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From Probiotic Screening to Postbiotic Potential: An Integrated In Vitro Assessment of Endogenous Non-*Saccharomyces* Yeast Isolates

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Abstract

Yeasts isolated from fermented foods have attracted increasing attention for their probiotic potential; however, studies on yeast-derived postbiotics remain limited. In this study, endogenous yeast strains belonging to *Kluyveromyces marxianus* (*n* = 3), *Yarrowia lipolytica* (*n* = 3), *Pichia fermentans* (*n* = 3), and *Debaryomyces hansenii* (*n* = 3) were evaluated for their in vitro probiotic properties. A multi-criteria decision-making analysis using the Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) identified *K. marxianus* ETP12, *P. fermentans* SJ2023, and *Y. lipolytica* ARTP9.2 as the most promising strains for postbiotic production. Among them, *K. marxianus* ETP12 exhibited the highest functional potential and was subjected to comprehensive postbiotic characterization. The postbiotic of *K. marxianus* ETP12 was further characterized for total phenolic content, antioxidant capacity (DPPH and ABTS scavenging activities), phenolic compound profile, biofilm inhibition capacity, free amino acid composition, and fatty acid profile. The results revealed a diverse phenolic composition, primarily consisting of fumaric acid, quercetin, gallic acid, and quinic acid. A total of 29 essential, non-essential, and bioactive amino acids were identified, with lysine, leucine, and glycine as the predominant components. Fatty acid profiling indicated the predominance of palmitic and stearic acids, accompanied by medium-chain fatty acids. Notably, it exhibited strong biofilm-inhibition activity against *S. aureus* ATCC 25923 and *C. sakazakii* ATCC 29544. Overall, these findings demonstrate that *K. marxianus* ETP12 represents a valuable source of multifunctional postbiotics with potential applications in the development of functional foods and nutraceuticals.

Keywords: yeasts; non-*Saccharomyces*; probiotics; postbiotics; *Kluyveromyces marxianus*

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1. Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host [1]. Although research on probiotics is frequently focused on bacteria, yeasts have gained increasing attention as promising probiotic candidates. Among them, *Saccharomyces boulardii* is the most extensively used and clinically applied probiotic yeast species, with proven efficiency in the prevention and treatment of gastrointestinal disorders [2]. Beyond *S. boulardii*, several yeast species, including *Kluyveromyces marxianus* [3], *Pichia fermentans* [4], *Yarrowia lipolytica* [5], and *Debaryomyces hansenii* [6], have been reported to exhibit promising probiotic characteristics.

The functional efficacy of probiotics depends on cell viability. Challenges to cell viability arise during food processing, storage, and gastrointestinal transit. Environmental

and technological stressors can significantly reduce cell survival, leading to inconsistent functional outcomes. Moreover, the use of live microorganisms raises safety concerns, particularly in immunocompromised individuals, due to the potential risk of opportunistic infections, horizontal gene transfer, and the dissemination of antibiotic resistance determinants [7,8].

In response to these limitations, increasing attention has been directed toward non-viable microbial preparations such as postbiotics and parabiotics. Postbiotics, also called cell-free supernatant or cell-free extracts, are bioactive metabolites or cellular components produced by microorganisms that confer health benefits independently of cell viability [7]. These bioactive constituents include short-chain fatty acids, peptides, polysaccharides, amino acids, and organic acids [9]. Compared to live cells, postbiotics exhibit greater stability, improved safety, and enhanced suitability for standardization and industrial applications, while retaining biological activities, including antioxidant, immunomodulatory, and anti-inflammatory effects [10].

To date, a growing number of studies have investigated the biological effects of postbiotics derived from various lactic acid bacteria (LAB) [11] and yeasts [4]. The characterization of postbiotic preparations, particularly their metabolic composition, has been more extensively explored for LAB-derived postbiotics [12,13]. On the other hand, studies addressing yeast-derived postbiotics have predominantly focused on *S. cerevisiae* and its probiotic subspecies *S. boulardii* [14,15]. Endogenous yeasts possess substantial metabolic capacity, enabling the synthesis of a diverse range of bioactive compounds with potential functional relevance [16]. Among non-*Saccharomyces* yeasts, *K. marxianus* is of particular interest due to its metabolic versatility, rapid growth, and capacity to produce a wide range of bioactive compounds [17]. Nevertheless, comparative and systematic evaluations of *K. marxianus*-derived postbiotics in relation to other endogenous non-*Saccharomyces* yeasts remain limited. This study systematically evaluates the preliminary in vitro probiotic properties of different non-*Saccharomyces* yeast species and identifies the most promising candidates using an integrated multi-criteria decision-making approach. Specifically, the Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) method was applied to rank strains based on their overall in vitro probiotic performance across all evaluated criteria. The postbiotic preparations obtained from the top-ranked strains were subsequently screened for antioxidant activity, and the most prominent postbiotic was selected for further characterization, including phenolic compound content, biofilm inhibition capacity, free amino acid profile, and fatty acid composition. Through this integrated approach, this study seeks to expand current knowledge on postbiotics derived from non-*Saccharomyces* yeasts and to elucidate their potential as functional bioactive ingredients.

2. Materials and Methods

2.1. Yeast Isolates

The yeast cultures used in this study comprised 12 isolates previously obtained from various traditionally produced fermented foods: *D. hansenii* (n = 3), *K. marxianus* (n = 3), *P. fermentans* (n = 3), and *Y. lipolytica* (n = 3). All isolates had been molecularly identified in earlier studies using SCoT-PCR-based DNA fingerprinting, with well-characterized reference strains employed as controls. Following molecular confirmation, the isolates were deposited and preserved in the Aksaray University Food Microbiology Laboratory culture collection. All strains grow at 37 °C, exhibit high viability indices, and are non-hemolytic. Additionally, *S. boulardii* ATCC MYA-796 was included as a reference strain and used as a positive control in the preliminary in vitro probiotic characterization assays.

2.2. Molecular Confirmation

Genomic DNA extraction was performed according to the method reported by Aydın et al. [18]. DNA quantity was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), standardized to 50 ng/µL, and stored at −20 °C until further analysis. To amplify the D1/D2 domain of the large subunit NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-TCC TCC GTC TAT TGA TAT GC-3′) primer pairs were used [19], as previously described [20]. PCR products were purified and sequenced, and the resulting sequences were aligned using MEGA X software (12.0) [21]. Species identification was achieved by BLAST (2.17.0) analysis against the GenBank database, with ≥99% sequence similarity to type strains. All sequences were deposited in GenBank under accession numbers PX775536–PX775547.

