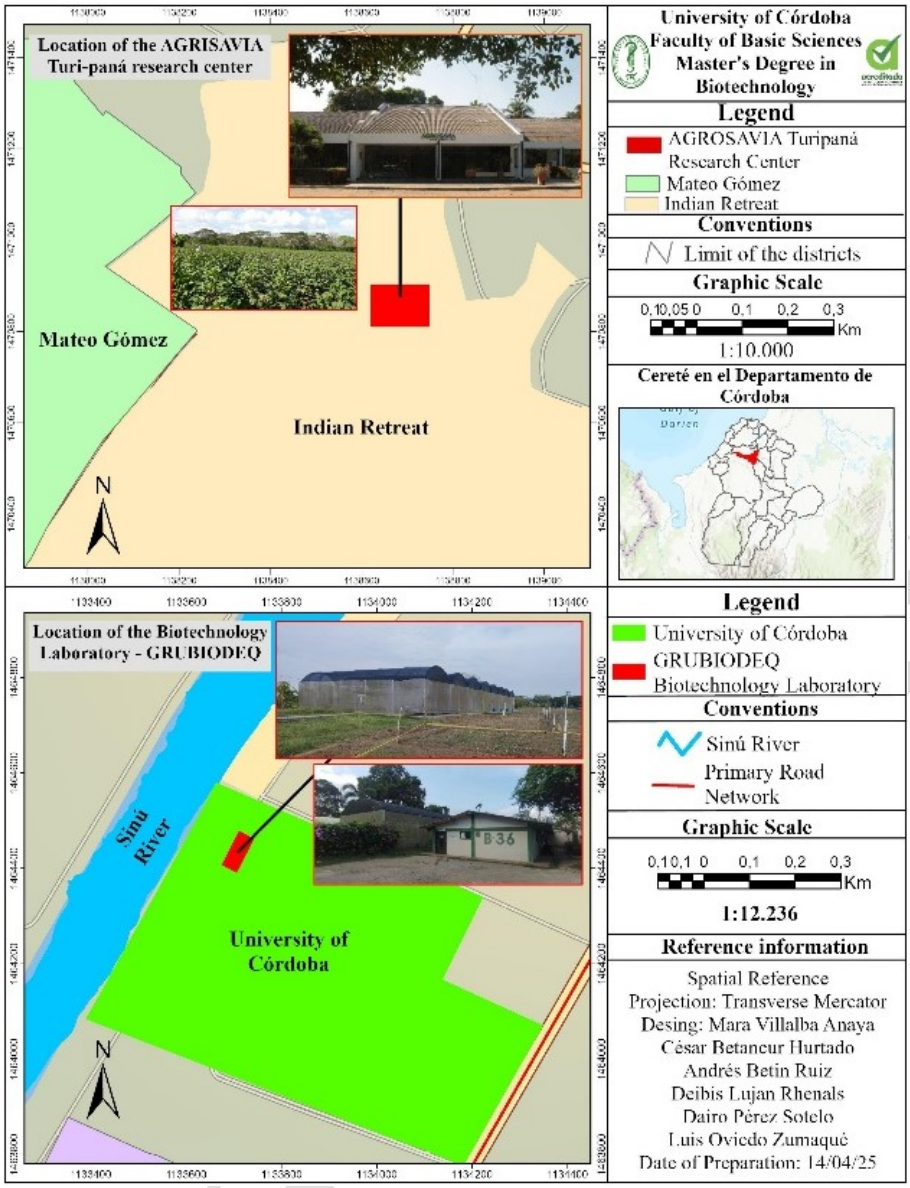

Fig. 1: Area of study

Growth at Different Sodium Chloride Concentrations

YPD broth was supplemented with NaCl at concentrations of 2.0, 4.0, 7.0, and 10% (w/v) and sterilized at 15PSI and 121°C for 15minutes (Anuarbekova et al., 2024). Cultures were inoculated and incubated at 30°C for 24hours. Growth was assessed visually, and viable cell counts were performed to determine survival percentages using Equation 1.

