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Quantitative analysis of the three main genera in effective microorganisms using qPCR

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Abstract−Effective microorganism (EM) cultures have been applied in many research fields such as agriculture, environment and bioremediation. EM is a mixed culture of microorganisms including predominant populations of lactic acid bacteria and yeasts with smaller numbers of photosynthetic bacteria, actinomycetes and other types of microorganisms. Quantitative analysis of EM is requisite for the applications of EM, as its efficiency varies depending on the composition of its main genera of EM. In this study, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae*, the main genera of EM were quantified by quantitative real time polymerase chain reaction, (qRT-PCR). By using selected specific primers, photosynthetic bacteria, lactic acid bacteria and yeast were quantified with high sensitivity and specificity. The ability of viable cell count by qRT-PCR was compared with agar plate cell count, showing linear relationship. Thus, PCR based quantification system is a rapid and highly specific and sensitive tool for the quantification of EM.

Keywords: Effective Microorganisms, qRT-PCR, Quantitative Analysis, Photosynthetic Bacteria, Lactic Acid Bacteria, Yeast

INTRODUCTION

The concept of effective microorganisms (EM) was developed by Professor Teruo Higa, University of the Ryukyus, Okinawa, Japan. EM consists of mixed cultures of beneficial and naturally occurring microorganisms that can be applied as inoculants to increase the microbial diversity of soils and plants.

EM contains five families, ten genera and more than 80 types of aerobic and anaerobic microbes including photosynthetic bacteria, lactic acid bacteria, yeast, actinomycetes, fungi and so on (Table 1). The main species of EM are yeast, lactic acid bacteria and photosynthetic bacteria [1].

EM was first developed to be used in organic farming, but currently, EM is earning an increasing attention with applications in agriculture, forestry, livestock industry, fisheries, environment and medicine among others [1-8]. But its effectiveness varies with the ratio of main genera [9]. Thus, quantitative analysis of EM is essential for its application. Therefore, to produce and use EM efficiently and in wider application requires quantitative analysis and modeling of mixed culture EM system.

Table 1. EM international standard in 1997

Type of microorganisms	Basic species	Works
Lactic acid bacteria	*Lactobacillus plantarum* (ATCC8014)	Lactic acid production
	Lactobacillus casei (ATCC7469)	Breakdown of lignin and cellulose
	Streptococcus lactis (IFO12007)	
Photosynthetic bacteria	*Rhodopseudomonas palustris* (ATCC17001)	Antioxidant synthesis
	Rhodobacter sphaeroides (ATCC17023)	CO2, N2 fixation
		Amino acid nucleic acids, bioactive substance and sugars synthesis
Yeasts	*Saccharomyces cerevisiae* (IFO0203)	Bio active substance synthesis (hormones and enzymes)
	Candida utilis (IFO0619)	
Actinomycetes	*Streptomyces albus* subsp. *albus* (ATCC3004, KCTC1082)	Antimicrobial activity of the soil
	Mucor hiemalis (IFO3358, KCCM12630)	
Fungi	*Aspergillus oryzae* (IFO5770)	Alcohol, esters and antimicrobial substances
	Mucor hiemalis (IFO8567)	
Others	Beneficial microorganisms in nature combine into EM in the manufacturing process and survive in the mixture of EM at pH level under 3.5.	

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Quantitative real time polymerase chain reaction (qRT-PCR) is a tool that can detect both culturable and non-culturable microbes in quick fashion [10,11]. By choosing a primer that can amplify the nucleic acid sequence of a specific gene, we can quantitatively analyze the target microbe in EM and mixed culture by amplifying the DNA of the microbe. Quantitative real-time PCR provides a highly sensitive and specific method for tracing and quantifying the PCR products formed during the exponential phase of the reaction. The detection of PCR products is monitored by measuring the increase in fluorescence caused by the binding of SYBR green dye to double-stranded DNA (dsDNA) [12], which can provide both quantitative results and differentiation of PCR products by analysis of melting curves [13].

Our objective was to evaluate the use of real-time PCR to quantify total photosynthetic bacteria, lactobacilli and yeast, which are the main microorganisms in EM samples.

