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ORIGINAL RESEARCH

Identification of new yeast strains, *Candida zeylanoides* Y12-3 and *Hyphopichia pseudoburtonii* Y12-1, from the intestinal tract of rainbow trout, *Oncorhynchus mykiss*, with potential probiotic characteristics

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Abstract

In this study, 2 yeasts, *Candida zeylanoides* isolate Y12-3 (MN073455.1) and *Hyphopichia pseudoburtonii* isolate Y12-1 (MN073489.1), were isolated from rainbow trout hindguts. Their identity was confirmed by amplification of internal transcribed spacer (ITS) gene regions. Their in vitro probiotic properties showed that they were hydrophobic and did not inhibit bacterial growth. However, they displayed positive co-aggregation with several bacterial pathogens. It was interesting that Y12-1 and Y12-3 isolates survived and grew well in bile salt concentrations ranging from 0.6% to 1.5% and at low pH conditions. After a 35-day feeding trial, the results of dietary incorporation of the yeast isolates showed that serum myeloperoxidase activity was unchanged in the treated and control groups. Meanwhile, serum lysozyme activity in yeast groups was decreased significantly compared to the control. However, fish fed with Y12-1 had higher total protein, albumin, and globulin than other groups. Total cholesterol levels were decreased significantly in yeast groups compared to the

control. Interestingly, the Y12-1 and Y12-3 groups showed higher survival (69.05% and 80.95%) after the challenge with *Lactococcus garvieae* compared to the control (45.24%). In brief, the aforementioned findings showed that Y12-1 and Y12-3 strains could be potential probiotic candidates for use in rainbow trout farming.

KEYWORDS
disease resistance, immunity, probiotic yeasts, rainbow trout

1 | INTRODUCTİON

In aquaculture, the use of probiotics is one of the most common and significant advancements because of their ability to promote growth and enhance the overall health and physiological responses of farmed fish and shrimp (Abdel-Latif et al., 2022; Yilmaz et al., 2022). Yeast probiotics gain their special attractiveness for application in aquafeed because of their β-glucan content, which can effectively boost fish's immune system (El-Saadony et al., 2021; Martínez et al., 2018; Sahoo & Mukherjee, 2001). A variety of yeast species have been isolated from the fish intestines and have been identified as candidate probiotic yeasts: for example, *Debaryomyces hansenii*, *Candida zeylanoides*, *Saccharomyces cerevisiae*, *Leucosporidium* sp., and *Rhodotorula mucilaginosa* from rainbow trout (Gatesoupe, 2007; Martínez et al., 2018; Raggi et al., 2014), *Pichia kudriavzevii* CM 02 strain from mrigal carp (*Cirrhinus mrigala*), *Candida parapsilosis* LR 01 strain from rohu (*Labeo rohita*), and *Candida tropicalis* OM 01 (Mandal & Ghosh, 2013) and *Candida rugosa* ONF19B (Banerjee & Ghosh, 2014) from Mozambique tilapia (*Oreochromis mossambicus*). Recently, four autochthonous yeasts, including *Cutaneotrichosporon jirovecii*, *D. nepalensis*, *Blastobotrys proliferans*, and *Diutina catenulata*, have also been retrieved from the intestines of goldfish (*Carassius auratus*) with potential probiotic properties (Taha et al., 2023). However, *Saccharomyces cerevisiae* (Abdel-Tawwab et al., 2008; Banu et al., 2020; Hoseinifar et al., 2011) and *D. hansenii* (Angulo et al., 2017; Reyes-Becerril et al., 2011, 2021; Reyes-Becerril, Salinas, et al., 2008; Reyes-Becerril, Tovar-Ramírez, et al., 2008) have been mostly investigated as probiotics in aquaculture.

There are several characteristics that should be found in the candidate strains to be classified as potential probiotic organisms, such as their capability to adhere to intestinal epithelial cells and bile tolerance in fish guts (Chabrillón et al., 2006; Fjellheim et al., 2010; Lazado et al., 2010; Nikoskelainen et al., 2001). Probiotic organisms with high tolerance to bile salts and hydrophobic properties have a higher chance of passing through the gastrointestinal tract (GIT) and forming colonies in the host intestines (Nikoskelainen et al., 2001; Zhou et al., 2007). Moreover, the probiotic capacity to co-aggregate with pathogenic bacteria can build a barrier that may delay or prevent harmful bacteria from colonizing the gut (Collado et al., 2007; Jankovic et al., 2012; Kos et al., 2003). There are several studies investigating the co-aggregation abilities of yeasts isolated from fish with bacterial pathogens. For instance, it was found that the yeast strains *Kazachstania exigua* RC037 and RC038 isolated from the intestines of rainbow trout are known to have the ability to co-aggregate with the pathogen *Pseudomonas aeruginosa* (Martínez et al., 2018). Later, Taha and coauthors uncovered four yeast strains isolated from goldfish with the potential ability to co-aggregate with several fish-associated bacterial pathogens (Taha et al., 2023).