Glucose Fermentation

YPD broth containing 0.2% (v/v) bromocresol purple solution (0.5% v/v) was inoculated into Durham tubes and incubated at 37°C for 48hours. Glucose fermentation was indicated by the presence of gas in the inverted Durham tubes.

Molecular Identification

DNA Extraction

Yeast was cultured on YPD agar and incubated at 30°C for 24hours. Biomass was collected directly from the plates, frozen, and ground with liquid nitrogen. Genomic DNA was extracted using the GeneJET™ kit (Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions.

Amplification of the ITS Region

PCR (polymerase chain reaction) was used to amplify the ITS region using universal primers ITS4 (5'-TCCTCCGCTTATTGATATATGC-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') from Sigma-Aldrich (Sevgili et al., 2023). The reaction mixture (50µL) contained 25µL of DreamTaq Hot Start PCR Master Mix, 1µL of each primer (10mM), 2µL of DNA (~157ng μL^{-1}), and molecular-grade water. PCR conditions included initial denaturation (5min at 95°C), 30cycles of denaturation (30s at 95°C), annealing (40s at 60°C), extension (1min at 72°C), and a final extension (10min at 72°C). Amplicons were verified by 1% agarose gel electrophoresis and visualized under UV light (Enduro GDS Labnet, Tewksbury, MA, USA). The purification and sequencing of the fragment was carried

out through the Sequencing and Molecular Analysis Service (SSiGMol), of the Institute of Genetics of the National University of Colombia.

Phylogenetic Analysis

Sequences were analyzed using 4Peaks v1.8 and compared with GenBank sequences via BLAST. Multiple sequence alignment was performed using ClustalW in MEGA11 (Tamura et al., 2021), and a phylogenetic tree was generated using the maximum likelihood method with 1000 bootstrap replicates.

Yeast Growth in a Mango-Based Medium
Growth Assessment

Mango-based liquid media were prepared at different pulp concentrations (5, 10, 15, 20, and 25% w/v), supplemented with 1% yeast extract and 1.5% peptone. Cultures were incubated in Erlenmeyer flasks at 2.2VVM aeration and 150rpm, using a 0.2µm filter for contamination control (Lara et al., 2010). Growth was assessed macroscopically, microscopically, and through viable cell counts at 24, 48, and 72hours.

Chemical Analysis of the Mango (*Mangifera indica*) Pulp Extract in the Medium

The chemical composition of the culture medium containing 10% (w/v) mango pulp, supplemented with 1% yeast extract and 1.5% peptone, was analyzed. Total sugar content was determined using the phenol-sulfuric acid method described by Dubois et al. (1956) (DuBois et al., 1956), while reducing sugars were quantified following Miller's method (Miller, 1959). Protein content was assessed via the Kjeldahl method (Di Marzo et al., 2021), and ash content was measured according to the AOAC standard method 942.05 (Helrich, 1990). Mineral composition was analyzed as follows: sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca) were determined according to ICONTEC standard NTC 5349-2016; copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe) following NTC 5526-2024; sulfur (S) according to NTC 5402-2006; and phosphorus (P) in accordance with NTC 5350-2020. Additionally, moisture content and pH were measured following AOAC method 930.15/90. All analyses were conducted at the Soil and Animal Nutrition Laboratory of the Universidad de Córdoba.

Experimental Design and Statistical Analysis

A completely randomized design (CRD) was used with a 5x3 factorial arrangement and three replicates per condition. Data were analyzed via ANOVA using SPSS v20, and Tukey's test was applied ($P<0.05$). Graphs were created using SigmaPlot v12.0.

RESULTS

Yeast Growth under Stress Conditions

Fig. 1 illustrates the percentage survival of *K. marxianus* under various stress conditions, demonstrating its adaptability and resilience. In acidic pH conditions (Fig. 2A), *K. marxianus* exhibited robust growth, with the highest survival rate observed at pH 5.6 (94.52±0.71%), followed by pH 4.0 (94.36±0.58%), pH 3.0 (91.51±0.68%), and pH 7.0 (90.95±0.95%). Statistical analysis revealed significant differences ($P<0.05$) between pH 7.0 and pH 4.0/5.6, while pH 3.0 showed no significant differences compared to other pH levels, indicating the yeast's broad pH tolerance.