Until now quantification of specific bacteria in mixed culture was commonly achieved by selection, using culture environment differentiation or antimicrobial compounds, such as antibiotics. These methods utilize much more material and are time consuming compared to real-time PCR based identification and quantification. Using real-time PCR for quantification of the microorganisms in an EM consortia, is a quick and accurate method for microbial analysis; this also allows to observe the symbiosis cycle of the main genera in EM.

MATERIALS AND METHODS

1. Microbial Strains and Growth Conditions

The four strains used in this study as the main genera of EM are *Rhodobacter sphaeroides* (ATCC17023), *Rhodopseudomonas palustris* (ATCC17001), *Lactobacillus plantarum* (ATCC8014), and *Saccharomyces cerevisiae* (KCCM35053).

R. sphaeroides and *R. palustris* were grown in van Niel's yeast medium (K_2HPO_4 1.0 g/*l*, $MgSO_4$ 0.5 g/*l*, yeast extract 10.0 g/*l*) for 3 days at 37 ºC in anaerobic light condition. *L. plantarum* was grown in MRS broth (Difco, U.S.A.) for 24 h at 37 ºC. *S. cerevisiae* was grown in YM broth (Difco, U.S.A.) for 16 to 18 h at 28 ºC. Solid media were prepared by the addition of 2% agar.

2. Primer Selection

PCR primer pairs used in this study are listed in Table 2. To quantify the total number of photosynthetic bacteria, standard curves were generated by plotting the threshold cycle (C_T) versus log CFU/ml. The partial fragment of *pufM* gene was amplified with the primers based on the previous report [14], which is specific for anoxygenic phototrophic purple bacteria. This DNA was extracted from cell samples and serially diluted from 2.2×10^5 CFU/ml to 10^{-8}. For enumeration of total lactic acid bacteria, standard curves were also created by plotting threshold cycle (C_T) versus log CFU/ml. The sample concentration was serially diluted from 0.4 ng/μl to $0.4/10^4$ ng/μl. The qPCR amplifications were performed using the primer pair LactoF and LactoR, which are almost identical in sequence to the primary Lactobacillus primers designed by Byun et al. [15]. For quantification of total *S. cerevisiae*, amplification for qPCR was performed using *S. cerevisiae* specific primer pairs SCDF/SCDR [16].

3. Genomic DNA Extraction of Pure Culture and Commercial EM Samples

For the real-time PCR experiments, genomic DNA was prepared using the SolGent™ Genomic DNA Prep Kit. For this purpose, 500 μl of culture sample was used. Samples were centrifuged and pellet was washed twice with sterile water. The sample was stored at −70 ºC until further use. The DNA was then extracted according to the DNA prep kit manufacturer's protocol followed by a precipitation step to minimize inhibitions. DNA was resuspended in 50 μl of DNA hydration buffer, and its concentration was measured with a spectrophotometer (Agilent 8453 UV-visible Spectroscopy System). For EM products, three commercial preparations of effective microorganisms were used. EM was produced from the basic EM concentrate EM-1 by the manufacturer (Bionova Hygiene/GmbH, Stans, Switzerland). Each 500 μl of commercial EM product solution was centrifuged at 10,000 rpm for 1 min and the supernatant was removed. Each sample was stored at −70 ºC and DNA was extracted as described above.

4. Temperature Gradient Real-time PCR

The PCR condition was optimized to an equal annealing temperature of 60 ºC. Conditions for all PCRs were optimized in a Chromo4™ Real-Time PCR gradient cycler (Bio-Rad laboratories, USA) with various annealing temperatures (50-70 ºC).

5. Quantitative PCR

After amplification and detection with the Chromo4™ Real-Time PCR detector, the results were analyzed by Opticon Monitor 3.1.32 program (Bio-Rad Laboratories, USA). Duplicate samples were used. The PCR mixture contained 10 μl of 2X realtime PCR premix (SolGent, Korea), 2 μl of DNA template and 1 μl of each primer (concentrated 10 pm/μl), 1 μl of 20X evagreen and distilled water in a 20 μl final volume. The amplification program was carried out as described above. Duplicates of each sample and the various controls were processed. The reaction conditions were 95 ºC for 5 min and 40 cycles of 95 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min. Fluorescence was measured at the end of the annealing-extension phase of each cycle. A threshold value for the fluorescence of all samples was set automatically. The reaction cycle at which the PCR product exceeded this fluorescence threshold was identified as the