Herein, we isolated two host-derived yeast strains from the hindguts of rainbow trouts, namely, *Candida zeylanoides* (isolate Y12-3) and *Hyphopichia pseudoburtonii* (isolate Y12-1). Their identity was confirmed using molecular diagnosis. To test the probiotic hypothesis and functionality, the in vitro probiotic screening characteristics of the identified yeast strains have been evaluated in terms of antagonism to bacterial strains, hydrophobic behavior, adhesion, co-aggregation ability, and tolerance to different pH values and bile salt concentrations. Finally, their

in vivo probiotic effects have also been assessed on the fish immunity, serum biochemistry, and disease resistance capacity after conducting a feeding experiment. The present study findings may pave the way for finding novel yeast strains with potential probiotic activity for application in aquaculture.

2 | MATERIALS AND METHODS

2.1 | Sampling and isolation of yeasts

For sampling, two fish farms in Turkey were chosen. The Suleyman Demirel University's Local Ethical Committee approved the study SDU HADYEK 10/06). Yeast strains were isolated from 30 apparently healthy rainbow trout (approximately 250 g). After the fish were euthanized with 50 mg/L clove oil, their hindguts were aseptically sampled. Each sample was homogenized in a phosphate-buffered saline (PBS) solution. Tenfold serial dilutions of the test samples were conducted, spread on chloramphenicol glucose yeast extract agar (YGC) plates (BioLife Italiana srl., Milan, Italy), and then incubated at 25°C for 72 h. The grown yeast colonies were chosen, purified, and then streaked twice onto 15% Yeast Mold (YM) broth (BioLife Italiana srl., Milan, Italy) supplemented with 15% glycerol (v/v) as a cryo-preservative agent. All yeast isolates were then stored at −20°C for subsequent in vitro analysis.

2.2 | ITS gene region sequence analysis

Molecular identification of isolates was performed by amplification of ITS (internal transcribed spacer) gene regions and subsequent sequence analysis. For this purpose, the genomic DNAs of the isolates were obtained according to the manufacturer's instructions using the commercial Yeast DNA extraction kit (Thermo Fisher Scientific), whose principle is based on spin column filtration. The concentrations of the extracted DNAs were measured at 260 nm with a spectrophotometer (NanoDrop 2000 spectrophotometer, Thermo Scientific). The ITS gene regions of candidate probiotic yeasts were amplified by PCR using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (Gago et al., 2014). 50 µL PCR master mix containing DEPC-treated water, 1xPCR buffer, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 2.5 U Taq polymerase, 0.5 µM each primer, and 5 µL template DNA were prepared. This PCR mixture was amplified in a thermal cycle using the following program: initial denaturation step at 94°C followed by 35 cycles at 94°C 30 s, at 56°C for 45 s, and at 72°C for 2 min and finally, one cycle at 72°C for 5 min. The PCR products were visualized on the Gel imaging system after 1% agarose gel electrophoresis containing ethidium bromide (2 µg/mL).

PCR product purification and DNA sequence analysis were performed by Macrogen Inc. (Seoul, Korea) with the Sanger method in Applied Biosystems 3130 Genetic Analyzer, using ITS1 and ITS4 primers as double-stranded. The forward and reverse sequences of each isolate were aligned using Contig Express' Vector NTI Advance 11.5 (Invitrogen) program and then compared with the reference sequences in the GenBank data library using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). The identity percentages of the sequences were determined. Data belonging to the unique sequences obtained from the study were registered to GenBank, and accession numbers were obtained. ITS results of the present study revealed two yeast strains named *C. zeylanoides* isolate Y12-3 (NCBI GenBank Accession No. MN073455.1) and *H. pseudoburtonii* isolate Y12-1 (NCBI GenBank Accession No. MN073489.1).

2.3 | In vitro antagonistic activities against bacterial strains

Four bacterial strains: *Lactococcus garvieae* (Didinen et al., 2014), *Vagococcus salmoninarum* (Didinen et al., 2011), *Yersinia ruckeri* (Onuk et al., 2019), and *Aeromonas hydrophila* (Onuk et al., 2013), were previously isolated,

identified, and characterized by molecular tools by our research team. These strains were provided from the culture collection of Egirdir Fisheries Faculty, Isparta, Türkiye. They were used to determine the in vitro antagonism against the isolated yeast isolates. A well diffusion agar test was used for antagonistic effects of yeast isolates against pathogen bacterial strains. For this purpose, yeasts grown in YM agar were taken into YM broth, and 48-h yeast cultures were obtained at 25°C. Pathogen bacteria were inoculated into Tryptic Soy Broth (TSB), and a 24-h bacterial culture was obtained at 25°C. 100 µL of 24-h cultures of pathogen strains were added to 100 mL of Tryptic Soy Agar (TSA) at 25°C. Then, the media were left to solidify for 15 min, and wells of approximately 3 mm in diameter were opened. 25 µL of 48-h cultures of candidate probiotic yeast isolates in YM broth were added to the wells and incubated at 25°C for 24–48 h. After incubation, clear zones of inhibition around the wells were measured (Hjelm et al., 2004).