In bile salt tolerance assays (Fig. 2B), *K. marxianus* demonstrated significant growth variations depending on bile salt concentration. Optimal growth was observed at low concentrations (0.05% and 0.1%), with survival rates of 76.29±0.56% and 74.94±0.40% at 0.15% and 0.3%, respectively. No significant differences were detected between 0.15% and 0.3%, suggesting the yeast's ability to adapt to higher bile salt concentrations.

Temperature tolerance tests (Fig. 2C) revealed that *K. marxianus* thrived across a range of temperatures, with the highest survival rate at 43°C (97.45±0.59%), followed by 37°C (95.7±0.29%) and 30°C (94.63±0.38%). Significant differences ($P<0.05$) were observed between 30°C and 43°C, while 37°C showed no significant differences compared to other temperatures, highlighting the yeast's thermotolerance.

In sodium chloride tolerance assays (Fig. 2D), *K. marxianus* exhibited optimal growth at 2% NaCl (92.46±0.40%), with survival decreasing to 42.59±0.32% at 10% NaCl. Significant differences ($P<0.05$) were observed between 2% and higher NaCl concentrations, although no significant differences were detected between 4% and 7%, indicating the yeast's ability to adapt to elevated salt concentrations.

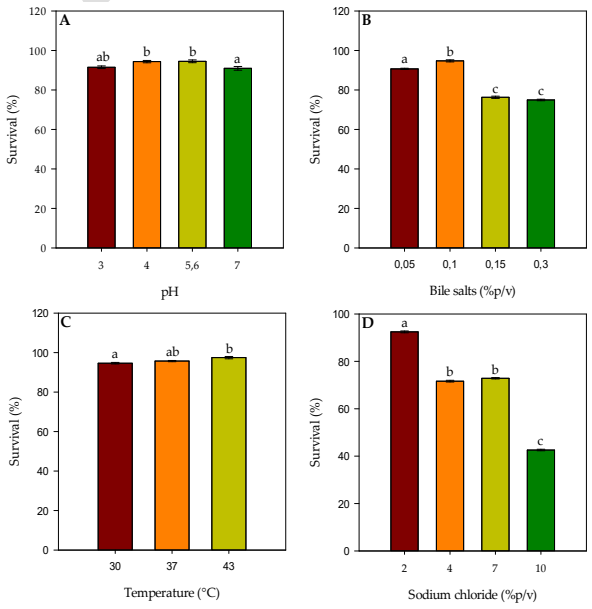

Fig. 2: Survival percentage of *K. marxianus* (strain CLARA-E) under varying stress conditions: (A) different pH levels, (B) bile salt concentrations, (C) temperatures, and (D) sodium chloride concentrations. Lowercase letters above the bars denote statistically significant differences ($P<0.05$). Results are based on triplicate experiments (n=3), with error bars representing the standard error of the mean.

These results collectively underscore the resilience of *K. marxianus* under diverse stress conditions, supporting its potential as a robust probiotic candidate for industrial and animal feed applications.

Molecular Identification

The ITS sequence of strain CLARA-E, with a size of 405bp, was analyzed using the GenBank (NCBI) megablast algorithm against the Fungi-type ITS database. The alignment revealed a highly significant match (E-value<10^{-157}), with a maximum alignment score of 551, query coverage exceeding 350 nucleotides, and 93% identity with the reference sequence NC_036029.1, corresponding to chromosome 5 of *K marxianus* DMKU3-1042. The sequence of strain CLARA-E has been deposited in the NCBI GenBank database under the accession code PP726659.1 (available at: https://www.ncbi.nlm.nih.gov/nuccore/PP726659.1, accessed on January 9, 2025.

Phylogenetic analysis further confirmed the classification of the wild yeast strain within the *K. marxianus* clade (Fig. 3), supporting its identification as a member of this species. These molecular findings validate the strain's taxonomic placement and provide a foundation for its characterization as a potential probiotic candidate.

