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Research Article

Detection of Viable *Zygosaccharomyces rouxii* in Honey and Honey Products via PMAXX-qPCR

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In order to establish a fast detection method for the living *Zygosaccharomyces rouxii* (*Z. rouxii*) cells in honey and honey products, the performance of propidium monoazide bromide (PMA) and enhanced propidium monoazide bromide (PMAXX) combined with real-time PCR for detecting living cells of *Z. rouxii* was compared. PMAXX was chosen as the added agent because of its better performance. The optimal concentration of PMAXX was found to be 76.92 μM in cell solution (the cell concentration was 1.0×10^8 CFU/mL). The LODs of PMAXX-qPCR in detecting *Z. rouxii* in pure MEA and honey solution were found to be 10^3 and 10^1 CFU/mL, respectively. Living *Z. rouxii* cells in 18 real honey samples were detected using this PMAXX-qPCR method and compared with the plate count method. The two methods showed consistent detection results in ten negative samples. In the other eight plate count zero but PMAXX-qPCR-positive samples, further verification experiments showed that six of the PMAXX-qPCR-positive samples contained viable but nonculturable (VBNC) *Z. rouxii*, while the other two PMAXX-qPCR-positive samples may have contained DNA contamination of *Z. rouxii*. This method is not only fast and sensitive but also can detect both culturable and viable but nonculturable *Z. rouxii*. This study provides a promising fast and culture-independent method for the detection of living *Z. rouxii* cells in honey and honey products.

1. Introduction

Honey is usually used as a sweetener, and in some regions of Mexico and other countries, it is also used as a therapeutic agent [1]. It has a high sugar content, mainly fructose and glucose, low water activity ($a_w = 0.50–0.60$), high osmotic potential with a humidity lower than 18%, and low pH (3.4–6.1) [1, 2]. Honey also contains some substances with antimicrobial activity such as hydrogen peroxide and several phytochemical compounds such as flavonoids, phenols, organic acids such as cinnamic acid, methyl syringate, and methylglyoxal, which limit the number of microorganisms present and allow only a few to remain viable, such as yeasts [1, 3–6]. There are two major categories of yeasts, osmophilic yeasts and osmotolerant yeasts, that significantly influence the quality of honey [1,5,6].

Zygosaccharomyces rouxii (*Z. rouxii*) is a common type of osmotolerant yeast that is extensively distributed in foods with high sugar and salt contents [5–10]. The osmotolerant and halotolerant food yeast *Z. rouxii* is known for its ability to grow and survive in the face of stress caused by high concentrations of nonionic (sugars and polyols) and ionic (mainly Na + cations) solutes. This ability leads to spoilage of high-sugar and high-salt foods [10]. To adapt to a high-sugar and high-salt environment, *Z. rouxii* adjusts the permeability and liquidity of its cell wall or plasma membrane and the dynamic balance of cations, sugar transfer, biosynthesis, and the pathway for accumulation glycerinum through genetic or metabolic pathways (high-salt and high-sugar environment may induce high expression of glycerol metabolism-related genes in osmophilic and osmotolerant yeasts, leading to the increase of glycerol content in the cell

structure of osmophilic and osmotolerant yeasts under high osmotic pressure, thus enabling osmophilic and osmotolerant yeasts to adapt to high osmotic pressure environment) [10]. *Z. rouxii* has been found in honey [11], concentrated grape juice [12], and apple juice [13]. Chen et al. isolated 60 yeast strains from honey and identified 21 strains belonging to *Z. rouxii* through real-time PCR [11]. This meant that 35% of the 60 strains of yeast isolated from honey were *Z. rouxii*. *Z. rouxii* can result in the deterioration of many kinds of foods and drinks, including honey and its products, concentrated grape juice, apple juice, and even ice cream [5, 8, 12–16]. It also adversely affects the shelf life, quality, and stability of these foods [5, 8, 12–16].

Osmotolerant yeast contamination in honey has two main sources. One source includes nectar, bees, soil from bee farms, and honeycombs. This type of source involves multiple species and complicated kinds of microorganisms that are difficult to control in practical production. The other source comes from the production and processing after raw honey collection, including contacted air, contacted operators, and equipment and container contamination during production. This source can theoretically be controlled through strict management measures [1, 5–7].