2.4 | Co-aggregation test

Yeast strains were tested for their abilities to co-aggregate the previously mentioned pathogenic bacterial pathogens. Yeast strain suspension (1 mL, 10^7 CFU mL^{-1} in PBS) was mixed with each bacteria culture (1 mL, 10^9 CFU mL^{-1}) and incubated for 2 hr at 25°C. The presence or absence of aggregation was confirmed by gram staining (Martínez et al., 2018; Mastromarino et al., 2002).

2.5 | Hydrophobicity test

Congo red was added to YM agar (0.03%) for a hydrophobicity test. On YM agar, various yeast strains were dispersed and incubated for 48 h at 25°C. White or colorless colonies were classified as negative (non-hydrophobic) and red colonies as positive (hydrophobic) (Sharma et al., 2006).

2.6 | Bile salts and pH tolerance

To determine the tolerance of the yeast strains to bile salts and pH values, a cell suspension of 24-h YM cultures of each yeast strain was first prepared. Bile samples were taken from rainbow trout and stored at −20°C to prepare bile salt concentrations. A volume of 0.5 mL was taken from 24 h of each yeast culture (10^7 CFU mL^{-1}) and inoculated in PBS (4.5 mL) containing 0.6%, 1.0%, and 1.5% bile salt concentrations. After the samples were incubated at 25°C for 1.5 h, they were inoculated on YM agar by dilution plate method, and the yeast cells in each sample were counted after 24–48 h of incubation at 25°C (Pérez-Sánchez et al., 2011). To determine the tolerance of the identified yeasts to different pH conditions, 0.5 mL of 24-h yeast culture was taken and inoculated into PBS (4.5 mL) with different pH values (pH 2, 3, and 7.5) with HCl. After the incubation at 25°C for 1.5 h, yeast cells were counted using YM agar with a dilution plating (dilution plate method) (Pérez-Sánchez et al., 2011).

2.7 | Pathogenicity of yeasts in rainbow trout

To investigate their pathogenicity in rainbow trout, 24-h pure cultures of the identified strains were obtained in YM Broth at 25°C. The culture was centrifuged at 5000 g for 15 min at 4°C, and yeasts were collected in the falcon tube and then re-suspended with an equal volume of PBS. The yeast count was determined by the dilution plate method in YM agar. After preparation, yeast suspension was injected intraperitoneally (IP) at a dose of 10^7 CFU per fish. A total of 20 trout fingerlings (with approximately 50 g) were grouped in duplicate and were IP injected with the

previously prepared yeast suspension. Fish were then observed for 15 days for any clinical findings, and mortalities were recorded daily.

2.8 | In vivo (feeding) experiment

2.8.1 | Preparation of the test diets

The isolates Y12-3 and Y12-1 were cultured in YM agar for 48 h at 25°C and harvested from plates. Each strain was added to commercial rainbow trout feed with sunflower oil at 10^5 yeast cells/g feed. For the control group, only sunflower oil was added to the feed. The prepared diets (Y12-3, Y12-1, and control diets) were stored in the refrigerator at 4°C. After feed preparation, the yeast counts in each diet were determined on YM agar at 7, 14, and 21 days during storage. Hence, we found that it is better to prepare diets every 14 days to keep the required yeast count.

2.8.2 | Experimental conditions

After the acclimation (14 days), 60 trout fingerlings (approximately 70 g) were divided into three duplicate groups (defined as control, Y12-3, and Y12-1). Fish were reared in concrete ponds (depth 98 cm, length 345 cm, width 135 cm). Afterward, the feeding experiment (35 days) was performed in the flow-through system (1–1.5 L water/min, 13°C).

2.8.3 | Blood sampling and serum collection

After feeding with candidate probiotic yeasts, three fish were randomly sampled from each group and euthanized with phenoxyethanol. Then, blood samples were taken from a fish's caudal vein and placed in non-heparinized tubes. After clotting, blood samples were centrifuged at 3000 *g* for 15 min at 4°C. Until analysis, the serum samples were stored at −80°C.