Table 2. List of primer pairs used for the qPCR assays

Specific target	Primer name	Sequence (5′→3′)	PCR product size
Photosynthetic bacteria	*pufM*557F	CGCACCTGGACTGGAC	229 bp
	*pufM*750R	CCCATGGTCCAGCGCCAGAA	
Lactic acid bacteria	LactoF	TGGAAACAGRTGCTAATACCG	231-233 bp
	LactoR	GTCCATTGTGGAAGATTCCC	
Yeast	SCDF	AGGAGTGCGGTTCTTTG	310 bp
	SCDR	TACTTACCGAGGCAAGCTACA	

threshold cycle (C_T). Melting curve analysis was performed with temperature increased from 65 to 95 °C at a rate of 0.2 °C/sec, with continuous detecting of fluorescence.

RESULTS

1. Annealing Temperature

To determine the conditions for qPCR a temperature gradient PCR was used. Fig. 1 shows the PCR data of photosynthetic bacteria and lactic acid bacteria. The PCR condition was optimized to an equal annealing temperature at 60 °C.

2. Primer Specificity

The primer's specificity was analyzed by melting curve analysis of the amplified products. The list of the primers, their targets and the sequences are in Table 2. Table 3 shows that the selected primers specifically amplify the target gene and are also specific in mixed

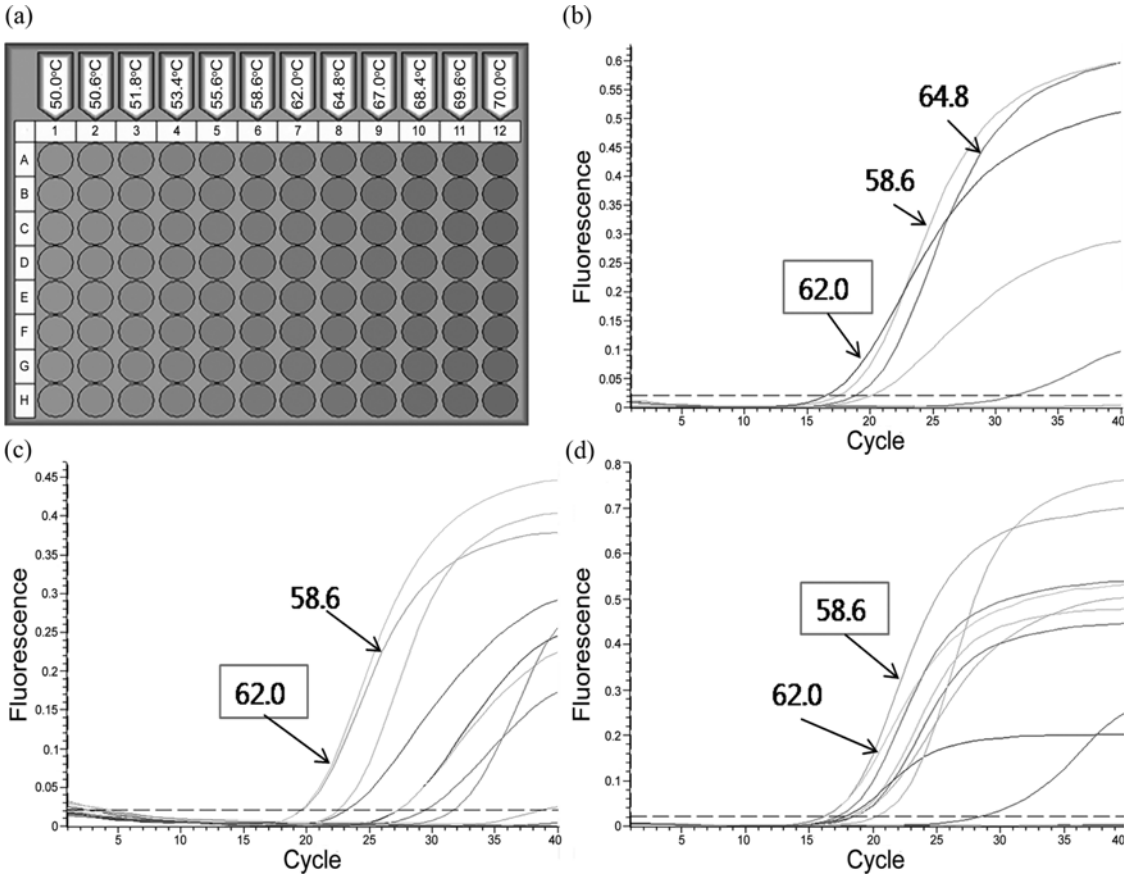

Fig. 1. Temperature gradient PCR. (a) Temperature profile on 96 well plate, PCR data graph of (b) *L. plantarum*, (c) *R. palustris*, (d) *R. sphaeroides*.