Fig. 3: Phylogenetic tree of the *K. marxianus* strain CLARA-E (•) and related sequences retrieved from the NCBI GenBank database. The tree illustrates the taxonomic placement of strain CLARA-E within the *K. marxianus* clade, based on ITS sequence analysis. Bootstrap values (where applicable) indicate the robustness of the nodes, and the scale bar represents genetic distance.

Assessment of *K. marxianus* Growth in a Mango (*Mangifera indica*)-Based Culture Medium

Table 1 presents the growth of *K. marxianus* expressed in colony-forming units per milliliter (CFU mL^{-1}) across varying concentrations of mango pulp (5%, 10%, 15%, 20%, and 25% w/v) in the culture medium.

Table 1: Growth of *K. marxianus* (CFU mL^{-1}) at varying concentrations of mango pulp in the culture medium and different incubation times.

Mango Pulp (% w/v)	24Hours (CFU mL^{-1})	48Hours (CFU mL^{-1})	72Hours (CFU mL^{-1})
5%	$5.0\times10^{8}\pm1.8\times10^{8a}$	$2.2\times10^{9}\pm6.9\times10^{8a}$	$3.0\times10^{9}\pm0.\ 0^{a}$
10%	$5.0\times10^{8}\pm1.5\times10^{8a}$	$2.4\times10^{10}\pm1.3\times10^{10b}$	$3.7\times10^{9}\pm1.8\times10^{9a}$
15%	$4.3\times10^{8}\pm8.8\times10^{7a}$	$4.7\times10^{9}\pm8.8\times10^{8a}$	$4.7\times10^{9}\pm8.8\times10^{8a}$
20%	$1.0\times10^{8}\pm3.3\times10^{6a}$	$8.0\times10^{8}\pm5.8\times10^{7a}$	$5.0\times10^{8}\pm2.1\times10^{8a}$
25%	$6.3\times10^{8}\pm8.8\times10^{7a}$	$7.0\times10^{7}\pm5.8\times10^{6a}$	$5.0\times10^{7}\pm5.8\times10^{6a}$

* Data are presented as mean ± standard deviation of triplicate experiments (n=3). Different superscript letters within rows and columns indicate significant differences (Tukey's test, P<0.05).

At 5% (w/v) mango pulp, the cell density was $5.0\times10^{8}\pm1.8\times10^{8}$CFU mL^{-1} at 24hours, increasing to $2.2\times10^{9}\pm6.9\times10^{8}$CFU mL^{-1} at 48hours and reaching $3.0\times10^{9}\pm0.0$CFU mL^{-1} at 72hours. Although growth was gradual, no significant differences were observed between 24 and 48hours, and the increase from 48 to 72hours was minimal (Table 1).

In the medium with 10% (w/v) mango pulp, initial growth at 24hours ($5.0\times10^{8}\pm1.5\times10^{8}$CFU mL^{-1}) was similar to that at 5% However, a marked increase to $2.4\times10^{10}\pm1.3\times10^{10}$CFU mL^{-1} was observed at 48hours, representing a significant difference compared to other concentrations (5%, 15%, 20%, and 25% w/v) and incubation times (24 and 72hours). By 72hours, cell density decreased to $3.7\times10^{9}\pm1.8\times10^{9}$CFU mL^{-1}, with no significant differences between 24 and 72hours (Table 1).

At 15% (w/v) mango pulp, growth was moderate, with cell densities of $4.3\times10^{8}\pm8.8\times10^{7}$CFU mL^{-1} at 24hours, increasing to $4.7\times10^{9}\pm8.8\times10^{8}$CFU mL^{-1} at 48 and 72hours, where it remained stable. For 20% (w/v) mango pulp, initial growth was limited ($1.0\times10^{8}\pm3.3\times10^{6}$CFU mL^{-1} at 24hours), stabilizing at 48hours ($8.0\times10^{8}\pm5.8\times10^{7}$CFU mL^{-1}) and decreasing at 72hours ($5.0\times10^{8}\pm2.1\times10^{8}$CFU mL^{-1}). At 25% (w/v), initial growth was $6.3\times10^{8}\pm8.8\times10^{7}$CFU mL^{-1} at 24hours, declining to $7.0\times10^{7}\pm5.8\times10^{6}$CFU mL^{-1} at 48hours and $5.0\times10^{7}\pm5.8\times10^{6}$CFU mL^{-1} at 72hours. No significant differences (P>0.05) were observed between incubation times (24, 48, and 72hours) for mango pulp concentrations of 15%, 20%, and 25% (Table 1).