2.3. Preliminary In Vitro Probiotic Characterization

2.3.1. Acid and Bile Tolerance

The yeast cultures were activated in Yeast Peptone Dextrose Broth (YPDB; Merck, Darmstadt, Germany) at 28 °C for 24–48 h. The YPDB supplemented with 0.3% bile salts and adjusted to pH 2.5 with 1 M HCl was inoculated with the yeast strains (10^6 CFU/mL). Non-fortified YPDB inoculated with yeast strains samples were used as control. The samples were incubated at 28 °C for 4 h in an ELISA plate reader (Allsheng AMR-100 T, Hangzhou, China). The final absorbance at 600 nm was recorded, and the data were arranged as growth index (GI) as follows, as suggested by Aktepe et al. [22]:

$$\text{GI (\%)} = \frac{\text{Abs}_y}{\text{Abs}_c} \times 100, \quad (1)$$

where Abs_y and Abs_c represent the absorbance measured for the fortified YPDB and the control after incubation.

2.3.2. In Vitro Digestion Tolerance

Survival under simulated gastrointestinal (GI) conditions was assessed using oral digestion (OD), gastric digestion (GD), and pancreatic digestion (PD) assays, with synthetic oral, gastric, and pancreatic fluids prepared as previously described by Alkalbani et al. [23] and Perricone et al. [24]. Viable cell counts were determined by plating appropriate dilutions on Yeast Extract Glucose Chloramphenicol (YGC; Merck, Darmstadt, Germany) Agar before and after treatment. Survival after each digestion step was expressed as a viability index (VI), calculated using the following equation:

$$VI(\%) = \frac{\log N(\text{after treatment})}{\log N(\text{before treatment})} \times 100 \quad (2)$$

2.3.3. Autoaggregation Capacity and Hydrophobicity

Autoaggregation capacity (AAC) and cell surface hydrophobicity (CSH) were determined following previously reported procedures with slight modifications [25]. Briefly, overnight yeast cultures were harvested by centrifugation (10,000 rpm, 10 min), washed twice with phosphate-buffered saline (PBS), and resuspended to get approximately 10^8 CFU/mL. For the AAC, the cell suspensions were incubated at 37 °C, and the absorbance of the upper layer was measured at 600 nm after 4 h and 24 h. The AAC was expressed as the percentage decrease in absorbance over time, as given in the following equation:

$$\text{AAC (\%)} = \frac{A_0 - A_t}{A_0} \times 100 \quad (3)$$

where A_0 is the absorbance at the beginning, and A_t is the absorbance at later time points (4 and 24 h). The CSH was assessed using the xylene method. Aliquots of the standardized yeast suspension (approximately 10^8 CFU/mL) were combined with 1 mL of xylene and allowed to equilibrate for 15 min, then vortexed for 2 min. The mixtures were then incubated at 37 °C for 20 min to allow phase separation. The aqueous phase was collected, and the absorbance reduction was recorded at 600 nm using the following equation:

$$\text{CSH (\%)} = \frac{A_1 - A_2}{A_1} \times 100 \quad (4)$$

where A_1 is the absorbance at the beginning, and A_2 is the absorbance of the aqueous phase at the end.

2.3.4. Multi-Criteria Decision Analysis and Visualization

A multi-criteria decision analysis was conducted using the Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS), adapted from Gul and Dervisoglu [26], to integrate multiple in vitro probiotic attributes of the yeast isolates. Briefly, a decision matrix was constructed using seven in vitro probiotic criteria, which were then normalized and weighted. The weighting coefficients (w_j) were assigned based on a viability > colonization priority, reflecting evidence that survival under gastric and intestinal stress represents the most critical determinant of probiotic performance [27]. Accordingly, simulated gastric fluid and simulated intestinal fluid tolerance were assigned the highest weights (0.20 each), while colonization-related parameters (AAC and CSH) and stress-related traits (simulated salivary fluid, low pH tolerance, and bile salt tolerance) were assigned equal weights (0.10). A weighted normalized matrix was then generated, and Euclidean distances to the positive and negative ideal solutions (d_i^+ and d_i^-) were calculated. The relative closeness coefficient (C^*) was subsequently determined as follows:

$$C_i^* = \frac{d_i^-}{d_i^+ + d_i^-} \quad (5)$$

As a complement to the decision analysis, a performance heatmap was created to visualize the strain-specific profiles of all evaluated parameters. In this matrix, the rows are ordered by TOPSIS ranking in descending order to facilitate direct comparison between composite scores and individual phenotypic performances. Cell values representing average performance scores are color-coded using a gradient scale to highlight functional strengths and weaknesses according to the ranking. The TOPSIS algorithm was implemented and the heatmap was constructed in Python (Version 3.13.9) using the Pandas library (Version 2.3.3) for data management and Seaborn (Version 0.13.2) for graphical representation.

2.4. Postbiotic Characterization

2.4.1. Postbiotic Preparation

The methodology proposed by Garcia et al. [28] was followed with minor modifications. The top-ranked yeast strains were reactivated in YPDB at 28 °C for 24 h and then heat-inactivated at 70 °C for 30 min in a water bath to ensure complete loss of viability and obtain a non-viable cell preparation. Cells were harvested by centrifugation (2150× *g*, 10 min), and the supernatants were filtered through 0.22 µm membranes (MF-Millipore, Merck, Germany). The sterile supernatants were designated as 'postbiotics samples' for further analyses. To verify the absence of viable cells, the samples were plated on YGC Agar and incubated at 28 °C for 48 h. The samples (non-viable) were stored at −18 °C.

2.4.2. Total Phenolic Content and Antioxidant Activity of Postbiotics

Total phenolic content (TPC) of postbiotics was quantified using the Folin–Ciocalteu colorimetric assay. Briefly, 0.5 mL of each sample was combined with 0.2 mL of Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 10 min. Subsequently, 0.6 mL of a 20% (*w*/*v*) sodium carbonate solution was added, and the reaction mixture was incubated at 30 °C for 30 min. Absorbance was then recorded at 765 nm using a UV–Vis spectrophotometer (Scilogex, Rocky Hill, CT, USA). Quantification was performed using a standard curve prepared with gallic acid in the range of 0–200 mg/L, and results were expressed as mg gallic acid equivalents (GAE) per gram of sample [29]. The free radical scavenging capacity of postbiotic and paraprobiotic samples was first evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, St. Louis, MO, USA) assay. For this purpose, 800 µL of each sample was mixed with 1.0 mL of a 0.2 mM DPPH solution prepared in methanol. The mixtures were incubated at room temperature in the dark for 30 min. Gallic acid was included as a reference antioxidant. Following incubation, absorbance was measured at 517 nm, and DPPH radical scavenging (DPPHScv) activity was calculated as the percentage inhibition [30].

$$DPPHScv(\%) = (1 - \frac{A_{517}(\text{yeast suspension})}{A_{517}(\text{control})}) \times 100 \quad (6)$$

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich, St. Louis, MO, USA) radical scavenging activity (ABTSScv) was assessed using a modified ABTS assay. The ABTS radical solution was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature for 12–16 h. Prior to analysis, the solution was diluted with 0.1 M sodium phosphate buffer (pH 7.4) to obtain an absorbance of 0.750 ± 0.025 at 734 nm. Then, 100 µL of postbiotic was mixed with 900 µL of ABTS solution to be incubated for 5 min at room temperature. The absorbance was measured at 734 nm. Ascorbic acid solution was used as a positive control, and absorbance was recorded at 734 nm. The ABTSScv (%) was calculated as:

$$ABTSScv(\%) = (1 - \frac{A_{734}(\text{postbiotic sample})}{A_{734}(\text{control})}) \times 100 \quad (7)$$

