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Characteristics of *Malassezia pachydermatis* Isolated from Dogs and Antifungal Effect of Essential Oils

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Abstract : This work describes the characteristics of *Malassezia pachydermatis* isolated from dog ear canals and the effect of essential oils on the growth of this organism. Sterile cotton swabs were used to collect specimens from the external ear canal and culture tests were performed to detect the population size of *Malassezia* yeast. Using three different isolation media, included Sabouraud dextrose agar (SDA) to isolate common *M. pachydermatis*, and SDA supplemented with olive oil (SDAO) and Leeming's medium (LM) to detect lipophilic yeast, *Malassezia* spp were isolated from 14 of 18 dogs (77.8%); isolation rates were 33.3% in SDA, 72.2% in SDAO and 66.7% in LM media. All *Malassezia* spp isolates were identified as *M. pachydermatis* according to results of PCR amplification, but gross colony morphology and SDA growth rates suggested four different subtypes. Large (LC) and medium colony (MC) types respectively describe large colony (diameter > 3 mm) and medium colony (around 2 mm) after 72 hour incubation, and small (SC) type refers to smaller colony (< 1 mm) even after 5 days incubation; lipid dependent colonies did not grow onto SDA. Large Colony type strains were isolated from 4, 11, and 11 samples, MC type strains from 2, 3 and 1 and SC type strains from 1, 2 and 1 in SDA, SDAO and LM, respectively. Lipid-dependent *M. pachydermatis* (Lipo) were isolated from 3 samples each in SDAO and LM. Anti-*M. pachydermatis* activity testing was done using disc-diffusion assays and well diffusion tests. Most essential oils inhibited the growth of *M. pachydermatis* in a range from 0.5% to 1.0% of essential oils. MIC90 and MIC50 were variable depending upon the nature of essential oils. Thyme oil was found to be highly effective in inhibiting the growth of *M. pachydermatis* in a range from 0.125% to 0.0625% while marjoram and then tea tree oil exhibited lower inhibitory capacity.

Key words **:** *Malassezia pachydermatis,* Dog, Antifungal effect, Essential Oil.

Introduction

Yeast of the genus *Malassezia* is commonly isolated from the skin of a variety of mammals and birds (30). This yeast belongs to the normal cutaneous microflora, but they may also behave as opportunistic pathogens whenever alterations of the skin or microclimates occur. *Malassezia*-related diseases include pityriasis versicolor, seborrheic dermatitis, folliculitis and systemic infections in humans (29), and otitis externa and seborrheic dermatitis in animals, expecially in dogs (17). In the past, the only recognized species in this genus were *M. furfur* in 1889 and *M. pachydermatis* in 1935 (16). Following improvements in molecular typing methods, the genus *Malassezia* was found to encompass additional species. Recently, ten species have been described by means of morphological, ultrastuctural, physiological and genetic characteristics (17,34): *M. dermatis, M. furfur, M. nana* sp. nov., *M. pachydermatis, M. sympodialis, M. slooffiae, M. obtusa, M. globosa, M. restricta.* and *M. sympodialis* have been isolated from cats with otitis externa (9), whilst *M. globosa, M furfur, M. slooffiae* and *M. sympodialis* have been isolated from cattle with otitis externa (11,12). The genus *Malassezia* can be identified by morphological, biochemical and molecular characteristics (17,18). Molecular typing methods have also been applied to these species (8,14,20). Sequences of the D1/D2 domains of the large-subunit (26S) rDNA and nucleotide sequences of the internal transcribed spacer 1 (ITS1) region, which is located between 18S and 5.8S rDNA, have been utilized for the characterization of these species (28). *M. pachydermatis* is the only species in the genus that does not require lipid supplementation for development in culture medium. This yeast, classically considered to be zoophilic, is frequently found on wild and domestic carnivores including dogs, cats, bears, ferrets and foxes (3,19). However, lipid-dependent *Malassezia* spp. are also associated with otitis externa of dogs (9) and some *M. pachydermatis* also have been reported as lipophilic (16). *M. pachydermatis* is highly susceptible to ketoconazole (27), but because this yeast usually share their habitat with other bacteria, such as *Staphylococcus* spp, treatment for *Malassezia* infections usually calls for both antibiotic and

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antifungal combinations.