Osmotolerant yeasts in honey, including *Z. rouxii*, are generally detected through traditional culture methods. However, these methods are time consuming (1–2 weeks) and involve complicated operation steps [15, 16]. Moreover, osmotolerant yeasts may be underestimated because traditional methods can only be used to detect cultivable microorganisms and not uncultivable microorganisms in samples. Therefore, such methods cannot completely meet the requirements of the real-time quality and safety monitoring of honey and honey products under emergency conditions. Moreover, because of the underestimation of the target microorganism, culture-based methods may lead to the risk of food corruption and even the risk of food-borne disease outbreaks. Molecular biological techniques can provide a new alternative to rapidly detect *Z. rouxii* in honey and its products. Real-time PCR (qPCR) offers a highly sensitive culture-independent quantification method. It can be combined with DNA-intercalating agents, such as propidium monoazide bromide (PMA) and enhanced PMA (PMAXX), which can enter dead cells and crosslink to DNA, thereby impeding DNA amplification during PCR. In this way, viable cells with an intact membrane can be differentiated from dead cells [17–26]. The efficiency of PMAXX differs from that of PMA. For example, some studies have shown that PMAXX, an enhanced type of PMA, has a higher activity and a stronger ability to distinguish dead and living cells [27–31].

In this study, PMAXX and PMA were combined with qPCR based on our previous studies [11, 32, 33] to set up a method for rapidly detecting living *Z. rouxii* cells in honey and its products. The performance of these two dyes was compared. Our results showed that PMAXX was superior to PMA. PMAXX was therefore selected in the following assay. The added concentration of PMAXX was optimized, and the limits of detection (LODs) of PMAXX-qPCR in pure Malt Extract Agar (MEA) [34], 55% honey solution, and 70% honey solution were determined. The proposed PMAXX-qPCR method was applied to detect living *Z. rouxii* in 18 real honey samples. The detection results were then compared with those of the plate counting method.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions. *Zygosaccharomyces rouxii* CGMCC 2.1915 was purchased from the China General Microbiological Culture Collection Centre (CGMCC), and *Z. rouxii* CICC 1417 and CICC 31259 were bought from the China Centre of Industrial Culture Collection. They were used as testing strains. All strains were stored at −80°C and were streaked onto Malt Extract Agar (MEA) (Beijing Land Bridge Technology Co., Ltd., China) [34] in an incubator at 28°C for 48 h. Then, the fresh live cells were washed with sterile water from the MEA (the cell concentration was adjusted to 1.0×10^8 CFU/mL and was determined by the plate counting method) before use. The other osmotolerant yeasts used in this paper were isolated from honey samples which were randomly bought from supermarkets in Beijing.

2.2. DNA Extraction and Quantitative Real-Time PCR. DNA was extracted using a TIANamp yeast DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). DNA concentration and quality were estimated with NanoDrop ND-1000 (Thermo Scientific, USA), and the samples were stored at −20°C.

qPCR was performed in a QuantStudio 7 Flex (Applied Biosystems of Life Technologies, USA). Real-time PCR was carried out in a total volume of 20 μL containing 25–50 ng of DNA template, 10 μL of TaqMan Gene Expression Master Mix (Applied Biosystems, USA), 0.2 μM forward primer, 0.2 μM reverse primer, and 0.1 μM of *Z. rouxii* specific probe. The sequences of the primers and the probe were the same as in our previous studies: forward primer, 5'-CCA CGA TAG TCG TAT TAG G-3'; reverse primer, 5'-TGA GGT CAA ACT TTG AGA A-3'; and probe, 5'-FAM-CCA GAC GCT GCC TGC TTC TA-TAMER-3' [31]. The qPCR conditions were as follows: 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 60 s, which were optimized in our previous studies [11,35].

2.3. Heat Treatment Conditions. Based on our previous study [32], the heat treatment conditions were as follows: 20 min of heat treatment at 90°C in a metal bath, which was performed with a constant-temperature mixing instrument (TS100, Hangzhou RuiCheng Instrument Co., Ltd.) to obtain dead *Z. rouxii* cells needed for this study.

2.4. Comparison of PMA-qPCR and PMAXX-qPCR Effects. PMA (Biotium, USA) solution (2 mM) was prepared using 20% dimethyl sulfoxide (DMSO; Tianjin Fuyu Fine Chemical Co., Ltd., China) [17] and stored at −20°C. Then, 20 mM PMAXX (20 mM PMAXX in water, a new type of enhanced PMA, Biotium Company, USA) was stored in the

Table 1: Comparison of the effect of PMA and PMAXX on detection of viable *Z. rouxii*.