2.8.4 | Immunity and serum biochemical indices

In brief, the serum myeloperoxidase (MPO) activity and lysozyme (LYZ) activity were determined according to the method by Sahoo et al. (2005) and Nudo and Catap (2011), respectively. Serum biochemistry analyses (triglycerides, total cholesterol, glucose, albumin, globulin, total protein) were performed using biochemistry kits (IMPROGEN, Istanbul/Basaksehir) using OPTIZEN brand spectrophotometer.

2.8.5 | Bacterial challenge experiment

After the feeding experiment ended, 14 fish per group were anesthetized with phenoxyethanol. These fish were IP injected with a pathogenic *L. garvieae* at a dose of LD50 (1.2×10^7 CFU/fish), according to Kubilay et al. (2008). Dead fish were collected, and mortality was recorded daily for an additional 15 days. The cumulative survival rates (SR, %), the mortality rate (MR, %), and relative percent survival (RPS) were calculated according to the equations described in our recently published study (Yilmaz et al., 2023).

2.9 | Statistical analysis

The experiment's data were analyzed with one-way ANOVA in the SPSS 17.0 program. Serum biochemical and immune parameters were examined by GraphPad Prism X8. When comparing the significance levels of the data, Duncan's test was utilized ($p < 0.05$).

3 | RESULTS

3.1 | Isolation and characterization of yeasts

In this study, two yeast strains, Y12-1 and Y12-3, were isolated and identified from the hindguts of rainbow trout. According to Table 1, it was found that the ITS gene region sequence of the retrieved yeast isolate (Y12-1) was 99.27% similar to the *H. pseudoburtonii* isolate makgeolli strain (CP024753.1, CP024756.1, and CP024757.1) and *H. pseudoburtonii* culture CBS:5510 (KY103609.1). The isolate Y12-1 was identified as *H. pseudoburtonii* and registered in GenBank with Accession Number MN073489.1. The other yeast isolate Y12-3 was 100% similar to sequences of *C. zeylanoides* (GenBank Accession Nos. MH459420.1, KY102543.1, KY102541.1, KY102537.1, EF687774.1, AY497688.1, AB278160.1, and AB278159.1). This isolate was named *C. zeylanoides* and registered in GenBank with Accession Number MN073455.1.

3.2 | The in vitro probiotic activities of the retrieved yeast strains

The in vitro antagonistic activities of the retrieved yeasts against bacterial strains using the agar well diffusion method showed that all identified yeast strains did not cause in vitro antagonism and did not inhibit the growth of

TABLE 1 The isolated yeast species, their closest relatives (yeasts belonging to each species), and their accession numbers were retrieved from NCBI GenBank.

Retrieved yeast species and closest relatives	Isolates	NCBI GenBank Accession Numbers	References
Hyphopichia pseudoburtonii	Isolate Y12-1	MN073489.1	Current study
Hyphopichia pseudoburtonii	Isolate makgeolli chromosome 3	CP024753.1	Unpublished
Hyphopichia pseudoburtonii	Isolate makgeolli chromosome 6	CP024756.1	Unpublished
Hyphopichia pseudoburtonii	Isolate makgeolli chromosome 7	CP024757.1	Unpublished
Hyphopichia pseudoburtonii	Culture CBS:5510	KY103609.1	(Vu et al., 2016)
Candida zeylanoides	Isolate Y12-3	MN073455.1	Current study
Candida zeylanoides	Strain Husl_FF18	MH459420.1	Unpublished
Candida zeylanoides	Culture CBS:6409	KY102543.1	(Vu et al., 2016)
Candida zeylanoides	Culture CBS:5122	KY102541.1	(Vu et al., 2016)
Candida zeylanoides	Culture CBS:5447	KY102537.1	(Vu et al., 2016)
Candida zeylanoides	Strain TJY13a	EF687774.1	Unpublished
Candida zeylanoides		AB278159.1	(Published only in NCBI Database, 2006)
Candida zeylanoides		AB278160.1	
Candida zeylanoides	Strain CBS 619	AY497688.1	(Diezmann et al., 2004)

the tested bacterial pathogens. These yeast isolates showed positive co-aggregation ability with all tested pathogens. Of interest, the cell surface hydrophobicity of identified yeast isolates revealed that these isolates were highly hydrophobic. According to Tables 2 and 3, it was found that the identified yeast isolates (Y12-1 and Y12-3) survived and grew well to bile salt concentrations ranging from 0.6% to 1.5% for 1.5 h at 25°C (Table 2) and also can grow well at low pH conditions ($p < 0.05$) (Table 3).

3.3 | The in vivo probiotic activities of the retrieved yeast strains

3.3.1 | Pathogenicity of yeast isolates in rainbow trout

The clinical examination of the identified yeast isolates (Y12-1 and Y12-3) revealed no pathogenic effects (no clinical signs or PM lesions) following injection into rainbow trout.

