Research Article

Application of PCR–DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil

Abd El-Latif Hesham^{1,2}, Sardar Khan¹, XinChun Liu¹, Yu Zhang¹, Zhenyu Wang¹ and Min Yang^{1*}

¹Research Centre for Eco-environmental Sciences, Chinese Academy of Sciences, PO Box 2871, Beijing 100085, People's Republic of China

²Genetics Department, Faculty of Agriculture, Assiut University, Assiut, Egypt

*Correspondence to:

Min Yang, Research Centre for
Eco-environmental Sciences,
Chinese Academy of Sciences,
PO Box 2871, Beijing 100085,
People's Republic of China.
E-mail:
yangmin@mail.rcees.ac.cn

Abstract

Slurry-phase reactors have been used to investigate the biodegradation feasibility of polycyclic aromatic hydrocarbons (PAHs) in weathered crude oil, by mixed culture containing five PAHs-degrading yeast strains. Yeasts were isolated from the oily soil by enrichment culture, using phenanthrene as a sole carbon source, and identified based on the 26S ribosomal DNA (rDNA) sequence. Yeast strains belonged to the genera *Candida*, *Pichia*, *Rhodotorula* and *Sporidiobolus*. The experiment was carried out for a period of 6 weeks at room temperature with a solid:liquid ratio of 50% w/w. The results showed that high removal efficiency was obtained for all PAHs, including low molecular weight (LMW) and high molecular weight (HMW) compounds (89.3–98.6% and 66.3–89.4% within 6 weeks, respectively). The higher removal efficiency for HMW-PAHs obtained in this work suggested that yeast strains mixture could play an important role to reclaim oil-contaminated sites. Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 26S rRNA genes was used to follow the changes of yeast populations during the slurry reactor process. The results of DGGE indicated that *Candida maltosa*-like and *Pichia guilliermondii* were the dominant species but *Rhodotorula dairenensis* appeared as a weak band and *Sporidiobolus salmonicolor* and *Pichia anomala* disappeared during the study. Moreover, the results showed that all of the five strains, including the two belonging to the same genus, could be differentiated from each other in the DGGE profile. Sequences of yeast isolates reported in this study have been deposited in the GenBank database under Accession Nos DQ302753, DQ303392, DQ303393, DQ350841 and DQ432635. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: degradation; PAHs; slurry reactor; isolation; identification; 26S rRNA gene; PCR-DGGE

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Introduction

Recently, oil-contaminated soil has become a focus point for research and there have been many studies on the source, fate and remediation of oil-contaminated soil (Barakat *et al.*, 2002; Meysami and Baheri, 2003; Trindade *et al.*, 2005). Oil is a complex mixture of hydrocarbons, basically composed of aliphatic and aromatic hydrocarbons. The

aromatic fractions, especially polycyclic aromatic hydrocarbons (PAHs), are compounds of intense public concern, owing to their persistence in the environment and potential deleterious effects on human health. Although indigenous microorganisms could utilize crude oil as a source of energy and break it down into non-toxic compounds (Atlas, 1981; Cerniglia, 1992), they could not degrade the high molecular weight

PAHs (HMW-PAHs), which are more harmful than the lower molecular weight PAHs in weathered oil-contaminated soils (Trindade *et al.*, 2005).

Numerous efforts have been dedicated to the search for proper remediation technologies to remove as many contaminants as possible from the environment. The most widely used technologies for the remediation of oil- or PAH-contaminated soils and sediments include land farming, bioventing, air sparging and the bioslurry reactor (US EPA/540/290/016, 1990; Wilson *et al.*, 1993; Katsivela *et al.*, 2005). A very high removal efficiency for some PAHs can be obtained in slurry phase reactors (EPA/540/A5-91/009, 1993; Woodhull and Jerger, 1995; Saponaro *et al.*, 2002), where contact between microorganisms, contaminants, nutrients and oxygen is more intimate than with solid-phase systems. Various recent studies have investigated the possibility of bioremediation of PAH-polluted soil with PAH-degrading bacterial consortia or pure strains, and enhanced PAH degradation in soil slurries and soil microcosms has indeed often been observed (Johnsen *et al.*, 2005). Although yeast has been found to be active in oil degradation (Palitapongarnpim *et al.*, 1998), information regarding yeast degradation of PAHs has been minimal. The ability of yeast to degrade HMW PAHs has therefore become of interest.