2.4.3. Phenolic Compound Analysis by LC-MS/MS and GC-MS

Phenolic profiling was conducted using an Agilent 6480 Triple Quadrupole LC–MS/MS system (Agilent Technologies, Santa Clara, CA, USA) following a modified method of Dikici et al. [31]. Filtered extracts (0.22 µm PTFE) were analyzed on a reversed-phase C18 column (ZORBAX Eclipse XDB, 4.6 × 150 mm, 5 µm) using a 25 min gradient of water and methanol, both containing 0.1% formic acid. Analyses were performed at a flow rate of 0.3 mL/min, injection volume of 5 µL, and column temperature of 35 °C, with ESI operated in both positive and negative modes. Quantification was based on external calibration curves of phenolic standards ($R^2 \geq 0.99$), and LOD and LOQ were determined using signal-to-noise ratios. Complementary GC–MS analysis was performed using a Shimadzu QP2010 Ultra system (Shimadzu, Kyoto, Japan) as described by Proestos and Komaitis [32]. Methanol and ethyl acetate extracts were analyzed in splitless mode on a DB-5MS column, with helium as the carrier gas. Mass spectra were acquired in EI mode (70 eV, m/z 40–600), and compounds were identified by comparison with the NIST library, retention indices, and authentic standards. Results were expressed as mg/kg (ppm).

2.4.4. Biofilm Inhibition Capacity

The assay was conducted according to Aydin et al. [4]. Overnight cultures of *Cronobacter sakazakii* ATCC 29544, *Listeria monocytogenes* ATCC 13932, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 were adjusted to 0.5 McFarland, and 60 µL were dispensed into 96-well microtiter plates. Subsequently, 60 µL of yeast postbiotic and 60 µL of sterile Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) were added. Pathogen cultures in TSB and postbiotic cultures in TSB served as positive and negative controls, respectively. After incubation at 37 °C for 24 h, wells were washed twice with PBS, fixed with methanol for 10 min, stained with 0.1% crystal violet for 30 min, rinsed with distilled water, and air-dried. The stained biofilm was solubilized in 200 µL ethanol, and absorbance was measured at 595 nm. Biofilm inhibition capacity (BIC) was calculated as follows:

$$\text{BIC (\%)} = \frac{OD_{595}(\text{positive control}) - OD_{595}(24\text{ h}) - OD_{595}(\text{negative control})}{OD_{595}(\text{positive control})} \times 100 \quad (8)$$

2.4.5. Free Amino Acid Profile

The amino acid composition of the selected postbiotic was analyzed using an Agilent 6460 Triple Quadrupole LC–MS/MS system (Agilent Technologies, Santa Clara, CA, USA) operated in multiple reaction monitoring mode. Quantification was carried out with a commercial JASEM Amino Acid Kit (JSM-CL-508, Istanbul, Turkey) following the manufacturer's instructions, with minor modifications adapted from Tabak et al. [33]. Chromatographic separation was performed using the kit-specific column, and all solvents and reagents were of analytical or chromatographic grade. For sample preparation, 500 µL of the postbiotic was subjected to acidic hydrolysis by adding 4 mL of the JASEM amino acid hydrolysis reagent and incubating the mixture at 110 °C for 24 h to ensure complete protein breakdown. After hydrolysis, samples were allowed to cool to room temperature and subsequently centrifuged at 4000× *g* for 5 min. An aliquot of 100 µL from the resulting supernatant was diluted to a final volume of 1 mL with deionized water, corresponding to an overall dilution factor of 800. From this diluted hydrolysate, 50 µL was transferred into an autosampler vial, followed by the addition of 50 µL of the internal standard solution and 700 µL of the JASEM acidic reagent. The mixture was vortexed for 10 s to ensure homogeneity, and the clarified supernatant was transferred into a clean vial for analysis. Finally, a 3 µL aliquot was injected into the LC–MS/MS system. In total, 39 amino acids were quantified. Amino acid concentrations were initially expressed in parts per million (ppm) and subsequently converted to g/100 g free amino acids (FAA) for data interpretation and comparison. The essential amino acid (EAA) index was calculated by comparison with the reference amino acid pattern established by the FAO/WHO [34]. The protein efficiency ratio (PER) value was calculated using the following equation [35].

$$PER = 0.780(Leu) + 0.435(Met) + 0.211(His) - 0.944(Tyr) - 1.816 \quad (9)$$

2.4.6. Fatty Acid Composition

The free fatty acid (FFA) composition of the selected postbiotic was determined using GC-MS according to İncili et al. [36] with minor modifications. Briefly, 2 mL of postbiotic was mixed with 2.5 g anhydrous Na_2SO_4 and acidified with 300 µL 4N H_2SO_4. The mixture was vortexed for 1 min, allowed to stand in the dark for 1 h, and centrifuged (1110× *g* for 5 min at 2 °C). Then 5 mL of hexane was added and vortexed for 3 min, and centrifuged (1610× *g* for 10 min at 2 °C). The resulting supernatant was allowed to equilibrate at room temperature for 2 h and passed through aluminum oxide columns for purification. The FFAs were eluted from the column using 5 mL of a hexane/diethyl ether mixture (1:1, *v*/*v*). After drying, the aluminum oxide was transferred to a clean tube, and 2 mL of

4% formic acid prepared in diisopropyl ether was added. The mixture was centrifuged (710× *g* for 10 min at 2 °C), and the supernatant was collected into GC vials. The FFAs were analyzed using GC-MS (Shimadzu QP2010, Kyoto, Japan) equipped with a DB-FFAP capillary column (60 m × 0.25 mm i.d. × 0.25 µm film thickness; Agilent). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature program was initiated at 90 °C for 3 min, increased to 200 °C at 20 °C/min, then to 245 °C at 10 °C/min, and held for 30 min. The FFAs were identified by comparing mass spectra with reference libraries, covering 30 FFAs ranging from butyric acid (C4:0) to heneicosanoic acid (C21:0). The results were expressed as relative peak area percentages.

2.4.7. Statistical Analysis

For each strain, two independent cultures were prepared and analyzed to ensure biological reproducibility. Each biological replicate was analyzed in triplicate. Data are presented as mean ± standard deviation (SD). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's PostHoc for probiotic characteristics, and the Bonferroni-corrected paired *t*-test was used for antioxidant assay. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using SPSS Statistics Version 31.0.1.0 (49) Full Version Trial (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Species Confirmation

The previously identified yeast strains (n = 12) were identified by DNA fingerprinting using the D1/D2 domain of 26S rDNA. Accordingly, the sequencing confirmed the previous species identification and resulted in *D. hansenii* (n = 3), *K. marxianus* (n = 3), *P. fermentans* (n = 3), and *Y. lipolytica* (n = 3). The BLAST algorithm indicated that the sequence similarity for each strain was at least 99.12% sequence similarity with the reference strains (*D. hansenii* NRRL Y-7426, *K. marxianus* CBS:458, *P. fermentans* CBS:246, and *Y. lipolytica* CBS:10150) on the NCBI database.