These results indicate that *K. marxianus* exhibits optimal growth at 10% (w/v) mango pulp, with significant biomass production at 48 hours, while higher concentrations (15%–25%) result in reduced or stabilized growth, suggesting potential substrate inhibition at elevated pulp levels.

Considering that the culture medium prepared with 10% (w/v) mango pulp, supplemented with 1% (w/v) yeast extract and 1.5% (w/v) peptone, yielded the optimal growth of *K. marxianus*, a chemical analysis of the medium was conducted. The results of this analysis are presented in Table 2. This evaluation aimed to characterize the nutritional composition of the medium, providing insights into the factors contributing to the enhanced growth of *K. marxianus* under these conditions.

Table 2: Chemical composition of the culture medium: 10% (w/v) mango pulp, 1% (w/v) yeast extract, and 1.5% (w/v) peptone.

Parameter	Concentration	Unit
Total Sugars	10.3	g
Reducing Sugars	2.1	g L^{-1}
Proteins	2.0	%
Ashes	2.6	%
Calcium (Ca)	44.0	mg L^{-1}
Magnesium (Mg)	11.0	mg L^{-1}
Sodium (Na)	265.0	mg L^{-1}
Potassium (K)	820.0	mg L^{-1}
Copper (Cu)	0.05	mg L^{-1}
Zinc (Zn)	1.4	mg L^{-1}
Iron (Fe)	0.3	mg L^{-1}
Manganese (Mn)	0.05	mg L^{-1}
Sulfur (S)	28.6	mg L^{-1}
Phosphorus (P)	3.0	mg L^{-1}
pH	5.5	pH units

Table 2 shows that mango is a fruit rich in soluble carbohydrates (*10.3g*), making it a valuable carbon source. It contains sugars such as glucose, fructose, and sucrose, along with other carbohydrates like starch and pectins.

Additionally, the protein content reported in this study (2%) is relatively high, providing sufficient nitrogen for yeast growth. The ash content of mango pulp (2.6%) is also considerable, likely due to its high mineral composition, including Ca, Na, K and Mg, as well as trace elements.

DISCUSSION

Yeast Growth under Stress Conditions

While the majority of probiotic research has historically focused on bacterial systems, recent studies have highlighted the potential of certain yeast species, such as *K. marxianus*, as promising probiotic candidates (Hu et al., 2023; Buonanno et al., 2025). Yeasts have emerged as model organisms in various biological processes, and their derivatives or by-products have garnered increasing scientific interest (Staniszewski & Kordowska-Wiater, 2021; Carrera Marcolin et al., 2024). Despite their critical role in maintaining gastrointestinal tract balance, particularly through antagonistic interactions with harmful microbiota, yeasts have been comparatively understudied as probiotics relative to their bacterial counterparts (Zahoor et al., 2021). A key criterion for evaluating a microorganism's probiotic potential is its ability to adapt to the specific conditions of the gastrointestinal tract (Menezes et al., 2020). To this end, the *K marxianus* strain CLARA-E was investigated for its tolerance to diverse stressors, including varying temperatures, high bile salt concentrations, sodium chloride, and acidic pH. One of the primary challenges for probiotic yeasts is their capacity to withstand the low pH conditions typical of the stomach environment (Pereira et al., 2012; Alkalbani et al., 2022a; 2022b). Survival in acidic pH, such as that found in gastric juice, is therefore a critical attribute for probiotic selection (Pereira et al., 2012). In this study, *K. marxianus* demonstrated no significant differences in average growth across a pH range of 3.0 to 7.0, maintaining a consistent population magnitude of 10^8 CFU/mL^{-1}. This indicates robust pH tolerance, aligning with findings by Merchán et al. (2020), who reported similar high resistance in *K. marxianus*. Galli et al. (2022) further corroborated these results, noting the strain's viability after exposure to acidic conditions. Similarly, Nag et al., (2023) observed that *K. marxianus* strain PCH397 exhibited a survival capacity of 78–99% under low pH conditions. Fadda et al. (2017) and Moradi et al. (2018) also reported high survival rates for *K. marxianus* strains S97, under simulated gastric conditions (pH 3.0), with survival percentages of 83%. The ability of yeasts to tolerate low pH is attributed to a regulatory mechanism involving ATPase in the cytoplasmic membrane, which establishes an electrochemical proton gradient. This gradient facilitates secondary solute transport, helping to maintain intracellular pH near neutrality (Arias et al., 2017). Additionally, yeasts employ strategies such as cell wall adjustment and activation of cell wall integrity pathways to resist acidic and general stress conditions (Lucena et al., 2020).