3.3.2 | Results of the feeding experiment

After a 35-day experiment, the blood protein profile of rainbow trout showed a significant increase in total protein (Figure 1a), albumin (Figure 1b), and globulin (Figure 1c) values in the Y12-1 group than in the Y12-3 and control groups ($p < 0.05$), whereas their values in control and Y12-3 displayed non-significant differences (Figure 1, $p > 0.05$). The serum biochemical parameters of rainbow trout revealed the lowest triglyceride concentrations were found only in the Y12-3 group than in other groups (Figure 2a). Moreover, there were significant decreases in total cholesterol values in the Y12-3 and Y12-1 groups than in the control group (Figure 2b, $p < 0.05$). On the other hand, glucose values (Figure 2c) were significantly higher in the Y12-3 and Y12-1 groups when compared to the controls. Of interest, the examination of serum immune indices showed a significant decrease in serum LYZ activities of the

TABLE 2 Effects of different bile salt concentrations (%) on the growth of the identified yeast strains cultured on YM agar at 25°C after 1.5-h incubation.

Bile salt (%)	Y12-3	Y12-1
0	4.95 ± 0.50^{ab}	4.62 ± 0.17^{a}
0.6	5.56 ± 0.03^{b}	4.49 ± 0.29^{a}
1	5.11 ± 0.03^{ab}	4.72 ± 0.29^{a}
1.5	4.62 ± 0.32^{a}	5.25 ± 0.25^{b}

Note: Data presented as log CFU/mL ± SD ($n = 3$). Values with different superscript letters in the same column denote statistically significant differences within experimental groups ($p < 0.05$).

TABLE 3 Effects of different pH values on the growth of the identified yeast strains cultured on YM agar at 25°C after 1.5-h incubation.

pH	Y12-3	Y12-1
7.5	4.95 ± 0.50	4.62 ± 0.17^{a}
3	5.08 ± 0.59	5.17 ± 0.31^{b}
2	4.75 ± 0.15	4.38 ± 0.37^{a}

Note: Data presented as log CFU/mL ± SD ($n = 3$). Values with different superscript letters in the same column denote statistically significant differences within experimental groups ($p < 0.05$).

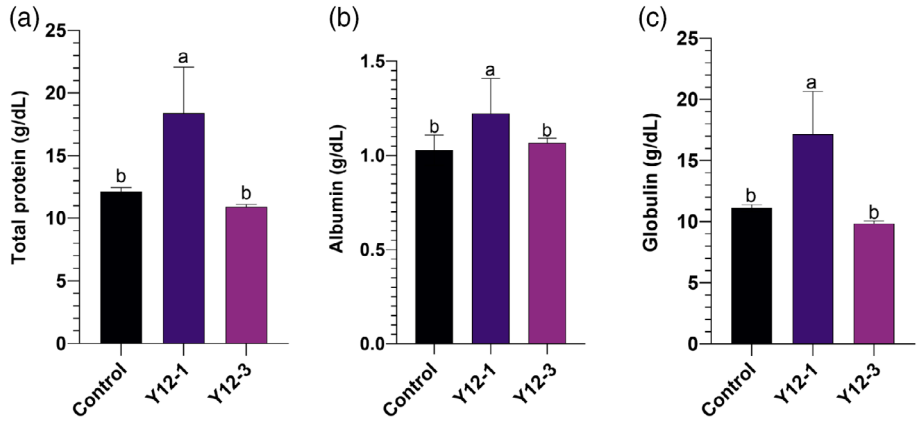

FIGURE 1 Blood protein profile includes (a) total protein, (b) albumin, and (c) globulin values of rainbow trout-fed diets supplied with the identified probiotic yeasts (Y12-1 and Y12-3) and those fed on the control diet for 60 days. Data presented as means ± SD ($n = 3$). Values with different letters denote statistically significant differences within experimental groups ($p < 0.05$).

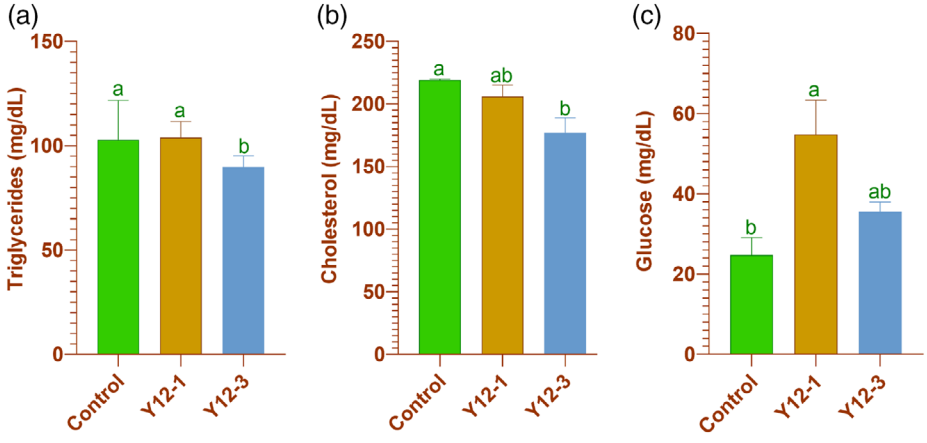

FIGURE 2 Serum biochemical measurements include (a) triglycerides, (b) cholesterol, and (c) glucose values of rainbow trout-fed diets supplied with the identified probiotic yeasts (Y12-1 and Y12-3) and those fed on the control diet for 60 days. Data presented as means ± SD ($n = 3$). Values with different letters denote statistically significant differences within experimental groups ($p < 0.05$).