Table 3. Strains tested in this study and the specificity of primers used of qPCR

Test strains		Primers and target species		
		pufM. 557F/*pufM*. 750R (PSB[a])	Lacto-16S-F/Lacto-16S-R (LAB[b])	SCDF/SCDR (yeast)
PSB	*R. sphaeroides*	+	–	–
	R. palustris	+	–	–
LAB	*L. plantarum*	–	+	–
Yeast	*S. cerevisiae*	–	–	+
Mixture	R+L	+	+	–
	R+S	+	–	+
	L+S	–	+	+
	R+L+S	+	+	+

[a]Photosynthetic bacteria

[b]Lactic acid bacteria

R: *R. sphaeroides*, *R. palustris*; L: *L. plantarum*, S: *S. cerevisiae*, +: presence of PCR product; –: absence of PCR product

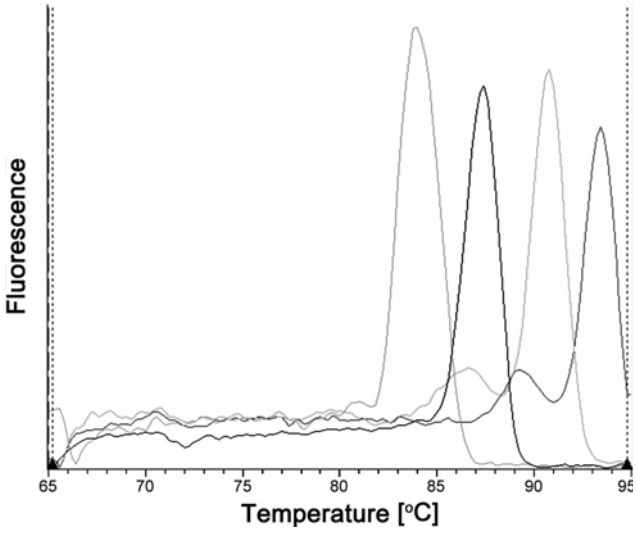

Fig. 2. Melting curve analysis. *S. cerevisiae* (Tm: 84.6 °C); *L. plantarum* (Tm: 87.8 °C); *R. palustris* (Tm: 90.6 °C); *R. sphaeroides* (Tm: 93.8 °C).

conditions of several microorganisms. From melting curve analysis in Fig. 2, we concluded that DNA amplification was achieved without non-specific products or primer dimers.

3. Standard Curve

The standard curve and sensitivity analysis of qPCR was obtained by diluting ten times the extracted DNA from pure cultured photosynthetic bacteria, lactic acid bacteria and yeast. The standard curve plotted as log CFU/ml times threshold cycle (C_T) by diluting 2.2×10^5 photosynthetic bacteria, 2.25×10^5 yeast and 1.6×10^5 lactic acid bacteria by the range of 1 to $1/10^7$ (Fig. 3). All the three bacteria showed a regression coefficient above 0.98 in regression analysis, which was reliable data and in the same condition all microbial DNA were able to amplify. From sensitivity analysis photosynthetic bacteria, lactic acid bacteria and yeast were able to be detected at 2.2 CFU/ml, 1.6×10^2 CFU/ml and 2.25×10 CFU/ml, respectively, by qPCR.

4. Quantification Correlation of Plate Count Method and qPCR

To confirm the viable cell count ability of qPCR, agar plate cell count was compared. Fig. 4 shows data of log CFU/ml (plate count) by log CFU/ml (qPCR data). Bacteria were separated and comparison experiments of plate count and qPCR were done two times each, displaying linear relationship.

5. qPCR Analysis of Commercial EM Products

The commercial EM products were quantitatively analyzed by qPCR method as shown in Table 4. Photosynthetic bacteria and lactic acid bacteria were detectable and able to undergo quantitative analysis. But the yeast was not detectable because the yeast used in commercial EM products of Jeju, Jeonju and Busan in Korea had *Candida utilis*, *Pichia deserticola* and *Candia versatilis*, respectively, while the primer used in this paper was specifically only for *S. cerevisiae*.