3.2. In Vitro Probiotic Characterization

The initial screening aimed to identify promising yeast candidates for postbiotic characterization by evaluating general in vitro probiotic characteristics, including tolerance to different digestion methods, AAC, and CSH. Strains demonstrating key in vitro probiotic traits may be considered promising functional reservoirs of bioactive compounds [22]. The preliminary screening was designed to identify yeast strains with high probiotic potential, with *S. boulardii* ATCC MYA-796 included as the reference strain. The in vitro probiotic characteristics of the strains are given in Table 1.

Exposure to low pH (2.5) and bile salts (0.3%), which form a simple model of digestive stress for probiotics, displayed a wide, strain-dependent survival. Strains that can withstand stress factors are likely to maintain their populations during gastric transit and reach the intestine at effective doses [37]. *Pichia fermentans* SJ2023 was the most acid-tolerant strain, with a viability of 85.59 ± 0.52%, followed by the reference strain MYA 796 (80.61 ± 0.84%), *K. marxianus* ETP12 (75.14 ± 2.57%), and *Y. lipolytica* ARTP9.2 (73.69 ± 0.45%); this matches reports that *Pichia* species often dominate the acidic fermented foods such as wine and pickles [38,39]. Under bile stress, SJ2023 again showed the highest survival rate (93.06 ± 2.96%) and was identified as the most resistant isolate. Similarly, *K. marxianus* ETP12 (90.54 ± 3.58%) and *Y. lipolytica* ARTP9.2 (88.84 ± 1.63%) also exhibited strong bile tolerance with no statistical significance with MYA-796 (88.54 ± 1.97%)

($p > 0.05$). The results agree with earlier findings showing high bile resistance in *K. marxianus* strains from traditional dairy products [40].

Table 1. Probiotic characterization of yeast isolates.

Strain	pH 2.5	Bile Salt	OD (%)	GD (%)	PD (%)	AAC (4 h)	AAC (24 h)	HPBI
Dh BO4	38.35 ± 1.46 ^{g}	75.21 ± 1.41 ^{f}	52.20 ± 0.93 ^{i}	31.85 ± 0.08 ^{j}	46.42 ± 1.13 ^{g}	24.15 ± 1.53 ^{def}	76.54 ± 3.96 ^{ef}	35.47 ± 2.62 ^{d}
Dh SC24.5	41.98 ± 1.02 ^{g}	79.12 ± 0.32 ^{def}	59.12 ± 1.12 ^{gh}	35.64 ± 0.12 ^{i}	42.71 ± 0.05 ^{h}	18.79 ± 0.91 ^{f}	55.78 ± 0.59 ^{g}	10.95 ± 0.53 ^{h}
Dh S42	18.23 ± 0.12 ^{h}	58.21 ± 1.53 ^{g}	63.51 ± 0.12 ^{fg}	23.50 ± 0.64 ^{k}	39.85 ± 0.64 ^{i}	20.61 ± 1.87 ^{ef}	69.33 ± 1.12 ^{f}	22.60 ± 1.09 ^{fg}
Km ETP12	75.14 ± 2.57 ^{c}	90.54 ± 3.58 ^{ab}	88.65 ± 1.63 ^{a}	78.64 ± 0.34 ^{b}	80.07 ± 0.78 ^{b}	58.67 ± 1.63 ^{a}	95.89 ± 4.54 ^{ab}	77.32 ± 3.51 ^{a}
Km KD1	58.52 ± 1.08 ^{e}	85.29 ± 2.44 ^{bc}	77.23 ± 2.52 ^{e}	65.03 ± 0.56 ^{f}	75.01 ± 1.50 ^{c}	33.75 ± 0.87 ^{c}	88.93 ± 3.96 ^{bc}	57.64 ± 3.25 ^{b}
Km TYY3.1	73.50 ± 1.47 ^{c}	86.39 ± 2.02 ^{bc}	89.64 ± 2.09 ^{ab}	68.12 ± 0.58 ^{e}	72.64 ± 0.56 ^{c}	43.86 ± 2.51 ^{b}	94.12 ± 3.25 ^{ab}	42.36 ± 0.64 ^{c}
Pf SJ2023	85.59 ± 0.52 ^{a}	93.06 ± 2.96 ^{a}	85.93 ± 2.50 ^{bc}	72.97 ± 1.03 ^{d}	81.21 ± 1.51 ^{b}	52.35 ± 1.62 ^{ab}	91.55 ± 2.84 ^{abc}	36.22 ± 0.27 ^{d}
Pf V14	61.60 ± 0.57 ^{de}	81.96 ± 1.52 ^{cd}	78.12 ± 2.76 ^{de}	53.12 ± 0.21 ^{g}	58.95 ± 0.85 ^{f}	27.62 ± 0.71 ^{cde}	75.63 ± 1.63 ^{ef}	20.10 ± 0.50 ^{g}
Pf CC13	72.85 ± 1.93 ^{c}	81.50 ± 2.48 ^{cde}	82.59 ± 1.54 ^{cd}	65.51 ± 0.76 ^{f}	74.87 ± 0.74 ^{c}	28.67 ± 1.48 ^{cde}	89.51 ± 2.12 ^{bc}	29.72 ± 1.57 ^{e}
Yl ARTP5.3	65.05 ± 0.87 ^{d}	75.60 ± 0.98 ^{ef}	66.42 ± 1.75 ^{f}	50.75 ± 0.95 ^{h}	73.91 ± 0.33 ^{c}	30.89 ± 2.01 ^{cd}	85.87 ± 1.41 ^{cd}	36.64 ± 1.64 ^{d}
Yl ARTP9.2	73.69 ± 0.45 ^{c}	88.84 ± 1.63 ^{ab}	91.98 ± 1.88 ^{a}	76.74 ± 0.72 ^{c}	69.74 ± 0.42 ^{d}	32.53 ± 0.46 ^{cd}	71.64 ± 1.52 ^{ef}	55.05 ± 0.96 ^{b}
Yl SVS16	54.50 ± 1.98 ^{f}	73.14 ± 1.22 ^{f}	55.74 ± 0.46 ^{hi}	51.25 ± 0.24 ^{h}	62.53 ± 0.75 ^{e}	22.72 ± 1.75 ^{ef}	79.26 ± 2.39 ^{de}	26.01 ± 0.16 ^{ef}
MYA 796	80.61 ± 0.84 ^{b}	88.54 ± 1.97 ^{ab}	85.45 ± 0.62 ^{bc}	88.65 ± 0.53 ^{a}	88.64 ± 0.41 ^{a}	49.74 ± 2.05 ^{b}	98.23 ± 1.59 ^{a}	38.06 ± 0.95 ^{cd}

Abbreviations: *Dh*: *Debaryomyces hansenii*, *Km*: *Kluyveromyces marxianus*, *Pf*: *Pichia fermentans*, *Yl*: *Yarrowia lipolytica*, *Saccharomyces boulardii*. OD Oral digestion, GD Gastric digestion, PD Pancreatic digestion. *Saccharomyces boulardii* ATCC MYA-796 strain was used as a control. Different letters in the same column indicate statistical significance ($p < 0.05$).