probiotic groups compared to the controls (Figure 3a; $p < 0.05$). Meanwhile, the serum MPO activities revealed no significant changes among the test groups (Figure 3b; $p > 0.05$).

Regarding the challenge experiment, it was found that the cumulative SR (%) after experimental infection with *L. garvieae* were 45.24%, 69.05%, and 80.95% in the control group, Y12-1, and Y12-3 groups, respectively (Table 4). This means that the SR (%) of the Y12-3 and Y12-1 groups were significantly higher than the control group ($p < 0.05$, Table 4). Interestingly, the highest RPS was found in the Y12-3 group.

4 | DISCUSSION

Molecular identification of fungal strains through sequencing of the ITS region is a universally and widely used method for characterization and comparing several fungi (Taha et al., 2023). It is desirable for taxonomy,

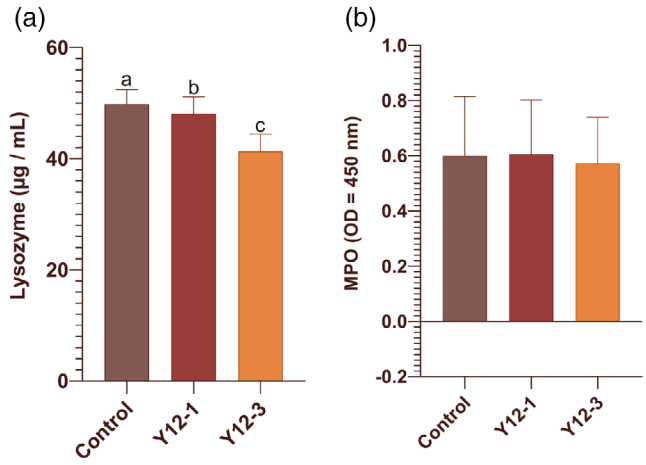

FIGURE 3 Serum immune parameters include (a) serum lysozyme activity and (b) serum myeloperoxidase (MPO) activity of rainbow trout-fed diets supplied with the identified probiotic yeasts (Y12-1 and Y12-3) and those fed on the control diet for 60 days. Data presented as means ± SD ($n = 3$). Values with different letters denote statistically significant differences within experimental groups ($p < 0.05$).

TABLE 4 Survival rate (SR, %), mortality rate (MR, %), and relative percent survival (RPS) of rainbow trout-fed diets supplied with probiotic yeasts and then experimentally challenged with *L. garvieae* infection.

Treatment group	SR (%)	MR (%)[1]	RPS[2]
Control	45.24^{c}	54.76^{a}	–
Y12-1	69.05^{b}	30.95^{b}	43.48
Y12-3	80.95^{a}	19.05^{c}	65.22

Note: Values with different superscript letters in the same column denote statistically significant differences within experimental groups ($p < 0.05$).
[1]MR (%) = (total number of fish died/total number of challenged fish) × 100.
[2]RPS (%) = [1 – (mortalities of experimental group/mortalities of control group)] × 100.

identification, and phylogenetic analysis (Sharma et al., 2023). It was successfully used to identify several fungal isolates retrieved from different fish species (Martínez et al., 2018; Reinoso et al., 2023; Taha et al., 2023). Herein, the yeast strains *C. zeylanoides* isolate Y12-3 and *H. pseudoburtonii* isolate Y12-1 have been identified for the first time from rainbow trout hindguts by sequencing of the ITS region. In a previous study, *C. zeylanoides* species was reported to be dominant in the intestines of cultivated rainbow trout, Atlantic salmon, and silver salmon (Raggi et al., 2014). In addition, different yeast species (from the genus Candida) have been previously isolated from the intestines of different fish species such as *C. nivariensis* (Pinheiro et al., 2018) from the intestines of Tambatinga fish (*Colossoma macropomum* × *Piaractus brachypomus*), *C. parapsilosis* from the intestines of *L. rohita* (Mandal & Ghosh, 2013), *C. tropicalis* (Mandal & Ghosh, 2013) and *C. rugosa* (Banerjee & Ghosh, 2014) from the intestines of Mozambique tilapia, and *C. parapsilosis* from the intestines of sturgeon (*Huso huso*) (Fami Zaghrami et al., 2021). However, no previous reports were found on the isolation and identification of *H. pseudoburtonii* from fish intestines.