The objective of this study was to investigate the feasibility of biodegradation of PAHs in synthetic weathered crude oil by a PAH-degrading yeast strain mixture inoculated into slurry reactors. PAH-degrading yeast strains were isolated from an oilfield soil site contaminated with oil and identified using a molecular technique. In order to trace the behaviour of the inoculated yeast strains in the slurry reactor system, the PCR–DGGE technique was applied.

Genetic fingerprinting techniques are able to provide a profile representing the genetic diversity of a microbial community from specific environment. The PCR–DGGE analysis represents a powerful tool to study the structure of microbial communities in environmental samples without cultivation and to determine the community dynamics in response to environmental variations. Although PCR–DGGE has been applied to the study of bacterial community structure associated with bioremediation of oil(PAH)-polluted soil (Macnaughton *et al.*, 1999; Ringelberg *et al.*, 2001; Zucchi *et al.*, 2003; Leys *et al.*, 2004; Vinas *et al.*, 2005), the

method using 26S ribosomal RNA (26S rRNA) gene has only been applied to the investigation of yeast populations in environmental samples such as milk (Cocolin *et al.*, 2002) and sourdough (Meroth *et al.*, 2003) and during coffee (Masoud *et al.*, 2004), wine (Cocolin *et al.*, 2000; Cocolin and Mills, 2003; Prakitchaiwattana *et al.*, 2004) and cocoa fermentations (Nielsen *et al.*, 2005). This was therefore the first time the PCR–DGGE technique was used to investigate yeast population dynamics in a slurry reactor system.

Materials and methods

Enrichment and isolation

Oil-contaminated soil samples were taken from Jidong Oilfield in Hebei Province, China. A soil enrichment technique was used for the isolation of PAH-degrading yeasts. Briefly, about 1 g soil was suspended in 10 ml sterile water and added to a mineral basal salts (MBS) medium containing, per litre distilled water, 1000 mg $(\text{NH}_4)_2\text{SO}_4$, 800 mg K_2HPO_4 , 200 mg KH_2PO_4 , 200 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, pH 5–6, and 1 ml vitamin solution sterilized by filtration, supplemented with 0.2% phenanthrene at 27 °C at 150 r.p.m. for 10 days. When growth was visible, enrichment was continued by serially subculturing several times in the same medium, using a 10% inoculum from the previous culture. Yeast strains were isolated from MBS agar plates coated with phenanthrene as the sole carbon source (Romero *et al.*, 1998).

DNA extraction

Two methods for the extraction of DNA were used. The first, for extracting DNA from pure cultures, was performed according to procedures described by Harju *et al.* (2004). The second, for extracting total DNA from soil, was performed by modified methods of Harju *et al.* (2004). Briefly, soil samples (0.5 g) were washed twice with 1 ml PBS buffer (containing, per litre distilled water, 8 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4 , 1.44 g Na_2PO_4 , pH 7.4) and centrifuged at 80 000 r.p.m. for 5 min. The pellets were resuspended in 1 ml lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) and 0.3 g glass beads. The tubes were placed

in a -80°C freezer for 5 min, and then immersed in a 95°C waterbath for 3 min to thaw quickly. This step was repeated once, and the tubes were vortexed vigorously for 60 s. Phenol:chloroform (1:1, 1 ml) was added and the tubes were vortexed for 2 min and then centrifuged for 3 min at room temperature at $20\,000 \times g$. The aqueous layer was transferred to a tube containing 1000 μL ice-cold 100% ethanol for 5 min at room temperature and then centrifuged for 5 min at room temperature at $20\,000 \times g$. Supernatants were discarded and DNA pellets were washed with 0.5 ml 70% ethanol, followed by vacuum drying for 20 min at room temperature, and then DNA was resuspended in 80 μL TE buffer.