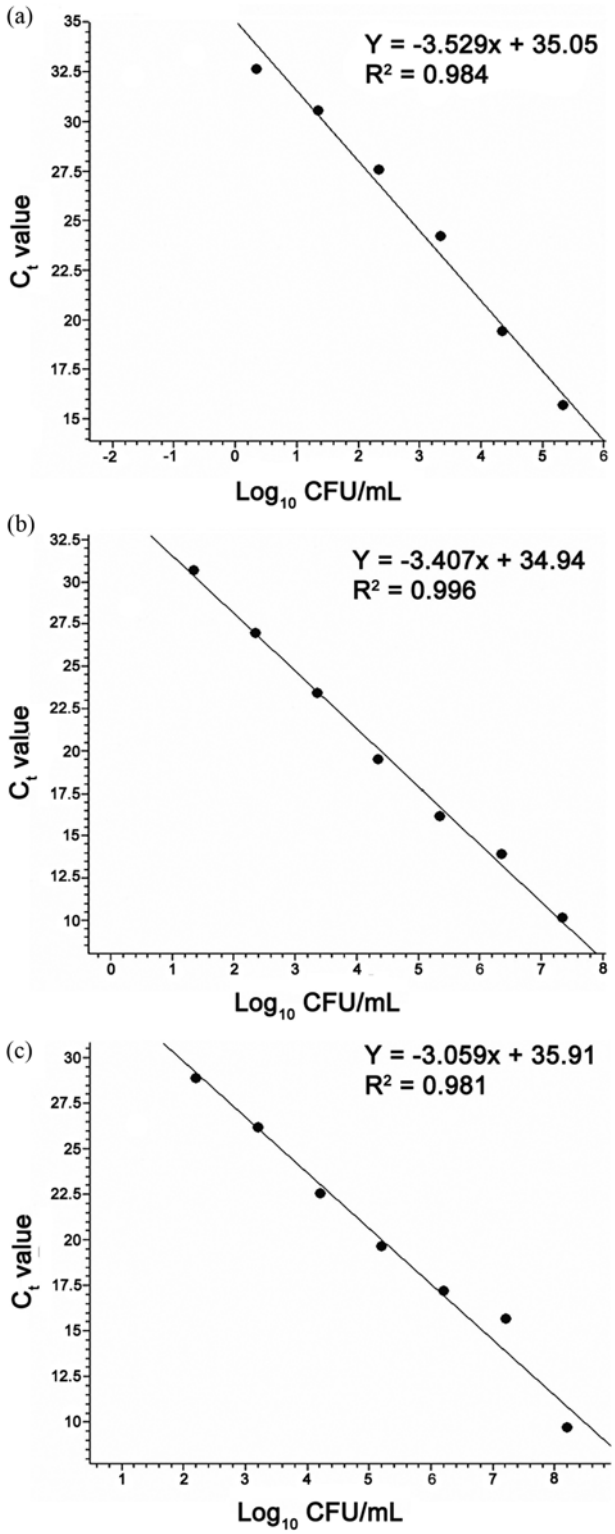

Fig. 3. Standard curve showing the correlation between threshold cycle (C_T) and initial CFU/ml. C_T values are averages of three replicates. (a) Photosynthetic bacteria, (b) Yeast, (c) Lactobacillus.

DISCUSSION

EM is a group of about 80 microbial species including photosynthetic bacteria, lactic acid bacteria, yeast, actinomyces and fungi which live together in a single culture medium [9]. But because of the difficulties in reproducibility, any culture medium where photo-