Essential oils are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (5). The essential oils have had a long history of use an antiseptic and the use of the oils in medicine gradually became secondary to their use for flavour and aroma (5). The effects of essential oils as antimicrobial and antifungal agents have been described qualitatively for many years (6,7). Antifungal activity of essential oils has been determined by agar diffusion assay (1), broth or agar dilution assay (31), but it appears that no standardized test has been developed for evaluating the antimicrobial activity, although the need for such harmonization has been indicated (5).

The aims of this work were to study the occurrence of *Malassezia* spp. from ears and skin of dogs, to investigate characteristics of these isolates and to compare the effect of several essential oils on the inhibition of *Malassezia* isolates using disk diffusion and well diffusion tests.

Materials and Methods

Sample collection

The samples were collected from 18 dogs at the Veterinary Teaching Hospital in the Jeju National University. All dogs exhibited mild to severe inflammatory skin lesions, and samples were taken from the right and left external ear canals of each dog using sterile cotton swabs. Otitis externa were evaluated in all animals and direct examination from swab samples was conducted to investigate yeast exhibitions.

Isolation of *Malassezia pachydermatis*

All samples were inoculated onto the following media: Sabouraud dextrose agar (SDA; BD, Sparks, MD) to isolate common *M. pachydermatis*, and SDA supplemented with 10 ml olive oil per liter (SDAO) and Leeming's medium (pH 6.2), including 10 g peptone, 5 g glucose, 0.1 g yeast extract, 4 g desiccated ox bile, 1 ml glycerol, 0.5 g glycerol monostearate, 0.5 ml Tween 60, 10 ml whole-fat cow's milk and 12 g agar per liter (LM) to detect lipophilic yeast. Plates were incubated at 37°C and examined after 3, 6, 9 days. A maximum of five colonies were selected from SDA, SDAO and LM to be subcultured and maintained on the same media for further investigations of their growth patterns. The yeast colonies on SDAO and LM were inoculated onto SDA to determinate their lipid-dependency. *M. pachydermatis* was identified by gross colony, microscopic morphology and by its ability to grow on SDA.

Identification of *Malassezia pachydermatis*

Malassezia spp. were first identified by gross colony and microscopic morphology, and all isolates were confirmed to be *M. pachydermatis* by a nested PCR method (34). Two sets of primers were ITSIF-N (5'-CCATCATTAGCCTTTATA-3' and ITS4-R (5'-TCCTCCGCTTATTGATATG-3') for all seven *Malassezia* species. and M.pa-F (5'-CTGCCATACGGATGCGCAAG-3') and 5.8S-R (5'-TTCGCTGCGTTCTTCATCGA-3') primers for *M. pachydermatis* (220bp) only. PCR mixtures were composed in a 20°C reaction volume containing 2 μl of a mixture of 200 μM of each deoxynucleoside triphosphate (each molar concentration of dATP, dCTP, dGTP and dTTP), 1.5 U Taq DNA polymerase (TaKaRa Ex Taz), 17 μl PCR buffer, 20 μM of each primer and 1 μl DNA template. PCR was performed in a thermocycler with an initial denaturation of 94°C, 1 min at 57°C, and 50 s at 72°C and a final extension at 72°C for 10 min. In the nested PCR step, 1 μl of the first amplification product was added to a new reaction mixture with the same composition as the first. The PCR procedure consisted of an initial denaturation of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 62°C, and 40 s at 72°C and a final extension at 72°C for 10 min.