The tolerance of *K. marxianus* to low pH is particularly relevant for industrial applications, as acidic conditions are common in fermentation and probiotic production processes. The strain's ability to thrive across a wide pH range enhances its potential efficacy in the gastrointestinal tract, where pH fluctuations are significant, making it a promising candidate for probiotic roduct development, particularly in animal feed.

In addition to acidic pH tolerance, resistance to bile salts is another critical factor in assessing probiotic potential (Alkalbani et al., 2022a). Bile salts, which act as lipid emulsifiers, are released into the duodenum post-ingestion and possess inherent antimicrobial properties (Urdaneta & Casadesús, 2017). To reach the intestinal tract in a viable state, ingested microorganisms must withstand bile salt exposure (Shruthi et al., 2022).

In this study, the CLARA-E strain of *K. marxianus* demonstrated excellent growth in the presence of 0.05–0.3% (w/v) bile salts, maintaining stable viable cell counts and metabolic activity without inhibition. The highest survival rate (94.74%) was observed at 0.1% bile salt concentration, though the strain also grew effectively at 0.3% (w/v), a concentration comparable to average intestinal bile levels (Shih-An & Jui, 2020). These findings align with those of Lama & Tamang (2022) and Merchán et al., (2020), who reported similar bile salt resistance in *K. marxianus*. Fadda et al. (2017) further noted survival rates exceeding 95% at 0.3% (w/v) bile salts. Bile salt resistance in yeasts is mediated by bile salt hydrolase activity, which mitigates the toxic effects of conjugated bile salts (Liu et al., 2012). Additionally, ATP-binding proteins in yeast membranes facilitate the translocation of conjugated bile salts, while the accumulation of polyols and glycerol helps regulate osmotic pressure, further enhancing bile salt tolerance (López et al., 2015; Arias et al., 2017).

Temperature is another critical factor influencing microbial growth, as it affects sugar metabolism, reproduction and cellular development (Manovacía Moreno et al., 2008). For probiotics, growth at 37°C (normal body temperature) is essential (Gil-Rodríguez et al., 2015; Menezes et al., 2020; Shih-An & Jui, 2020; Vergara Alvarez et al., 2023), but tolerance to elevated temperatures (e.g., 39°C and 42°C) is also advantageous (Vergara Alvarez et al., 2023). The CLARA-E strain of *K. marxianus* exhibited significant growth at 30°C, 37°C, and 43°C, maintaining stable population levels relative to the initial inoculum. This thermotolerance is particularly noteworthy, as lactic acid bacteria (LAB) typically exhibit greater heat resistance than yeasts (Romero-Gil et al., 2013). Similar findings were reported by Menezes et al., (2020) and Lama & Tamang (2022), underscoring the strain's adaptability to varying temperatures.