Dyes or compounds	Concentration (μM)	Ct[a] (viable cells)	Ct[b] (dead cells)	ddCt[c]
PMA	0.00	18.193 ± 0.054	19.824 ± 0.139	1.29
	19.80	18.438 ± 0.213	25.085 ± 0.560	7.09
	27.61	19.502 ± 0.100	25.241 ± 0.269	5.54
	31.50	19.081 ± 0.101	26.062 ± 0.019	19.23
	39.22	21.353 ± 0.051	26.908 ± 0.007	14.32
	58.25	19.980 ± 0.079	27.839 ± 0.133	9.99
	76.92	19.581 ± 0.023	27.021 ± 0.020	14.25
	113.21	20.076 ± 0.072	27.394 ± 0.026	12.24
	148.15	19.764 ± 0.090	27.612 ± 0.330	
PMAXX	0.00	18.193 ± 0.054	19.824 ± 0.139	8.52
	2.00	21.469 ± 0.064	22.581 ± 0.178	10.20
	2.80	21.892 ± 0.007	22.853 ± 0.601	16.50
	3.99	22.015 ± 0.220	24.142 ± 0.054	34.09
	23.72	22.279 ± 0.164	28.168 ± 0.256	34.05
	27.61	22.235 ± 0.085	28.249 ± 0.061	27.83
	31.50	21.184 ± 0.256	29.130 ± 0.094	27.63
	39.22	20.938 ± 0.247	29.888 ± 0.152	32.86
	58.25	21.276 ± 0.179	30.483 ± 0.223	37.29
	76.92	20.987 ± 0.380	33.171 ± 0.254	29.25
	113.21	20.496 ± 0.025	32.526 ± 0.014	96.09
	148.15	28.616 ± 0.035	29.043 ± 0.104	

Ct[a] and Ct[b]: mean Ct value ± standard deviation (SD); ddCt[c] = dCt (dead cells) × dCt (viable cells); dCt (dead cells) = Ct (dead cells with dye)–Ct (dead cells without dye); dCt (viable cells) = Ct (viable cells with dye)–Ct (viable cells without dye).

dark at −20°C and diluted to 2 mM by adding PCR water before use.

The added concentrations of PMA and PMAXX in a suspension with 500 μl/tube cells (heat treatment group D and nonheat treatment group L) are listed in Table 1. For PMA and PMAXX, the final concentrations in the cell suspension were from 0.00 μM to 148.15 μM.

Cell suspensions with PMA and PMAXX were mixed thoroughly and stored in the dark for 10 min. Then, the samples were transferred to ice and exposed to a 650 W halogen lamp twice for 10 min each with an interval of 1 min. The distance between the light source and the samples was 20 cm [32].

Sediments were centrifuged at 12,000 rpm for 3 min and collected; DNA was extracted by using a TIANamp yeast DNA kit for qPCR amplification. According to the PMAXX-qPCR amplification results, ddCts were calculated using the following equations: ddCt = dCt (dead cells) × dCt (viable cells); dCt (dead cells) = Ct (dead cells with dye) – Ct (dead cells without dye); and dCt (viable cells) = Ct (viable cells with dye) – Ct (viable cells without dye) [27]. The performance of PMAXX and PMA in distinguishing dead and living *Z. rouxii* cells was evaluated by comparing their ddCt, and the optimal reagent was selected. The added concentration of the chosen reagent was also optimized.

2.5. The LOD of PMAXX-qPCR in Distinguishing Pure Cultured Z. rouxii Living Cells. *Z. rouxii* on MEA was rinsed with sterile water, and the cell concentration was adjusted to 1.0×10^8 CFU/ml. The samples in 500 μl/tube were divided into a heat treatment group *D* (treated at 90°C, 20 min) and a nonheat treatment group L. To each tube, PMAXX was added to obtain the optimal concentration given in Section 2.4 (which was 76.92 μM). Then, all the sample tubes were kept in the dark for 10 min. Afterward, the sample tubes were placed on ice and exposed to a 650 W halogen lamp for 10 min for twice. Sediments were centrifuged at 12,000 rpm for 3 min and collected. Then, DNA was extracted with a TIANamp yeast DNA kit as described in Section 2.2. DNA was ten-fold serially diluted to 10^{-8} for qPCR amplification. The LOD of PMAXX-qPCR in detecting living *Z. rouxii* cells in pure MEA was obtained by analyzing the amplification results.