Identification of PAH-degrading yeasts by 26S rDNA sequences

DNA was isolated and fragments containing the D1/D2 region of about 600–650 bp at the 5' end of the 26S rDNA were amplified, using primers NL-1 and NL-4 (Kurtzman and Robnett, 1998). PCR was performed in a final volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , each dNTP at a concentration of 0.2 mM, 1.25 IU Taq polymerase (Takara, Dalian, China), each primer at a concentration of 0.2 mM and 1 μL DNA template. The amplification was carried out by polymerase chain reaction (PCR) under the following conditions: initial denaturation at 95°C for 5 min, followed by 36 cycles at 94°C for 2 min, 52°C for 1 min, 72°C for 2 min; final extension at 72°C for 7 min, holding at 4°C . The amplified products were then purified and sequenced (Shanghai GeneCor Biotechnologies Co., Ltd). The obtained sequence data were compared with those of all known yeast species, available at the GenBank database.

Soil characteristics

Clean soil sample (0–20 cm) was collected from a garden in Beijing. After transportation to the laboratory, the soil was air-dried, ground and sieved through a 2 mm mesh. Selected physicochemical properties of the sample were measured according to standard procedures (Table 3). Soil pH was measured by H_2O (1:2.5 ratio) and by CaCl_2 (1:10 ratio) (Kalra, 1995). The contents of extractable cations including K, Na, Mg and Ca were measured using inductively coupled plasma optical emission

spectrometry (ICP-OES), using an OPTIMA-2000 (Perkin-Elmer, USA; McKeague, 1981). The initial available (50 mM CaCl_2) Cd, Ni, Cu and Pb concentrations were below the detection limit. Organic C was measured by the modified Walkley-Black method (Houba *et al.*, 1989).

Biodegradation experiment

A bench-scale slurry reactor experiment was conducted using glass beakers in the laboratory control environment. Sterile uncontaminated soil (autoclaved at 121°C for 20 min) was mixed with synthetic weathered crude oil treated at 230°C according to Maki *et al.* (2001). In the slurry reactors, a 50% solid:liquid w/w ratio was obtained by using dry sieved soil (<2 mm) and deionized water. Macronutrients, including NH_4Cl (6.39 g), K_2HPO_4 (0.33 g) and KH_2PO_4 (0.25 g) were introduced in reactors to keep a w/w balance between C:N:P ratios (100:5:1). A mixed culture containing five PAH-degrading yeast strains was inoculated in the slurry reactors. In order to maintain aerobic conditions in the reactors, aeration was provided by a magnetic stirrer. Sterile controls were also used to quantify the abiotic losses of PAHs. The experiment was carried out for a period of 6 weeks at room temperature. Water losses due to evaporation were compensated for by daily addition of deionized water on a weight basis. Samples were taken for an initial concentration determination 3 h after the slurry reactor systems had been amended with weathered crude oil and yeast mixtures. Further samples were taken on a weekly basis. All results of biodegradation were obtained in reference to sterile controls: $R1 - R2/R1 \times 100$ (where $R1$ is the residual PAH concentration in the control, while $R2$ is the residual PAH concentration in the degradation beaker).

PAH extraction and analysis conditions

The PAHs were extracted by the method as used by Wu *et al.* (2005) with slight modifications. Briefly, the whole beaker soil (Villemur *et al.*, 2000) was used to extract PAHs by the Soxhlet extraction method with 200 ml dichloromethane (DCM) for 24 h. After filtration, the extract was evaporated to approximately 1 ml at 35°C , using a rotary evaporator. Silica chromatography columns were prepared with silica gel and Al_2O_3 and capped with Na_2SO_4 (activated before use at 180°C , 250°C