Salt tolerance is another desirable trait for probiotics, as sodium chloride is commonly used in food preservation and flavor enhancement (Zeng et al., 2019; Alkalbani et al., 2022b). The CLARA-E strain demonstrated tolerance to NaCl concentrations of 2.0%, 4.0%, 7.0%, and 10% (w/v), though growth rates declined at higher concentrations (7.0% and 10% w/v) (Fig. 1D). Most microorganisms adapt to osmotic stress through mechanically rigid cell walls, and the CLARA-E strain's

ability to tolerate up to 7% NaCl suggests a capacity for osmotic adjustment (Lara Mantilla & Burgos Portacio, 2012). Under hypertonic conditions, yeasts undergo rapid dehydration due to water loss from the cytosol, followed by compensatory mechanisms such as vacuolar ion accumulation and passive solute movement to maintain cellular hydration (Serrano, 1996; Tao et al., 1999; Blomberg, 2000). Stress resistance mechanisms, including increased intracellular glycerol levels and the induction of protective proteins, further support survival and growth under osmotic stress (Rep et al., 2000).

Finally, *K. marxianus* demonstrated the ability to ferment glucose without gas production during 24hour incubation, a valuable trait for probiotic applications. Gas production by probiotics can cause digestive discomfort in hosts and represents an energy loss, as the gas is derived from substrates that could otherwise be metabolized for energy (Arias et al., 2017). The strain's efficient glucose assimilation without gas production underscores its potential as a safe and effective probiotic.

Assessment of the Growth of *K. marxianus* in a Mango (*Mangifera indica*)-Based Culture Medium

In this study, statistical analyses revealed that the optimal growth of *K. marxianus* occurred in a culture medium containing 10% (w/v) mango pulp, with an incubation time of 48hours. A decline in cell density was observed at 72hours, suggesting that while the 10% (w/v) mango pulp concentration supports robust initial growth up to 48hours, nutrient depletion may occur at an accelerated rate, leading to reduced cell density thereafter. Interestingly, as the concentration of mango pulp in the medium increased to 20% and 25% (w/v), yeast growth decreased. This inhibitory effect can likely be attributed to the high concentrations of sugars, particularly glucose and fructose, present in mango pulp. This observation aligns with studies indicating that elevated levels of reducing sugars in fruit-based media can suppress yeast growth (Lara Mantilla, 2008).

In mango (*Mangifera indica),* the carbohydrate content typically ranges between 16.20% and 17.18%, though this may vary depending on the fruit's ripeness (Yahia et al., 2023). High sugar concentrations can force yeasts into a decline phase, where populations enter a survival state with reduced metabolic activity due to osmotic stress. This can result in prolonged or even incomplete fermentations (Ogidi et al., 2020). These findings underscore the importance of carefully optimizing nutrient concentrations in culture media, particularly when using sugar-rich substrates like mango pulp, to maximize yeast growth and productivity in industrial applications.

Analysis of the chemical composition of the mango-enriched medium (10% w/v) (Table 2), supplemented with yeast extract and peptone, revealed that it provides the essential nutrients required for optimal growth of *K. marxianus*. These nutrients include carbohydrates, primarily in the form of total and reducing sugars, which serve as an energy source (carbon source), as well as proteins that supply nitrogen for microbial biomass synthesis (Lara Mantilla, 2008; Ezugwu et al., 2023).

In this study, a culture medium composed of 10% (w/v) mango pulp, 1.0% (w/v) yeast extract, and 1.5% (w/v) peptone supported satisfactory growth of *K. marxianus*. This demonstrates that agricultural residues, such as mango pulp, can serve as effective carbon sources, providing essential nutrients for the growth of biotechnologically relevant microorganisms, with cell densities ranging from 10^7 to 10^{10}CFU mL^{-1}. These results are supported by the work of Ogidi et al., (2020), who explored the use of various agricultural residues, including banana peels, pineapple, mango, peanut shells, coconut fiber, and walnut shells, supplemented with glucose, yeast extract, peptone, and minerals, for the growth of microorganisms such as *Bacillus subtilis*, *Candida albicans*, *Candida tropicalis*, *Lactobacillus delbrueckii*, and *Streptococcus thermophilus*. Their findings, which reported growth magnitudes of 10^4CFU mL^{-1}, suggest that agricultural residues provide essential nutrients such as nitrogen, carbon, and minerals, which microorganisms utilize for metabolite and biomass production (Fuentes-Gutiérrez et al., 2022).