In this study, Y12-1 and Y12-3 isolates showed hydrophobic properties, suggesting their ability to colonize the fish intestinal mucosa and might play a role in the prevention of colonization of pathogenic organisms. Similar findings were observed in the yeast strain *Kluyveromyces lactis* M3, which was previously isolated from marine sediments and demonstrated high cell surface hydrophobicity (Guluarte et al., 2019). Moreover, the yeast strain *Sporidiobolus ruineniae* A45.2 obtained from traditionally fermented tea leaves also had high cell surface hydrophobicity

(Kanpiengjai et al., 2020). Lately, four host-derived yeasts have been characterized from the guts of goldfish with high hydrophobic behavior (Taha et al., 2023). Indeed, hydrophobicity is an important criterion for the investigation of candidate probiotic strains. It helped in the non-specific interaction between the probiotic organisms in their attachment to intestinal epithelial cells (Gut et al., 2019). This interaction can provide important benefits for the treated hosts because of their ability to colonize the host intestinal mucous membranes with probiotics and then help in the prevention of the adhesion of the challenging bacterial pathogens (Kavitha et al., 2018).

For a probiotic, tolerance to fish bile is a very important feature to survive in the fish intestine, where bile from the liver is secreted (Balcázar et al., 2008). In our study, the identified yeasts were tolerant to bile salts (0.6%–1.5%). Similarly, yeast strains *Sporidiobolus ruineniae* A45.2, *D. hansenii* BCS004, *Kluyveromyces lactis* M3, and *Pichia kudriavzevii* ONF7.1C have been reported to be tolerant to bile salts (Ghosh et al., 2017; Kanpiengjai et al., 2020; Reyes-Becerril et al., 2021). Furthermore, the yeast strains jpn01, jpn02, jpn05, and jpn06 isolated from the gut of goldfish grew well and tolerated low pH and bile salt concentrations (Taha et al., 2023). The pH of gastric juice is the main factor determining the survival of bacteria that pass from the stomach to the intestine (Balcázar et al., 2008). In our study, yeast strains were found tolerant to low pH conditions (pH 2 and 3). Similarly, it has been reported that *C. tropicalis* and *K. exigua* isolated guts of rainbow trout show high tolerance to pH 2 (Martínez et al., 2018). In addition, Kanpiengjai et al. (2020) reported that *Sporidiobolus ruineniae* A45.2 was tolerant to pH 2 (Kanpiengjai et al., 2020). It has been noted that the yeast strain *D. hansenii* BCS004 isolated from surface seawater can also survive under low pH conditions (Reyes-Becerril et al., 2021).

Another crucial factor to consider when choosing a new candidate probiotic is co-aggregation (Menezes et al., 2020). A potential probiotic's ability to co-aggregate with pathogenic bacterial strains aids in building a barrier and confers protection against the colonization of bacterial pathogens in the host gut (Collado et al., 2007; Jankovic et al., 2012). According to Kos et al. (2003), yeasts, especially those belonging to the genus *Saccharomyces*, may attach to the cell wall surface of pathogenic organisms, resulting in the creation of a yeast–bacteria complex that aids in the defense mechanisms against invasive pathogens. Pizzolitto et al. (2011) showed that the capacity of yeast to bind to a microorganism varies depending on the yeast strain and the related bacterium. In our study, Y12-1 and Y12-3 showed a high co-aggregation ability to all tested bacterial pathogens. Similarly, Martínez et al. (2018) also reported that *K. exigua* RC037 and RC038 strains isolated from the intestines of rainbow trout have the potential to co-aggregate with *Ps. aeruginosa*. Kanpiengjai et al. (2016, 2020) also noted that *Sporidiobolus ruineniae* A45.2 can co-aggregate with several bacterial pathogens such as *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Salmonella ser. typhimurium*.

By altering intestinal cholesterol absorption and metabolism, probiotics may change the amount of total cholesterol in fish (Vazirzadeh et al., 2020). In our study, the probiotic yeast strains caused a decrease in the total cholesterol in rainbow trout. Similarly, Vazirzadeh et al. (2020) reported that the addition of *S. cerevisiae* to the diet significantly reduced serum cholesterol in rainbow trout. Mocanu et al. (2022) also reported a decrease in cholesterol in Siberian Sturgeon (*Acipenser baerii*) after feeding a diet supplied with *Saccharomyces boulardii*.