and 450 °C for at least 12 h, respectively) and then washed with hexane. The concentrated extracts were loaded onto columns to separate the PAHs from other polar interfering compounds. These columns were eluted with hexane and DCM (15 : 70 ml); the eluted fractions were again evaporated to 1 ml using a rotary evaporator and transferred to a Kuderna–Danish concentrator and rinsed with 10 ml *n*-hexane. The extracts were dried to 1 ml under a gentle nitrogen flow and transferred to a vial capped with a Teflon-lined septum for GC–MS analysis. The concentrations of PAHs in the extracts were determined using an Agilent 6890 gas chromatograph equipped with a 5973 mass selective detector under the selected ion monitoring mode (SIM). An HP-5 silica fused capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) was used, with helium as the carrier gas at a constant flow rate of 1 ml/min. The GC oven temperature was programmed from 50 °C (2 min) to 200 °C (2 min) at 10 °C/min, then to 290 °C at 20 °C/min and held for 8 min. The injector and detector temperatures were 280 °C and 290 °C, respectively. Mass spectra were acquired at the electron ionization (EI) mode with an electron multiplier voltage of 1906 eV. Quantification was performed using the five-point calibration curve for individual components. The detection limit for all PAHs was 0.001–0.01 mg/l.

Recovery and quality control

Clean soil samples were spiked with 16 known PAHs (1–2 µg/g). The recovery and reproducibility of the extraction method was satisfactory, ranging from 91.3 ± 5.6% to 95.3 ± 3.9%.

PCR–DGGE and primers

The D1/D2 domain of the 26S rRNA gene was amplified by a two-step, nested PCR. The first PCR was conducted with the forward primer, NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'), and reverse primer, NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'). PCR was performed in a final volume of 50 µl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, each dNTP at a concentration of 0.2 mM, 1.25 IU Taq polymerase, each primer at a concentration of 0.2 mM, and 2 µl DNA template. PCR was run for 36 cycles with annealing at 52 °C, extension at 72 °C for

2 min, and denaturation at 94 °C for 1 min (Kurtzman and Robnett, 1998). The amplicon (approximately 600 bp) from the first PCR was diluted and further amplified with a second PCR, using the GC-clamp primer NL1 (5'-CGC CCG CCG CCG CCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3'; GC-clamp underlined) and reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin *et al.*, 2000). The conditions of this reaction were the same as described above. The PCR was run with an initial step at 95 °C for 5 min, and 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min, extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. The amplified mixture (5 ml) was analysed in a 1.5% 0.5× TBE agarose gel electrophoresis. The gel was stained with 0.5 mg/ml ethidium bromide, visualized under UV light and photographed.

DGGE analysis

The GC clamp products were separated according to their sequences, using DGGE, with a Bio-Rad Dcode System (Bio-Rad, USA). The PCR product was loaded on 0.8 mm thick polyacrylamide gel (8% w/v acrylamide : bisacrylamide 37.5 : 1) using a denaturing gradient from 30% to 50% of urea and formamide (100% corresponds to 7 M urea and 40% w/v formamide), being increased in the direction of the electrophoresis run. The electrophoresis was performed under a constant voltage of 120 V for 6 h and a constant temperature of 60 °C. After the run, the gels were stained for 20 min in 1× TAE containing 0.5 mg/ml ethidium bromide and destained with distilled water, then photographed with UV transillumination.

Results and discussion

Enrichment and isolation

A series of pure yeast strains were isolated from the soil sample and their ability to grow on agar plates coated with phenanthrene, chrysene, or benzo(a)pyrene as the sole carbon source was investigated (data not shown). Five of the best-growing isolated yeast strains were selected for the slurry reactor system and identified by 26S rDNA sequence.