2.6. The LOD of PMAXX-qPCR in Honey Solutions. Two 3-ml cell suspensions of fresh *Z. rouxii* standard strains (CGMCC 2.1915) with a cell concentration of 1.0×10^8 CFU/mL were cultured in 100 ml of 55% and 70% sterilized honey solutions (W/W) and cultivated at 28°C for 5 d. Next, 500 μl/tube of cell suspension was divided into two groups (heat treatment group *D* and nonheat treatment group L, 2 tubes each). Thalli were collected after the suspension was centrifuged at 12,000 rpm for 3 min. To each tube, 500 μl of sterilized water was added. The D group was initially treated under thermal conditions (90°C) for 20 min and subsequently treated with PMAXX (under the same conditions as in Section 2.4). Samples without PMAXX treatment were used as the control group. Later, DNA was extracted with a TIANamp yeast DNA kit. The extracted DNA was serially ten-fold diluted to 10^{-8} with DNA-free water for qPCR amplification (triplicates for each sample). The LOD of PMAXX-qPCR could be obtained on the basis of the results of qPCR. Cell concentrations in the culture solutions were also determined with the plate count method.

2.7. Detection of Z. rouxii Living Cells in Honey Samples. To assess the ability of the PMAXX-qPCR assay to distinguish viable and dead *Z. rouxii* in honey, we applied this assay to detect viable *Z. rouxii* cells in honey. Eighteen honey samples were purchased randomly from supermarkets in Beijing. Four tubes of honey (500 μl in each tube and divided into D and L groups, with two tubes in each group) were collected from each of the 18 honey samples. Then, the honey samples in each tube were washed twice using 1 ml of sterile water and centrifuged at 12,000 rpm for 3 min. Sediments were collected and mixed evenly with 500 μl of sterile water. The D group was treated at 90°C for 20 min. Both the D and L groups were treated with 76.92 μM PMAXX in the dark and exposed to intensive light. The honey sample without treatment with PMAXX was used as the control. Subsequently, DNA was extracted for qPCR amplification, and the plate count method was conducted in accordance with GB 14963–2011 [36] (a Chinese standard plate count method for osmotolerant yeasts, including *Z. rouxii* in honey). The sensitivity, accuracy, specificity, and application prospects of PMAXX-qPCR were evaluated on the basis of PMAXX-qPCR results and plate counts.

2.8. Verification of Suspicious Samples. Eight honey samples with negative results from the plate count method but with Ct values that ranged from 34.134 to 38.611 by the PMAXX-qPCR method were diluted with sterile water to 50% honey concentration and cultured at 28°C for 48 hours. Then, clonidine 18% glycerol (DG18) agar plates (Beijing Land Bridge Technology Co., Ltd., China) [36]) were used to detect the osmotolerant yeasts by the plate count method and determine whether the samples contained viable but unculturable osmotolerant yeasts. At the same time, 1 mL of a 50% diluted culture solution of the eight suspicious samples was taken from each sample and then treated with PMAXX, DNA was extracted with a TIANamp yeast DNA kit, and the samples were subjected to qPCR analysis. Therefore, PMAXX-qPCR detection was also carried out on the eight suspicious samples at a 50% dilution.

3. Results and Discussion

3.1. Comparison of PMA and PMAXX in Distinguishing Dead and Living Cells in Z. rouxii. The performance of PMA and PMAXX in distinguishing dead and living *Z. rouxii* cells is summarized in Table 1. The results showed that both PMA and PMAXX slightly affected the Ct value of living *Z. rouxii* cells when the concentrations were within 0.00–113.21 μM and the Ct values of PMAXX-qPCR were within 18.193–22.279 (Table 1). This result indicates that PMA and PMAXX within this concentration range could not penetrate living *Z. rouxii* because its cell wall was intact; thus, qPCR results were not affected. When the PMA or PMAXX concentrations were increased to the maximum value (148.15 μM), the Ct value of PMA in living *Z. rouxii* cells still remained essentially unchanged (19.764); however, the Ct value of PMAXX-qPCR increased significantly (28.616). This