In the OD assay, the reference strain MYA 796 (85.45 ± 0.62%) ranked fifth. In comparison, the top four positions were occupied by *K. marxianus* ETP12 (92.65 ± 1.63%), *Y. lipolytica* ARTP9.2 (91.98 ± 1.88%), *K. marxianus* TYY3.1 (89.64 ± 2.09%), and *P. fermentans* SJ2023 (85.93 ± 2.50%), indicating high amylase resistance and excellent oral stability. The findings are consistent with those of Alkalbani et al. [23], who reported high survival rates for *P. kudriavzevii* strains across various fermented foods. In the GD assay, where the most significant loss generally occurs due to low pH and a relatively long exposure time, the reference strain MYA 796 (88.65 ± 0.53%) showed the highest resistance, followed by *K. marxianus* ETP12 (78.64 ± 0.34%), *Y. lipolytica* ARTP9.2 (76.74 ± 0.72%), and *P. fermentans* SJ2023 (72.97 ± 1.03%). The high viability rates obtained for these strains are consistent with previous findings reported for *K. marxianus* [17], *Y. lipolytica* [41], and *P. fermentans* [4]. In the PD assay, containing bile salts and pancreatin, the reference strain MYA 796 (88.64%) and the isolates *P. fermentans* SJ2023 (81.21 ± 1.51%) and *K. marxianus* ETP12 (80.07 ± 0.78%) exceeded the 80% VI and were classified as having high intestinal tolerance [42]. In addition, *K. marxianus* KD1 (75.01 ± 1.50%) and *P. fermentans* CC13 (74.87 ± 0.74%) also exhibited high viability. The viability of the strains as measured by PD is in line with that reported for different non-*Saccharomyces* yeasts by Bonatsou et al. [43], but higher than that reported by Kanak and Öztürk-Yılmaz [44].

In vitro evaluation of probiotic strains includes their capacity to adhere to epithelial and mucosal surfaces, as this trait is vital for their persistence in the gastrointestinal tract and their interactions with the host. The HPBI, typically assessed using hydrocarbon adhesion methods, and auto-aggregation capacity are regarded as complementary assays. The former indicates a microbe's attachment potential [45], while the latter reflects the microorganism's tendency to cluster, a property indirectly linked to adherence to intestinal epithelial cells [46]. In the present study, *K. marxianus* ETP12 exhibited the highest CSH (77.32 ± 3.51%). According to Aydın [17], *K. marxianus* KD1 and *Y. lipolytica* ARTP9.2 had high CSH values (CSH > 50). On the other hand, six strains displayed moderate CSH (25 < CSH < 50), while the rest were poorly hydrophobic (CSH < 25). AAC significantly increased with increasing incubation period. Among all isolates, *D. hansenii* isolates generally had lower AAC than those of other species. *K. marxianus* ETP12, TYY3.1, and *P. fermentans* SJ2023 stood out along with the reference strain MYA-796 at 4 h and 24 h of

incubation. Although the results are slightly lower than those reported by Goktas et al. [47] and Merchán et al. [39], they are generally comparable to those reported by Kanak and Öztürk-Yılmaz [44]. These findings give a preliminary idea of how the isolates might interact with mucosal surfaces.

Selecting a functionally prominent candidate may be challenging when multiple parameters are supposed to be evaluated simultaneously. Assessing a strain based on a single trait does not adequately reflect its overall performance. Therefore, an integrated multivariate decision-making framework is required to objectively compare candidate strains. The TOPSIS, a multi-criteria decision-making method, was applied to rank strains based on their overall performance across all evaluated criteria, considering their relative closeness to ideal and non-ideal solutions [48]. The TOPSIS rankings are given in Table S2. Accordingly, *K. marxianus* ETP12 reflected the highest score (C_i^* = 0.8761), outperforming the reference strain MYA 796 (C_i^* = 0.7535) with stronger gastric survival and surface adhesion, and thus emerged as the best candidate overall. *Pichia fermentans* SJ2023 was ranked as the third isolate with a C_i^* value of 0.6842, followed by *Y. lipolytica* ARTP9.2 (C_i^* = 0.6782).

In addition to the TOPSIS evaluation, a heat map analysis was performed to assess probiotic potential broadly and to cluster strains based on their cumulative performance across all stress and colonization parameters. According to the heatmap in Figure 1, MYA 796 shows the darkest red in the GD and PD columns; that is, it survives best in stomach and intestinal fluids. However, in the CSH column, it appears more blue-orange and is weaker than *K. marxianus* ETP12, which ranks first among the probiotics in the TOPSIS analysis. *Kluyveromyces marxianus* ETP12 is colored orange/red in all columns. This visual confirmation suggests that ETP12 is balanced and strong across both stress tolerance and colonization parameters. *P. fermentans* SJ2023 and *Y. lipolytica* ARTP9.2 show most cells as red at pH 2.5, in bile salts, and in GI fluids; these strains are also seen to be in the prominent group for stress resistance. Conversely, many other columns show blue/light tones, indicating that their overall probiotic performance appears lower. These two analyses were consistent with and mutually confirmed each other. Accordingly, *K. marxianus* ETP12, *P. fermentans* SJ2023, and *Y. lipolytica* ARTP9.2 were selected for the subsequent analyses.

3.3. Total Phenolic Content and Antioxidant Activity of Postbiotics

The TPC and radical scavenging activities of the selected strains' postbiotics are presented in Table 2. The highest TPC activity was recorded for *K. marxianus* ETP 12, and the highest DPPHScv and ABTSScv also support this. The TPC measured in this study was lower than the amounts previously reported for postbiotics derived from LAB, such as *Pediococcus acidilactici* [13] and *Lactiplantibacillus plantarum* [49]. Conversely, our values were substantially higher than those observed in *S. cerevisiae* [50]. These differences are likely due to the unique metabolic characteristics of each strain, which influence the synthesis of phenolic compounds and other antioxidant metabolites.

Table 2. Antioxidant activity of selected strains.

Strain	Antioxidant Assay		
	TPC (mgGAE/L)	DPPHScv (%)	ABTSScv (%)
Km ETP12	859.61 ± 10.96 [a]	73.24 ± 1.32 [a]	86.02 ± 1.25 [a]
Yl ARTP9.2	623.42 ± 6.53 [b]	48.09 ± 0.62 [b]	73.66 ± 2.11 [b]
Pf SJ2023	613.64 ± 3.12 [b]	22.64 ± 0.26 [c]	74.77 ± 1.85 [b]

Abbreviations: *Km*: *Kluyveromyces marxianus*, *Yl*: *Yarrowia lipolytica*, *Pf*: *Pichia fermentans*. [a–c] Different letters in the same column are statistically significant by Bonferroni corrected paired *t*-test at the 0.05 level of significance. Data are presented as mean ± standard deviation.