Table 1. Identification of PAH-degrading yeast strains

Strain number	Identification and GenBank Accession No.	Identity origin (%)
AH1	<i>Candida maltosa</i> -like DQ302753	99
AH2	<i>Pichia anomala</i> DQ432635	100
AH3	<i>Sporidiobolus salmonicolor</i> DQ303392	99
AH4	<i>Pichia guilliermondii</i> DQ303393	100
AH5	<i>Rhodotorula dairenensis</i> DQ350841	100

Identification and differentiation of PAH-degrading yeasts

The sequencing results of the D1/D2 of the large subunit 26S rDNA were submitted to the GenBank database. The results obtained from the yeast identification by the sequencing approach and their Accession Nos are described in Table 1. A variety of yeast species were differentiated by analysis of partial sequences of large subunit rDNA encompassing the D1 and D2 expansion domains (Kurtzman and Robnett, 1998). When the isolated strains were subjected to DGGE analysis, following PCR using the primers NL1GC/LS2, a good differentiation was obtained, as shown in Figure 1. It is clear that all five strains, including the two belonging to the same genus, could be differentiated from each other in the DGGE profile. These results are in agreement with results obtained by Cocolin *et al.* (2002) and Prakitchaiwattana *et al.* (2004). DGGE analysis was therefore used for following population changes during biodegradation of the weathered oil.

Figure 1. DGGE profiles of amplified 26S rRNA D1/D2 regions obtained from different yeast strains isolated from oily soil. Lane 1, *Candida maltosa*-like (AH1); lane 2, *Pichia guilliermondii* (AH4); lane 3, *Rhodotorula dairenensis* (AH5); lane 4, *Pichia anomala* (AH2); lane 5, *Sporidiobolus salmonicolor* (AH3)

PAHs degradation

The aliphatic hydrocarbons present in weathered oil were not considered in this study because of their easy degradability (Zinjarde and Pant, 2002). The initial concentrations, residual and degradation ratios of all of the PAHs are described in Table 2, and the degradation processes of LMW-PAHs and HMW-PAHs by the yeast mixture are shown in Figure 2(A,B) and Figure 3 (A–D), respectively. The data indicate that all of the PAHs in the weathered oil were removed significantly, although

Table 2. Calculated mass balance of PAHs in slurry experiments

PAHs	No. of rings	Initial concentration (mg/kg)	Remaining (mg/kg)	Degradation (%)
Naphthalene	2	0.69	0.07	89
Acenaphthylene	3	0.15	0.01	95
Acenaphthene	3	0.47	0.01	98
Fluorene	3	1.89	0.17	91
Phenanthrene	3	1.89	0.05	97
Anthracene	3	2.63	0.18	93
Fluoranthene	4	1.62	0.17	89
Pyrene	4	1.96	0.49	75
Chrysene	4	4.05	1.36	66
Benzo(b)fluoranthene	5	2.12	0.43	79
Benzo(k)fluoranthene	5	0.63	0.12	81
Benzo(a)pyrene	5	1.99	0.46	76
Dibenzo(a,h)anthracene	5	4.19	0.68	83
Perylene	5	6.03	1.81	70
Indeno(1,2,3-cd)pyrene	6	2.53	0.39	84
Benzo(g,h,i)perylene	6	2.17	0.47	78

Table 3. Initial physicochemical properties of uncontaminated soil used for slurry reactors

Parameters	Values
pH (water)	8.12
pH (CaCl ₂)	7.68
Particles sizes	
Sand	46.80%
Silt	39.71%
Clay	13.49%
K	0.98 mg/kg
Na	12.01 mg/kg
Mg	210.54 mg/kg
Ca	164.82 mg/kg
Organic matter	1.02%

different individual PAHs demonstrated different degradation patterns. The removal efficiency for the LMW-PAHs was 89.3–98.6% and that for the HMW-PAHs was 66.3–89.4% after degradation for 6 weeks. According to the study conducted by Saponaro *et al.* (2002) using bacteria, the PAH removal efficiency was in the range 65–90% in slurry reactors, which also supported our results.