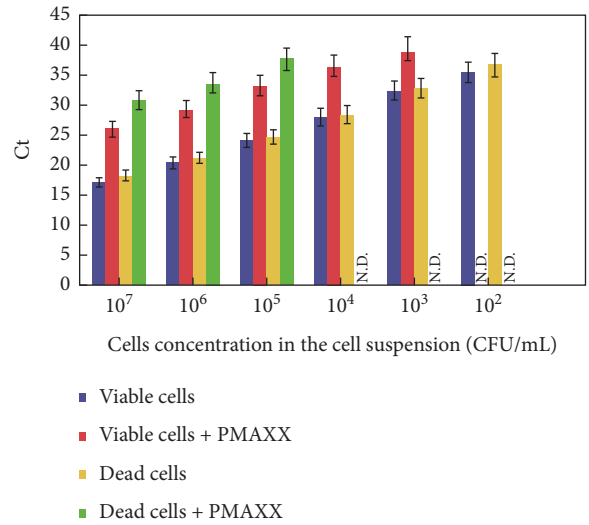

Figure 1: Detection limit of PMAXX-qPCR of *Z. rouxii* cultured in MEA. The cell concentration was 1.0×10^8 CFU/mL, and the PMAXX concentration was 76.92 μM.

variation might be caused by differences in the properties of PMA and PMAXX.

For the dead cell group, the Ct values of the PMA or PMAXX treatment group were higher than those of the control group (without treatment of PMA or PMAXX). When the PMA concentration was smaller than or equal to 58.25 μM and the PMAXX concentration was smaller than or equal to 76.92 μM, the Ct value was positively related to the dye concentration. The maximum Ct value (27.839) of the dead cells was achieved when the PMA concentration increased to 58.25 μM. After that, the Ct values remained stable as the PMA concentration continuously increased. This phenomenon was consistent with previous results [32], indicating the presence of a saturated PMA concentration in the dead cells. For PMAXX, the maximum Ct value (33.171) of the dead cells was achieved at 76.92 μM. Afterward, the Ct value began to decrease gradually as the PMAXX concentration increased. The differences in the performance of PMA-qPCR and PMAXX-qPCR in distinguishing dead and living *Z. rouxii* cells might be attributed to the different properties of PMA and PMAXX.

As the concentration of PMA increased, the ddCt value of PMA initially increased and then decreased (Table 1). When the PMA concentration was 39.22 μM, the ddCt value (ddCt = dCt (dead cells) × dCt (viable cells); dCt (dead cells) = Ct (dead cells with dye)–Ct (dead cells without dye); dCt (viable cells) = Ct (viable cells with dye)–Ct (viable cells without dye)) [27] was calculated to be 19.23, which was the maximum ddCt of PMA.

For PMAXX, after treatment with PMAXX at 148.15 μM, the ddCt value reached 96.09, which was the maximum ddCt of PMAXX. However, this concentration of PMAXX was too high for viable *Z. rouxii* cells (see Table 1; the Ct value of living *Z. rouxii* cells at this PMAXX concentration was 28.616, which was 10.423 higher than the control Ct value of living *Z. rouxii* cells, which was 18.193).

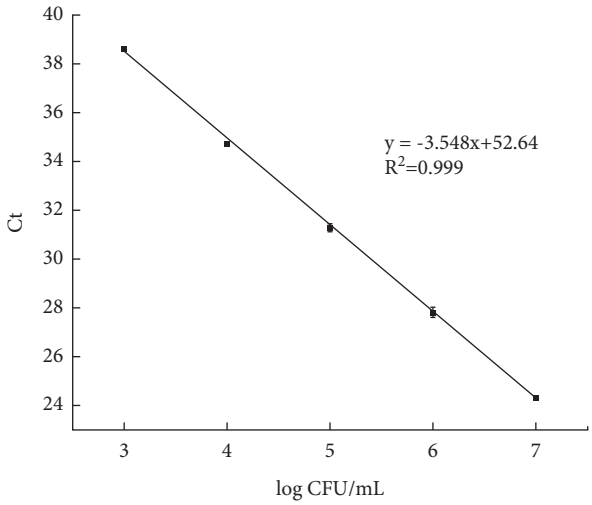

Figure 2: Correlation between population in MEA plate and the Ct of PMAXX-qPCR for purely cultured *Z. rouxii*. The cell concentration was 1.0×10^7 CFU/mL, and the PMAXX concentration was 76.92 μM.