In our study, the addition of Y12-3 to the feed of rainbow trout had no effect on the levels of triglycerides, total protein, albumin, or globulin. Similarly, Hoseinifar et al. (2011) declared that the addition of *S. cerevisiae* var. *ellipsoideus* to the feed of great sturgeon (*Huso huso*) had no effect on serum cholesterol, glucose, and total protein values. Reyes-Becerril et al. (2011) also noted that the addition of *D. hansenii* (CBS 8339) isolated from the intestines of rainbow trout to the feed of grouper (*Mycteroperca rosacea*) did not affect plasma protein values. Boonanuntanasarn et al. (2019) also reported that feeding striped catfish (*Pangasianodon hypophthalmus*) with *S. cerevisiae* in microcapsules had no effect on cholesterol, glucose, triglycerides, protein, and albumin. On the other hand, Abdel-Tawwab et al. (2008) observed that the addition of live baker's yeast *S. cerevisiae* at the rates of 0.025%, 0.5%, and 1% to the feed of Nile tilapia increased the total protein, glucose, albumin, and globulin values. In our study, glucose, total protein, and globulin values were also found to be higher in fish fed with Y12-1 compared to the control group. These results showed an improvement in fish health when fish were fed with a Y12-1 yeast supplement.

In this study, Y12-1 and Y12-3 strains significantly decreased serum LYZ activity alongside no effects on serum MPO activity in rainbow trout. Reyes-Becerril et al. (2011) reported that adding the *D. hansenii* (CBS 8339) strain from rainbow trout intestines to the feed of juvenile leopard grouper and feeding them for 4 weeks did not affect serum MPO activity. Li and Gatlin III (2003) also noticed that the addition of *S. cerevisiae* to the feed of hybrid striped bass did not increase serum LYZ activity. According to Boonanuntanasarn et al. (2019), feeding *P. hypophthalmus* with *S. cerevisiae* in microcapsule had no impact on their LYZ activity. On the other hand, Hernández-Contreras et al. (2021) found that the addition of *D. hansenii* CBS 8339 to the feed of yellowtail (*Seriola rivoliana*) did not affect MPO activity in mucus but increased LYZ activity. Reyes-Becerril et al. (2017) also noted that application of *Sterigmatomyces halophilus* 04 N-16 isolated from marine sediment was added to the feed of gilthead seabream at a rate of 0.55%, the activity of MPO determined in the mucus samples of the fish on the 15th and 30th days of feeding was higher than in the control, and LYZ activity in mucus increased with the addition of yeast strain to the diet at a rate of 1.1%. Different results can be related to the feeding duration, water conditions, yeast strain, size, and species of fish.

In this study, as a result of the fish challenge with *L. garvieae*, the survival rates of the fish fed with Y12-1 and Y12-3 were higher than the control. In a similar sense, Li and Gatlin III (2003) reported that a hybrid striped bass-fed diet with *S. cerevisiae* had higher resistance against challenge with *Str. iniae* infection. In contrast, Pinpimai et al. (2015) noted that dietary supplementation with encapsulated *S. cerevisiae* JCM did not provide disease resistance of Nile tilapia against *Str. agalactiae* infection. Differences between studies may be due to different fish species. Several hypotheses have been proposed to declare the role of probiotic yeasts in enhancing disease resistance in fish, such as potential antagonistic activity, production of antimicrobial substances, and/or enhancement of gut mucosal immunity (El-Saadony et al., 2021; Mahdy et al., 2022). However, the precise mechanisms by which the probiotic yeasts reduce the cumulative mortalities of fish experimentally challenged with bacteria pathogens are not fully understood.

5 | CONCLUSİONS AND PERSPECTİVES

In brief, the autochthonous yeast strains Y12-1 and Y12-3 isolated from the intestines of rainbow trout showed a co-aggregation to different bacterial pathogens, tolerance to different bile salts and pH values, and higher hydrophobicity criteria. After in vivo experimentation, the dietary application of these strains provided higher resistance of rainbow trout against *L. garvieae* infection. These results suggest that Y12-1 and Y12-3 strains could be candidate probiotic strains and may be beneficial for use in trout farming. This should be considered only as an initial preliminary step, and further experimentations should be considered. Several points should be carefully taken into account while considering the use of novel probiotic yeast strains in aquafeed. For instance, the in vitro tests of the identified yeasts do not truthfully manifest and mimic their actual conditions in the fish gut ecosystems, as elucidated by Taha et al. (2023). In addition, the physiological activities of the fish may also affect the survivability and responsiveness toward the tested yeast strains. Lastly, the food ingredients may protect probiotic yeasts from the negative effects of the host bile and gastrointestinal secretions (Begley et al., 2005). Hence, future experiments are still required to describe the host–probiotic interactions at molecular, nutrigenomic, and gut microbiome levels. Moreover, the effects of Y12-1 and Y12-3 strains on growth performance, feed utilization, and feed efficiency should also be studied after a longer feeding period.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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