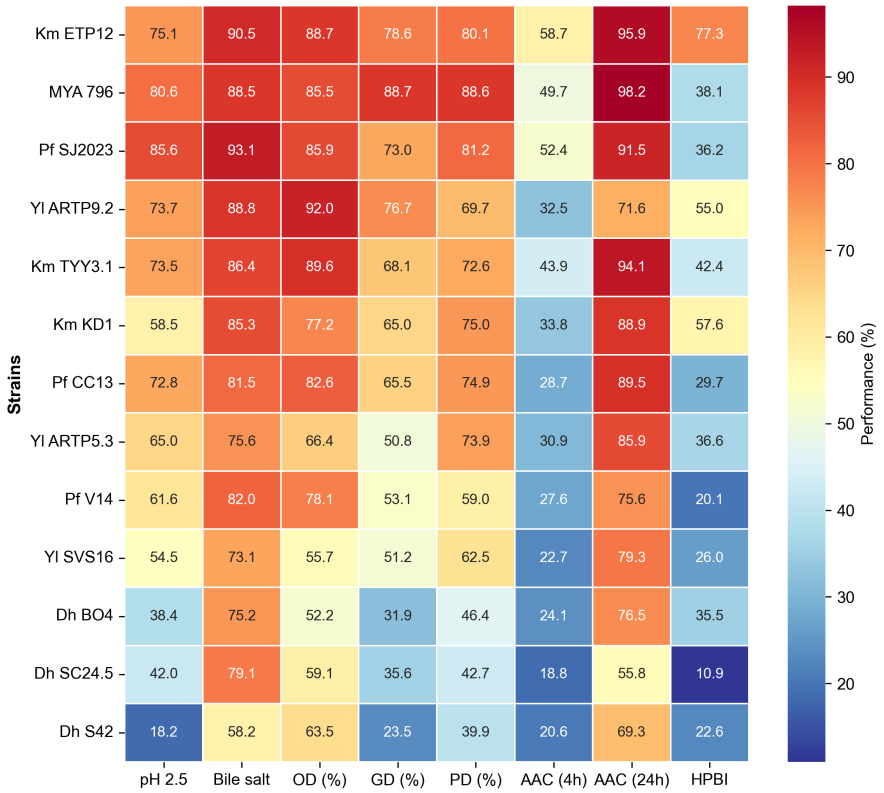


Figure 1. Performance heatmap visualizing the in vitro probiotic profiles of the evaluated yeast isolates. The matrix displays the mean performance percentages for critical functional traits. To visualize the consensus of the decision-making process, rows are ordered according to the TOPSIS ranking, descending from the most promising candidate (*Km* ETP12) to the lowest-ranked strain. The color gradient represents the performance magnitude, ranging from blue (low activity/survival) to red (high activity/survival). Abbreviations: OD: Survival in simulated salivary fluid; GD: Survival in simulated gastric fluid; PD: Survival in simulated intestinal fluid; AAC: Auto-aggregation capability; HPBI: Hydrophobicity index.

Postbiotics have gained increasing attention for their ability to enhance the functionality of food. They contribute to improved oxidative stability, prolonged shelf life, and the preservation of both nutritional value and sensory qualities through their antioxidant activity [51]. All yeast postbiotics showed high radical-scavenging activity, except for *P. fermentans* SJ2023, which had moderate DPPHScv (22.64 $\pm$ 0.26%) according to Gil-Rodríguez et al. [52]. *Kluyveromyces marxianus* ETP12 again ranked highest among the AOA, with DPPHScv of 73.24 $\pm$ 1.32% and ABTSScv of 86.02 $\pm$ 1.25%. There is limited information in the current literature on the ABTSScv of yeast-derived postbiotics. Nevertheless, Perpetuini et al. [53] and Aydin [17] reported higher ABTSScv than DPPHScv, consistent with our findings. This difference is likely related to the distinct chemical properties of the assays: while DPPH primarily reacts with lipophilic antioxidants, ABTS can detect both hydrophilic and lipophilic antioxidants [31]. Based on these findings, *K. marxianus* ETP12 was selected as the final strain for in-depth characterization, including phenolic profiling, BIC, FAA, and FFA analyses.

3.4. Phenolic Profile of ETP12 Postbiotic

The phenolic profile of *K. marxianus* ETP12 postbiotic is presented in Table 3, revealing a diverse composition of organic acids, phenolic acids, and flavonoids, which highlights the potential of yeast-derived postbiotics as a rich source of bioactive metabolites. Yeasts are known to transform and release phenolic compounds through enzymatic activity during

growth [54]. Accordingly, the phenolic profile observed in the ETP12 postbiotic is likely driven by yeast metabolic activity. Among the identified compounds, fumaric acid was the most abundant phenolic compound with a concentration of 19.13 ± 0.18 ppm. Organic acids such as fumaric and quinic acids are frequently detected in microbial metabolites and are known to contribute to antioxidant and antimicrobial activities [55]. The presence of quinic acid (3.95 ± 0.05 ppm) further supports the contribution of microbial metabolism to the phenolic profile of the ETP 12 postbiotic.

Table 3. The phenolic contents of the *K. marxianus* ETP12 postbiotic.

Number	Compounds	*K. marxianus* ETP12 Postbiotic (Mean ± SD)
1	Fumaric acid	19.13 ± 0.18
2	Quercetin	5.20 ± 0.21
3	Gallic acid	4.55 ± 0.16
4	Quinic acid	3.95 ± 0.05
5	4-dihydroxy benzoic acid	2.33 ± 0.05
6	Syringic acid	0.91 ± 0.05
7	Chlorogenic acid	0.58 ± 0.05
8	3-dihydroxy benzoic acid	0.44 ± 0.06
9	t-Cinnamic acid	0.28 ± 0.01
10	p-Coumaric Acid	0.11 ± 0.01
11	Luteolin	0.05 ± 0.01

Flavonoids constituted an important fraction of the detected phenolic compounds. Quercetin was identified at a relatively high level (5.20 ± 0.21 ppm), followed by luteolin at a lower concentration (0.05 ± 0.01 ppm). Flavonoids such as quercetin and luteolin are well-recognized for their potent antioxidant, anti-inflammatory, and antimicrobial activities [56]. Their occurrence in postbiotic preparations has been increasingly reported in recent studies [57,58].

Several phenolic acids, including gallic acid (4.55 ± 0.16 ppm), 4-dihydroxybenzoic acid (2.33 ± 0.05 ppm), syringic acid (0.91 ± 0.05 ppm), chlorogenic acid (0.58 ± 0.05 ppm), and p-coumaric acid (0.11 ± 0.01 ppm), were also identified. Phenolic acids are known to contribute to radical scavenging activity and to interfere with microbial quorum sensing and biofilm formation [59,60]. Cinnamic acid derivatives, including trans-cinnamic acid (0.28 ± 0.01 ppm), were detected at lower concentrations but remain biologically relevant. Even at low levels, cinnamic acid derivatives have been reported to exhibit antimicrobial and antibiofilm effects, particularly when acting synergistically with other phenolic compounds [61].