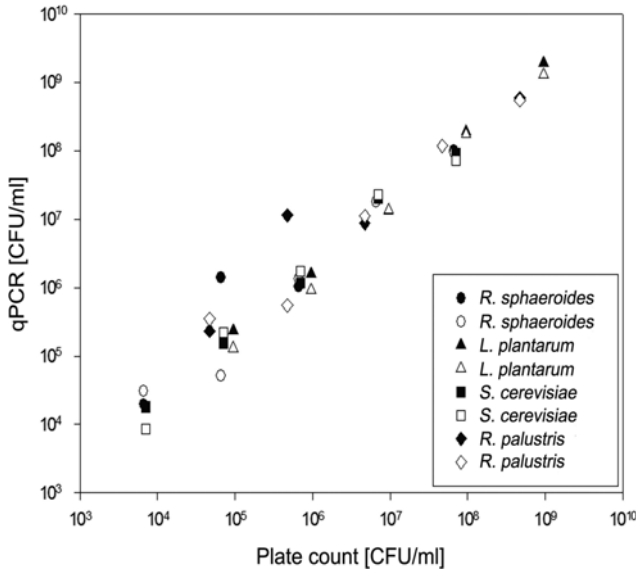

Fig. 4. Correlation between qPCR and agar plate count. The correlation was studied with two separate cultures of each optimal medium. Each organism was replcated twice (filled and open shapes) to show repeatability.

Table 4. Quantification of three main genera in commercial EM products by qPCR

Test sample	Target species		
	Photosynthetic bacteria	Lactic acid bacteria	Yeast
Jeju EM	3.60×10^4	1.37×10^7	ND[a]
Jeonju EM	2.87×10^4	1.43×10^6	ND
Busan EM	2.75×10^4	2.53×10^7	ND

[a]Not detected

synthetic bacteria, lactic acid bacteria and yeast can coexist at any ratio is known as EM [1]. EM technology has many applications in many industries including agriculture, environment, livestock industry, building industry and even the medical industry. EM is reported to have a positive effect especially in agriculture and waste treatment [9]. Even though these effects are shown, scientific research proving the mechanism for EM has not been approved. We know only simple facts like the characteristics of photosynthetic bacteria, which are known to be the most important factor of EM's antioxidant effect and play the main role for microorganisms to live together [17]. But it is hard to detect the amount of photosynthetic bacteria in an EM mixture.

Until now, no quantitative PCR studies of EM have been performed. Therefore, this study will be the basic research to clarify the symbiosis system of EM by overcoming the detection limitation of photosynthetic bacteria in a selective medium and monitoring the microbial growth change using a molecular technique called qRT-PCR. Because the ratio of microorganisms in EM is critical to the symbiosis system and characteristics of EM, knowing the amount of each microorganism will be a key for modeling the EM's characteristic behaviors.

In quantitative analysis by qPCR, specific primers were used for each bacterial genera, but the primer used for yeast, was specifically for *S. cerevisiae* [16]. Therefore, the DNA of yeast quantified in commercial EM was not detectable or amplified in this study. The *pufM* primer used for photosynthetic bacteria was able to detect specific microorganisms with purple bacterial-type reaction center like *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris*, which are generally used in EM. For lactic acid bacteria a lactobacillus specific primer was used that was able to amplify most lactobacillus [18]. Temperature gradient PCR was also carried out to decide the annealing temperature for the qRT-PCR primers of photosynthetic bacteria, lactic acid bacteria and yeast grown in both pure culture and mixed culture. All three primers were successfully amplified at 60 ºC, which showed that the DNA of all three microorganisms can be amplified in the same condition, making qRT-PCR an applicable tool for quantifying EM. The quantification of each organism was shown by plotting a log CFU/ml by C_T standard curve, which showed an acceptable R^2 value above 0.98. A particular T_m without non-specific PCR products was confirmed by analyzing the melting curve.

Based on these results we were able to analyze the number of microorganisms in three commercial EM which are currently being manufactured in Korea. Using qRT-PCR, photosynthetic bacteria and also lactic acid bacteria were successfully quantified, overcoming the difficulties of quantifying photosynthetic bacteria, which is one of the main genera in EM. However, yeast was not able to be detected or amplified because of the different genera used in the commercial EM products. This result can be overcome by using a yeast specific primer YEASTF and YEASTR to quantify all yeast including *Candida utilis*, *Pichia deserticola*, *Candia versatilis* and *S. cerevisiae* [19].

From the results of this study, direct detection of three main genera in EM by real-time PCR using the specific primer set was sensitive and reproducible, and it also allows makers to enumerate microorganisms in a short period of time (4 to 5 h). From this we can measure the amount and ratio of microorganisms in a certain culture and monitor the microbial change in EM, resulting in identifying the roles of the main microorganisms. Furthermore, using this quantification tool we can expect to prove the benefits of EM and even make a model of symbiotic culture with several organisms.

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