It is interesting that the five-ring PAHs, including benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, perylene and dibenzo(a,h)anthracene, and the six-ring PAHs, including indeno(1,2,3-cd)pyrene and benzo(g,h,i)perylene, were removed

Figure 2. Biodegradation of LMW-PAHs in slurry reactors: (a) naphthalene, acenaphthylene and acenaphthene; (b) fluorene, phenanthrene and anthracene

with a relatively high efficiency (70.1–84.2%). Previous studies demonstrated that the HMW-PAHs

Figure 3. Biodegradation of HMW-PAHs in slurry reactors: (a) fluoranthene, pyrene and chrysene (four rings); (b) dibenzo(a,h)anthracene and perylene (five rings); (c) benzo(b)fluoranthene benzo(k)fluoranthene and benzo(a)pyrene (five rings); (d) indeno(1,2,3-cd)pyrene and benzo(g,h,i)perylene (six rings)

could not be utilized as carbon sources for yeasts such as *Saccharomyces cerevisiae*, *Debaromyces hansenii*, *Candida lipolytica*, *Candida albicans*, *Candida guilliermondii*, *Candida maltosa* and *Pichia anomala* (Cerniglia and Crow, 1981; McGillivray and Sharis, 1993; Zinjarde and Pant, 2002; Pan *et al.*, 2004). The efficient removal of these compounds should be achieved through co-metabolism by the mixed yeasts, using the LMW-PAHs and aliphatic hydrocarbons as carbon sources. Hesham *et al.* (2006) found that benzo(a)pyrene (five rings) was decomposed by the yeast through co-metabolism only. This is the first report that the six-ring PAHs can be decomposed by a yeast mixture under co-metabolism. This study may encourage the use of mixed yeast consortia to reclaim oil-contaminated sites.

Changes of yeast community structure

Figure 4 shows DGGE profiles of 26S rRNA gene fragments of soil sampled from the slurry reactors at time 0, 2 weeks, 4 weeks and 6 weeks during biodegradation, together with the yeast isolates as the control. All of the five yeast strains could be clearly identified at time zero. *Sporidiobolus salmonicolor* (band 5) disappeared after 2 weeks' treatment, and then *Pichia anomala* (band 4) disappeared. *Rhodotorula dairenensis* appeared as a weak band (band 3) within 4–6 weeks. Only

Candida maltosa-like (band 1) and *Pichia guilliermondii* (band 2) remained in the system as the bright band at the end of the experiment, suggesting that these two species might have a more important role during PAH degradation than the other species. It is clear that the DGGE technique could be effectively used to follow the changes of yeast populations during biodegradation of weathered crude oil (PAHs).

PCR–DGGE has been applied successfully to the study of bacterial community structure associated with bioremediation of different kinds of environmental pollutants, especially oil(PAH)-polluted soil (Macnaughton *et al.*, 1999; Ringelberg *et al.*, 2001; Zucchi *et al.*, 2003; Leys *et al.*, 2004; Vinas *et al.*, 2005). The method using 26S rRNA gene has only been applied to investigate yeast populations in milk (Cocolin *et al.*, 2002), sourdough (Meroth *et al.*, 2003) and during coffee (Masoud *et al.*, 2004), wine (Cocolin *et al.*, 2000; Cocolin and Mills, 2003; Prakitchaiwattana *et al.*, 2004) and cocoa fermentations (Nielsen *et al.*, 2005). This study demonstrated that the DGGE technique could be effectively used to examine changes in yeasts.

In summary, slurry reactor systems inoculated with a yeast mixture removed all of the 16 PAHs contained in the weathered crude oil with high efficiency. Five- and six-ring PAHs could be effectively removed through co-metabolism. DGGE could differentiate different species from the same

Figure 4. DGGE profiles of the PCR products obtained from the DNA extracted directly from slurry reactors and yeast isolates: Lane 6, 0 time; lane 7, 2 weeks; lane 8, 4 weeks; lane 9, 6 weeks. Lanes 1–5 for yeast isolates, i.e. *Candida maltosa*-like, strain AH1 (lane 1); *Pichia guilliermondii*, strain AH4 (lane 2); *Rhodotorula dairenensis*, strain AH5 (lane 3); *Pichia anomala*, strain AH2 (lane 4); *Sporidiobolus salmonicolor*, strain AH3 (lane 5)

genus, and was successfully used for the tracing of yeast population changes during biodegradation. *Candida maltosa*-like and *Pichia guilliermondii* were shown to be the predominant yeasts over the whole 6 week treatment.

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