Although the antioxidant activity of yeast-derived postbiotics has been explored in several studies, comprehensive metabolite-level characterization has primarily been restricted to *S. boulardii* and a limited number of conventional yeasts. Previous reports on *S. boulardii* postbiotics mainly identified simple phenolic acids and aromatic compounds, such as vanillic acid, cinnamic acid, and phenylethyl alcohol [14,62]. In contrast, gallic acid and quercetin were detected at relatively low levels in biomass extracts of *S. cerevisiae*, *Kazachstania humilis*, and *Torulaspora delbrueckii*, with caffeic acid reported exclusively in *S. cerevisiae* [63]. The phenolic profile of the *K. marxianus* ETP12 postbiotic demonstrates a complex and diverse metabolite composition consisting of organic acids, phenolic acids, and flavonoids. These findings confirm that ETP12 postbiotic can serve as a source of bioactive compounds.

3.5. Biofilm Inhibition Capacity of ETP12 Postbiotic

ETP12 postbiotic has been tested for BIC against several food-related biofilm-forming pathogens. It exhibited a broad spectrum of BIC activity, with the highest inhibition

observed against *S. aureus* ATCC 25923 (87.81 ± 1.97%), followed by *C. sakazakii* ATCC (61.47 ± 1.05%) and *L. monocytogenes* ATCC 13932 (33.01 ± 2.52%). In comparison, the lowest activity was recorded against *P. aeruginosa* ATCC 27853 (27.52 ± 0.33%). The patterns may be linked to the specific phenolic and organic acid composition of the *K. marxianus* ETP12 postbiotic, since the phenolics and organic acids are known to interfere with key stages of biofilm development [64,65].

Fumaric acid was the dominant organic acid (19.13 ± 0.18 ppm), followed by quercetin (5.20 ± 0.21 ppm), gallic acid (4.55 ± 0.16 ppm), and quinic acid (3.95 ± 0.05 ppm). Fumaric acid has been reported to suppress biofilm formation in foodborne pathogens through environmental acidification and membrane destabilization, particularly under food-like conditions [66]. The strong inhibition observed against *S. aureus* is consistent with the well-documented antibiofilm activities of gallic acid and quercetin. Gallic acid has been shown to impair *S. aureus* adhesion and biofilm formation by disrupting cell wall integrity and suppressing *ica* operon expression [67], while quercetin reduces extracellular polymeric substance (EPS) synthesis and inhibits biofilm maturation in staphylococci [68]. At the detected concentrations, these phenolics likely contribute substantially to the pronounced BIC against *S. aureus*. Similar findings were also reported by Pernin et al. [69] against *L. monocytogenes*. On the other hand, the reports on the BIC of the detected phenolics to *C. sakazakii* are scarce, although various phenolics are reported to reduce biofilm formation in *C. sakazakii* [70].

The detectable amount of quinic acid (3.95 ± 0.05 ppm) has been shown to interfere with biofilm maturation and quorum-sensing pathways, including the *las* and *rhl* systems, even at sublethal concentrations. These mechanisms may explain the moderate, yet measurable BIC observed against *P. aeruginosa* [71].

Overall, the antibiofilm activity of the ETP12 postbiotic is best explained by its function as a multi-component biological matrix, in which organic acids (primarily fumaric acid) and phenolic compounds (gallic acid, quercetin, and quinic acid) act synergistically to disrupt bacterial adhesion, EPS production, and quorum sensing. In addition to these metabolites, yeast-derived exometabolites such as enzymes and bioactive peptides may further contribute to the observed BIC [72], as previously reported for yeast postbiotics against *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* [73–75]. Given the role of biofilms in persistent contamination and sanitizer resistance in food-processing environments, the observed BIC highlights the technological relevance of ETP12 postbiotic as a natural antibiofilm agent [76].

3.6. Free Amino Acid Profile of ETP12 Postbiotic

The FAA profile of the ETP12 postbiotic is summarized in Table 4. Because uninoculated YPDB was not analyzed for FAA, the FAA data represent quantitatively determined postbiotic profiles rather than background-corrected absolute net amino acid production. This approach is supported by previous studies demonstrating that FAA in yeast-derived products predominantly originates from proteolytic activity and cellular turnover rather than from passive diffusion from the culture medium [77]. The FAA content was evaluated to determine its essential amino acid (EAA) profile, overall protein quality, and potential health advantages. The branched-chain amino acid (BCAA) profile showed a relatively balanced distribution of leucine, isoleucine, and valine, with leucine as the predominant fraction. This amino acid is nutritionally important because it plays a crucial role in stimulating muscle protein synthesis pathways [78]. On the other hand, histidine was identified as the primary limiting amino acid, resulting in an EAA index of 96.4%, relative to the FAO/WHO [34] reference pattern. The overall amino acid distribution supports a high nutritional quality, as reflected by the elevated PER value (7.71), which surpassed the

previously reported PER values for yeast biomass of *S. cerevisiae*, *T. delbrueckii*, and *K. humilis* [63]. Overall, these results indicate that the protein fraction has a favorable EAA profile, supporting ETP12 postbiotic as a high-quality alternative protein source.

Table 4. Free amino acids profile of *K. marxianus* ETP12 postbiotic (Mean ± SE).

Number	FAA	Concentration (ppm)	g/100 g FAA (%)
1	Lysine	3195.61 ± 198.46	14.11 ± 0.89
2	Leucine	2810.56 ± 110.62	12.41 ± 0.51
3	Glycine	2252.01 ± 70.75	9.94 ± 0.33
4	Alanine	1706.43 ± 42.24	7.53 ± 0.21
5	Isoleucine	1623.85 ± 48.13	7.17 ± 0.23
6	Valine	1612.57 ± 35.13	7.12 ± 0.18
7	Phenylalanine	1267.98 ± 38.52	5.60 ± 0.18
8	Arginine	1087.63 ± 31.21	4.80 ± 0.15
9	Glutamic acid	958.55 ± 29.62	4.23 ± 0.14
10	Serine	854.71 ± 28.47	3.77 ± 0.13
11	Tryptophan	778.44 ± 14.22	3.44 ± 0.07
12	Threonine	614.87 ± 17.79	2.71 ± 0.08
13	Proline	542.96 ± 22.26	2.40 ± 0.10
14	Methionine	486.03 ± 20.92	2.15 ± 0.10
15	Sarcosine	458.25 ± 9.89	2.02 ± 0.05
16	Asparagine	424.63 ± 10.82	1.87 ± 0.05
17	Aspartic acid	412.60 ± 8.35	1.82 ± 0.04
18	Histidine	381.79 ± 9.62	1.69 ± 0.05
19	Tyrosine	342.92 ± 11.17	1.51 ± 0.05
20	Carnosine	202.13 ± 4.76	0.89 ± 0.02
21	Glutamine	185.14 ± 5.29	0.82 ± 0.03
22	Ornithine	124.25 ± 6.89	0.55 ± 0.03
23	Taurine	77.41 ± 1.74	0.34 ± 0.01
24	GABA	62.02 ± 1.44	0.27 ± 0.01
25	Ethanolamine	58.42 ± 2.49	0.26 ± 0.01
26	Anserine	47.63 ± 0.87	0.21 ± 0.01
27	AIB	35.29 ± 1.17	0.16± 0.01
28	Citrulline	28.01 ± 2.66	0.12 ± 0.01
29	Cystine	20.03 ± 1.49	0.09 ± 0.01

Abbreviations: AIB: 3-Aminoisobutyric acid; FAA: Free amino acid; GABA: Gamma-Aminobutyric acid.