The second highest ddCt value was calculated when the PMAXX concentration was 76.92 μM (Table 1.). This treatment did not affect viable *Z. rouxii* cells (the Ct value of living *Z. rouxii* cells at this PMAXX concentration was 20.987, which was very near the Ct value of the control living *Z. rouxii* cells, and the Ct value of dead *Z. rouxii* cells was 33.171, which was 13.347 higher than that of dead cell control Ct value (19.824). This meant that when PMAXX concentration was at 76.92 μM, the dead and living *Z. rouxii* cells could be clearly distinguished by this PMAXX-qPCR method.

Based on the ddCt values calculated from viable and dead cells, PMAXX was selected as the dye applied in this study, and the optimal concentration of PMAXX was selected to be 76.92 μM (related data are shown in Table 1).

3.2. The LOD of PMAXX-qPCR in Detecting Living Z. rouxii Cells in Pure Culture. The experimental results of the LOD of PMAXX-qPCR in pure cultures are shown in Figure 1. The trends of variations in Ct of qPCR were consistent for both living and dead *Z. rouxii* cells when no PMAXX treatment was adopted. That is, Ct values increased when the concentration of the cell suspension decreased within the range of 10^2–10^7 CFU/ml. This finding implies that qPCR amplification without PMAXX treatment could not be applied to distinguish dead and living *Z. rouxii* cells. Moreover, at the same cell concentration, the Ct of the dead group was higher than that of the living group possibly because the DNA in dead cells was partially damaged or degraded by the heat treatment.

For dead *Z. rouxii* cells, when the cell concentration was lower than 10^4 CFU/ml, the qPCR amplification of dead cells was thoroughly inhibited after the cell suspension was treated with PMAXX at a final concentration of 76.92 μM. However, for the living *Z. rouxii* cells, the LOD of PMAXX-qPCR was 10^3 CFU/mL which was higher than that of qPCR for dead or living *Z. rouxii* cells without PMAXX treatment (which was 10^2 CFU/mL).

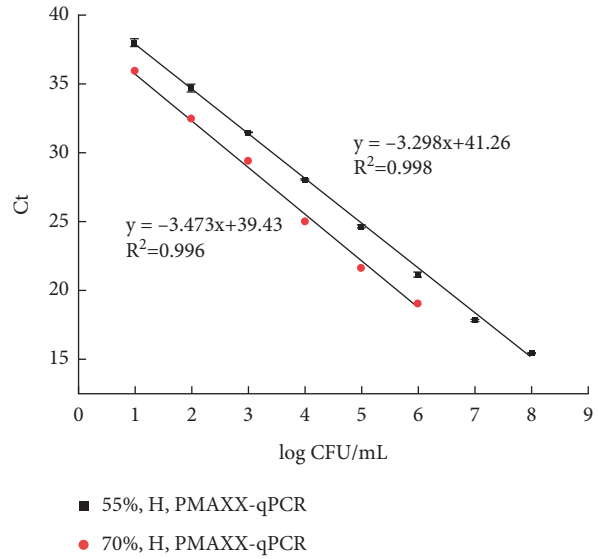

Figure 3: Detection limit of *Z. rouxii* in 55% and 70% honey solutions by PMAXX-qPCR. The PMAXX concentration was 76.92 μM. The cell concentration of *Z. rouxii* in 55% honey solution was 1.0×10^8 CFU/mL, and that in 70% honey solution was 1.0×10^6 CFU/mL.

The relationship between the logarithm of the cell concentration of the plate count (log CFU/ml) and Ct of PMAXX-qPCR is shown in Figure 2. Clearly, log CFU/ml showed a very good linear relationship with Ct of PMAXX-qPCR when the cell concentration of *Z. rouxii* was within the range of 10^3–10^7 CFU/mL and the linear equation was $y = -3.548x + 52.64$, $R^2 = 0.999$. This standard curve also indicates that the concentration of living *Z. rouxii* cells can be calculated on the basis of Ct of PMAXX-qPCR within a concentration range of *Z. rouxii* cells between 10^3 and 10^7 CFU/mL and under the detection conditions in this study.