The use of agroindustrial waste as a low-cost raw material represents a promising strategy for transforming waste into valuable compounds. In this context, mangoes are particularly advantageous due to their rich content of carbohydrates, proteins, vitamins, and minerals, which can meet the nutritional requirements of probiotic candidate strains like *K. marxianus* (Fuentes-Gutiérrez et al., 2022).

Research focused on biomass production from *K. marxianus* suggests that carbohydrate-rich agricultural wastes are a viable and cost-effective alternative to conventional carbon sources. The high cost of traditional carbon sources remains a significant barrier to large-scale biomass production, making the use of by-products an attractive solution. Specifically, underutilized mango residues have the potential to add value to these materials while producing high-quality protein-rich biomass, which could be beneficial for animal feed. Additionally, mango-derived growth factors such as citric and malic acids, along with essential minerals and trace elements (e.g., Mg, Ca, Fe, K, and Na), play critical roles in enzyme activation, cellular respiration, and other physiological functions (Fuentes-Gutiérrez et al., 2022).

Despite the identification of essential nutrients required by microorganisms in ruminants, optimizing total yeast growth remains an ongoing research challenge. This is due to the need to determine the optimal types, quantities, and combinations of these nutrients (Villamizar-Vargas et al., 2019). This approach not only offers a pathway to reduce resource waste but also generates valuable products from materials that would otherwise be discarded (Rivera et al., 2006). The results of this study highlight a promising avenue for sustainable biomass production, emphasizing the potential of agricultural residues as nutrient sources for microbial cultivation. This has significant implications for the biotechnology industry and agricultural waste management, paving the way for more sustainable and economically viable production processes.

Conclusion

This study highlights the potential of *Kluyveromyces marxianus* strain CLARA-E as a promising probiotic candidate for use as a feed additive in animal nutrition. The strain demonstrated robust tolerance to a range of environmental stressors, including acidic pH (optimal survival at pH 5.6), high bile salt concentrations (0.1% w/v), elevated temperatures (up to 43°C), and significant osmotic pressure from sodium chloride; additionally, the strain's ability to ferment glucose without excessive gas production. These attributes are critical for probiotic viability and functionality in the gastrointestinal tract, where conditions can be highly variable and challenging. The use of mango (*Mangifera indica*) pulp as a cost-effective and nutrient-rich culture medium further underscores the potential of agricultural residues in supporting microbial growth; this finding aligns with the growing interest in utilizing agroindustrial by-products as sustainable substrates for microbial cultivation, offering a dual benefit of waste valorization and cost reduction in probiotic production.

The stress tolerance, metabolic activity, and growth performance of *Kluyveromyces marxianus* CLARA-E position it as a viable candidate for modulating intestinal microbiota and enhancing immune responses in animals; however, while these *in vitro* results are promising, further in vivo studies are essential to validate the strain's efficacy, safety, and beneficial effects in animal models. This research contributes to the growing body of evidence supporting the use of native microbial strains, such as *Kluyveromyces marxianus* CLARA-E, in developing sustainable and effective alternatives for animal nutrition and health. By leveraging the strain's probiotic potential and the utilization of agricultural residues, this work paves the way for innovative approaches to improving animal welfare, productivity, and resource efficiency in the livestock industry.

Funding: The article processing charges (APC) was financed by the Universidad de Córdoba, Montería-Colombia.

Acknowledgment: The authors would like to thank the Universidad de Córdoba, the Biotechnology group of the Department of Chemistry and Department of Biology-GRUBIODEQ, Professor Juana Robles, Professor Luis Alfonso Rodríguez for having contributed significantly to the development of this manuscript.

Conflict of Interest: The authors declare that they have no competing financial interests or known personal relationships that could have influenced the work presented in this article.

Author's Contribution: First author (M.V.A): methodology, research, data analysis, conceptualization, writing, revising and editing. Second author (C.A.B.H): research, conceptualization, data analysis, writing, review and editing. Third author (D.E.L.R): visualization and revision. Fourth author (L.E.O.Z): review and editing. Fifth author (A.J.B.R): conceptualization, data analysis, writing, review and editing. (D.E.P.S): review and editing All authors have read and accepted the published version of the manuscript.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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