In total, 29 essential, non-essential, and bioactive amino acids were identified. Among them, lysine was the dominant amino acid (3195.61 ± 198.46 ppm, 14.11 ± 0.89%), followed by leucine (2810.56 ± 110.62 ppm, 12.41 ± 0.51%), glycine (2252.01 ± 70.75 ppm, 9.94 ± 0.33%), and alanine (1706.43 ± 42.24 ppm, 7.53 ± 0.21%). The predominance of lysine and BCAAs is consistent with previous reports on yeast-derived postbiotics and biomass extracts, in which leucine, lysine, and valine are frequently identified as major components [62,63]. They are closely related to protein synthesis, muscle metabolism, and nitrogen balance, suggesting potential nutritional and functional benefits [79].

In addition to EAA, several metabolically and physiologically relevant amino acids were also detected. They include arginine, glutamic acid, serine, and tryptophan. Arginine and glutamic acid play key roles in immune modulation and cellular metabolism. At the same time, tryptophan is particularly important because of its role in gut microbiota interactions and host signalling pathways [80]. Similar diversity in FAA profiles has been reported in postbiotics derived from LAB, although concentrations and composition were shown to be strain-dependent [81,82].

Notably, the ETP 12 postbiotic also contained bioactive amino acids, including γ-aminobutyric acid (GABA), taurine, ornithine, citrulline, carnosine, and anserine. The presence of GABA is particularly relevant, as yeast-derived GABA production has been reported less frequently than LAB production, despite its recognized roles in stress regulation,

neuromodulation, and antioxidant defence [83]. The co-occurrence of these bioactive amino acids with high levels of EAA may contribute synergistically to the functional properties of ETP12 postbiotic.

3.7. Free Fatty Acid Profile of ETP12 Postbiotic

Analysis of FFA in postbiotics derived from *K. marxianus* ETP12 revealed a distinctive composition dominated by long-chain saturated fatty acids (LCSFAs), with palmitic acid (C16:0, 43.14 ± 0.44%) and stearic acid (C18:0, 35.84 ± 0.34%) accounting for approximately 79% of the total fatty acids. This profile is notably different from previously reported *K. marxianus* and other non-*Saccharomyces* yeast lipid compositions, which generally exhibit higher proportions of unsaturated fatty acids such as oleic (C18:1) and linoleic (C18:2) acids [84–86]. The exceptionally high stearic acid content is particularly remarkable, as it exceeds values commonly reported by Ling et al. [87], where C18:0 typically ranges from 5–20%. Medium-chain fatty acids (MCFAs) constituted 8.26% of the profile, including caprylic acid (C8:0, 4.31 ± 0.17%) and capric acid (C10:0, 4.8 ± 0.04%). The presence of MCFAs may have contributed to the BIC.

Notably, cis-10-pentadecanoic acid (C15:1) was detected at 12.52 ± 0.22%, a level substantially higher than the trace amounts (<5%) typically reported for odd-chain fatty acids in *Rhodotorula toruloides* [88]. Emerging evidence suggests that this component may have anti-inflammatory and immunomodulatory properties [89], suggesting that it may contribute to the functional bioactivity of the ETP12 postbiotic.

The ratio of saturated to unsaturated fatty acids is 87:13, which is much higher than what is usually seen in yeast lipids. Even under stress conditions, *K. marxianus* generally exhibits lower saturation ratios, typically 70:30 to 75:25. This highly saturated profile is associated with increased lipid rigidity and enhanced resistance to oxidative degradation, a phenomenon previously linked to yeast growth under harsh environmental conditions [85]. It is important to note that the FFA composition observed in this study differs from that reported by Gientka et al. [86] and Guluarte et al. [85], who analyzed intracellular lipids from whole yeast biomass. In contrast, the present study focused on postbiotics obtained after centrifugation of a heat-inactivated yeast suspension. This methodological difference suggests that a substantial proportion of unsaturated fatty acids may have remained associated with the cellular biomass and were thus removed during centrifugation, whereas saturated fatty acids were preferentially retained or released into the supernatant. In addition, heat inactivation in an aqueous medium may have promoted the selective oxidation of unsaturated fatty acids, further shifting the profile toward a saturated fraction.

From a technological perspective, this elevated saturation ratio, largely driven by palmitic and stearic acids, may confer a functional advantage for postbiotic applications. Higher levels of saturated fatty acids are known to improve oxidative and thermal stability [90], thereby enhancing the robustness of postbiotic formulations during industrial processing steps such as heating, drying, and storage. Consequently, the observed FFA profile may help maintain postbiotic bioactivity under conditions that often compromise the stability of conventional probiotic products.

4. Conclusions

This study proposes an integrated and stepwise framework for the selection and functional evaluation of endogenous non-*Saccharomyces* yeasts, progressing from probiotic-related screening to postbiotic-oriented technological assessment. The findings provide a rational basis for prioritizing yeast-derived postbiotics with translational potential in food systems. Within this framework, the postbiotic derived from *K. marxianus* ETP12 emerged

as a particularly promising candidate, supporting its prospective use as a functional food ingredient or natural bioprotective agent.

From an application-oriented perspective, the compositional richness of the ETP12 postbiotic offers technological advantages in oxidative stability, antibiofilm activity, and nutritional enhancement. These multifunctional properties are well aligned with current industrial demands for clean-label, non-viable bioactive ingredients that can be incorporated into food matrices without the stability constraints associated with live cells. Accordingly, future studies should focus on evaluating the performance of this postbiotic in real food matrices and under processing and storage conditions to substantiate its technological feasibility.

Although *K. marxianus* holds Qualified Presumption of Safety (QPS) status by the EFSA Panel on Biological Hazards [91], and heat-killed *K. marxianus* biomass is classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, this study is limited by the absence of strain-specific cytotoxicity assays for ETP12. Therefore, targeted in vivo studies are required to confirm the safety of the ETP12 strain under the production conditions used. In addition, the metabolic characterization was limited to selected bioactive components; therefore, comprehensive metabolomic profiling would further strengthen the understanding of the functional potential of the ETP12 postbiotic and support its translation into food and industrial applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12020090/s1, Table S1: Yeast strains used in this study; Table S2: Final rankings of probiotic characterization of strains obtained by TOPSIS.

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