3.3. LOD of Living Z. rouxii Cells in Honey Solutions. The LOD of PMAXX-qPCR for *Z. rouxii* in 55% and 70% honey solutions is shown in Figure 3. The plate count results revealed that the concentration of viable cells could reach 1.0×10^8 CFU/mL when *Z. rouxii* was cultured in 55% honey solution and cultivated for 5 days at 28°C. Under the same culture conditions, the concentration of the cells in 70% honey solution was only 1.0×10^6 CFU/mL. Therefore, 55% honey solution was more beneficial to the growth and reproduction of *Z. rouxii* than 70% honey solution. This result also indicates that *Z. rouxii* is an osmotolerant yeast rather than an osmophilic yeast.

In Figure 3, the linear relationship between Ct of PMAXX-qPCR and log CFU/mL ($R^2 = 0.9988$) was good when the viable cell concentration of *Z. rouxii* was within 10^1–10^8 CFU/mL in 55% honey solution. In 70% honey solution, the linear relationship between Ct of PMAXX-qPCR and log CFU/ml ($R^2 = 0.9960$) was also good when the viable cell concentration of *Z. rouxii* was within 10^1–10^6 CFU/mL. In other words, Ct of PMAXX-qPCR and log CFU/mL maintained an obvious linear relationship in 55% and 70% honey solutions. This linear relationship with a negative slope was consistent with the trend of *Z. rouxii* in

Table 2: Detection results of viable *Z. rouxii* cells in honey samples by PMAXX-qPCR, qPCR, and plate count.

Sample no.	[e]Ct, PMAXX-qPCR	[f]Ct, qPCR	Plate count result (CFU/ml)
1	[d]N	28.346	0
2	36.337	33.303	0
3	36.465	25.272	0
4	[d]N	31.045	0
5	35.764	33.030	0
6	37.855	30.490	0
7	36.310	27.390	0
8	[d]N	35.731	0
9	38.611	36.180	0
10	34.134	24.198	0
11	[d]N	38.001	0
12	35.302	29.252	0
13	[d]N	29.252	0
14	[d]N	31.223	0
15	[d]N	34.472	0
16	[d]N	34.437	0
17	[d]N	33.082	0
18	[d]N	24.208	0
PC	—	20.673	—
NC	[d]N	[d]N	0

[d]N, undetermined; [e]Ct, PMAXX-qPCR: Ct value of qPCR with the treatment of PMAXX; [f]Ct, qPCR: Ct value of qPCR without the treatment of PMAXX.

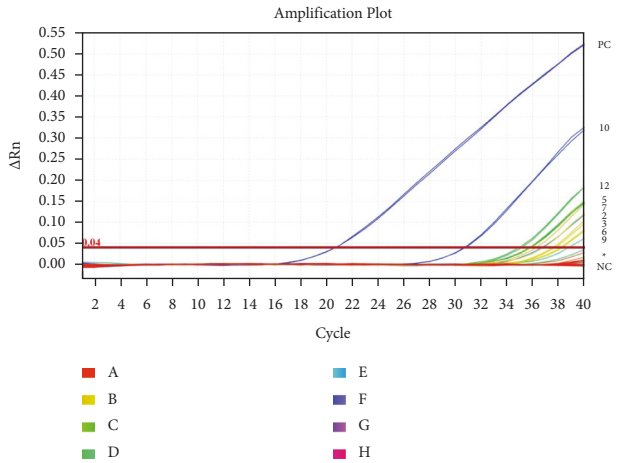

Figure 4: The PMAXX-qPCR amplification curves of living *Z. rouxii* in 18 honey samples. Note: the sample numbers on the right side (PC, 10, 12, 5, 2, 7, 3, 6, 9, and NC) are the same as those in Table 2. Among them, the asterisk (*) between 9 and NC represents the 10 samples whose amplification results were "undetermined."

pure MEA. However, the LODs in the honey solutions were lower than those in pure MEA, possibly because the ingredients in honey could protect the cell wall of *Z. rouxii*; therefore, PMAXX could not easily enter living *Z. rouxii* cells in honey solution compared to MEA. Thus, living cells were more easily detected in honey solution than in MEA.

3.4. Detection of Living Z. rouxii Cells in Real Honey Samples. The results of PMAXX-qPCR, qPCR, and plate counting of living *Z. rouxii* cells in 18 real honey samples are shown in

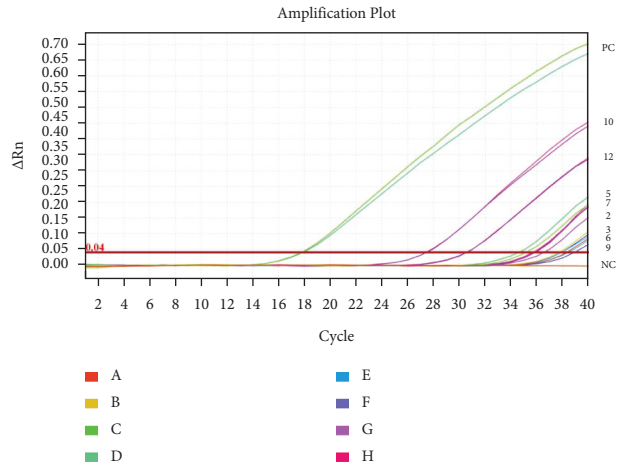

Figure 5: The PMAXX-qPCR amplification curves of living *Z. rouxii* in 8 suspicious honey sample cultures after diluted to 50%. Note: the sample numbers (PC, 10, 12, 5, 2, 7, 3, 6, 9, and NC) on the right side of the figure are the same as those in Table 2.

Table 2 and Figure 4. Although the plate counting results indicate that viable *Z. rouxii* cells were absent in all 18 honey samples, Ct of qPCR without PMAXX treatment implied that all 18 honey samples contained *Z. rouxii* DNA and ranged from 24.208 to 38.001. According to the detection results of PMAXX-qPCR, the eight suspicious samples still had amplification curves, as shown in Figure 4. However, we were unable to determine whether the DNA came from viable but nonculturable (VBNC) yeasts, dead yeast, or contamination. Therefore, qPCR without PMAXX treatment could not distinguish living and dead *Z. rouxii* cells.

The detection results of PMAXX-qPCR also showed that Ct of the ten samples was undetermined, which was consistent with the plate counting results. The Ct of the other eight samples (44% of the total tested samples) ranged from 34.134 to 38.611. This difference in Ct between PMAXX-qPCR and plate counting results might be a consequence of the existence of viable but nonculturable *Z. rouxii* cells or DNA contamination in these samples. Therefore, further studies should determine whether viable but nonculturable *Z. rouxii* cells existed in these samples or not to explain the differences in the results between the PMAXX-qPCR and plate counting methods.

3.5. The Verified Results of the Suspicious Samples. The eight suspicious honey samples that had negative results with the plate count method but had Ct values by the PMAXX-qPCR method were diluted into 50% honey concentration with sterile water and verified using DG18 agar three times. According to the requirements of the standard method [36], the DG18 agar plates spread with samples or sample dilutions were cultured at 25°C for 7 days. The results showed that six out of the eight suspicious samples actually contained osmotolerant yeasts; that is, the yeast colonies grew on DG18 agar plates.

Some typical yeast colonies from DG18 agar plates were picked, mixed with methylene blue staining solution and

coated onto slides, observed with a microscope, and compared with the standard strains. Based on the morphological characteristics, all were yeasts. The results of PMAXX-qPCR detection of 50% diluted cultures of the eight suspicious samples show that these eight samples still had amplification curves (see Figure 5). These results indicate that VBNC osmotolerant yeasts were present in six of the eight suspicious honey samples. There may be DNA contamination in the other two of the eight suspicious samples.

4. Conclusions

In this study, a rapid PMAXX-qPCR method for the detection of *Z. rouxii* living cells in honey and honey products was established for the first time. The method can shorten the detection of *Z. rouxii* from 1 to 2 weeks by the traditional culture method to about 6 hours. The detection limits of this PMAXX-qPCR method of *Z. rouxii* in MEA medium and 55% or 70% honey solution were 10^3 CFU/mL and 10^1 CFU/mL, respectively. Moreover, it can overcome the shortcomings of the traditional culture method, which can only detect culturable yeast and cannot detect viable but nonculturable yeast in samples. This study provides a promising and practical method for rapidly detecting living *Z. rouxii* cells in honey and its products. In the follow-up study, we will further classify and identify the osmotolerant yeast isolated from the suspected honey samples.

Data Availability

The data used to support the findings of this study can be available from the first author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

S. C., J. G., H. C., Y. L., and J. J. were involved in conceptualization. S. C., X. C., Y. R., K. L., and Y. C. were involved in validation. S. C. and Q. T. were involved in formal analysis. S. C., Q. T., and Y. W. were involved in writing—original draft preparation, review, and editing. All authors have read and agreed to the published version of the manuscript.

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