Biogrouping of *M. pachydermatis*

Biogroups were determined by examining colony morphology and growth rates. Large type *M. pachydermatis* was characterized by large colonies (LC), greater than 3 mm diameter, growing within 3 days after incubation. The medium type was represented by medium colonies (MC), about 2 mm in diameter, growing within 3 days of incubation. The small type was characterized by small colonies (SC), less than 1 mm diameter that took 5 days to grow. Lipophilic colonies (Lipo) were not able to grow onto SDA but grew onto SDAO and LM.

Essential oils

Six essential oils were used in the study: thyme (thymianol), geranium *(Pelargonium graveolens),* lavender *(Lavendula angustifolia),* tea tree *(Melaleuca alternifolia),* marjoram (*Origanum majorana*), and eucalyptus (*Eucalyptus globulus*); all were purchased from Styx Naturcosmetics Gmbh, Austria.

Disc diffusion test

Three nonlipophilic colony types of yeast were spread over the surface of the SDA plate using a sterile cotton swab. For preparing the test discs, 5 μl of each essential oil was pipetted onto 8 mm filter paper discs. After drying for 1h, the discs with oils were carefully transferred on to the surface of seeded agar plates. The diameter of the inhibition zone was measured followed by incubation at 37°C for 3 days. The net zone of inhibition was determined by subtracting the disc diameter from the total zone of inhibition shown by the clear halo surrounding the impregnated discs (1,7).

Well diffusion test

According to the method published by Inouye *et al* (22), MICs were measured to evaluate the antifungal effects of the various essential oils. *Malassezia pachydermatis* isolates tested included 22 LC types as faster growers, and 16 MC and SC types as slower growers.

In brief, each essential oil was prepared into 1.5 ml Eppendorf tube for final concentration of 2% to 0.03% by double

times dilution. In order to emulsify the oils, they were diluted in phosphate buffered saline (pH 7.4) containing 0.02% Tween 80. The diluted oils were mixed with the same volume of yeast suspension adjusted to the concentration of 1.5 to 3.5×10^6 CFU/ml. These oil and yeast mixtures (20 µl) were filled into the well of SDA plates. These plates were incubated for 5 days at 37°C, and the growth of yeast was determined around individual wells every day.

Results

Isolation characteristics

Malassezia spp. were isolated from 14 (77.8%) of 18 dogs in at least one of SDA, SDAO and LM media. The population of the organism growing from each sampled varied, with more than 100 colonies being recovered from most samples. The isolation rates of *Malassezia* spp. were 33.3% in SDA, 72.2% in SDAO and 66.7% in LM media (Table 1).

Five colonies were selected randomly from each sample for subculture and speciation. All *Malassezia* spp. isolates were identified as *M. pachydermatis* according to the results of PCR amplification (Fig 2), but colony morphologies were found to differ depending on the medium. All suspected colonies harbored 220 bp PCR product amplified by *M. pachydermatis* specific primer set, M.pa-F/5.8S-R in regardless of colony type. Of all isolates, 23 *M. pachydermatis* were selected to characterize colony type. These were divided in four different types based on gross colony morphology and growth rate onto SDA. Large colony strains were isolated from 4, 11, and 11 samples, MC strains, from 2, 3 and 1, SC strains, from 1, 2 and 1 in SDA, SDAO and LM, respectively. Lipid-dependent *M. pachydermatis* were isolated from 3 samples each in SDAO and LM, and their colony size was less than 1 mm (Table 2 and Fig 1). LC and MC type strains grew well within 3 days incubation, while SC and Lipo type strains were required more than 5 days to observe with naked eyes.

Table 1. Isolation rates and population of *Malassezia pachydermatis* from 18 canine ear swabs

Population	SDA	SDAO	LM
TNTC[a)]	3	8	7
100-300	2	1	1
10-100	1	3	2
< 10	0	1	2
Total	6 (33.3)	13 (72.2)	12 (66.7)

[a)]Too numerous to count

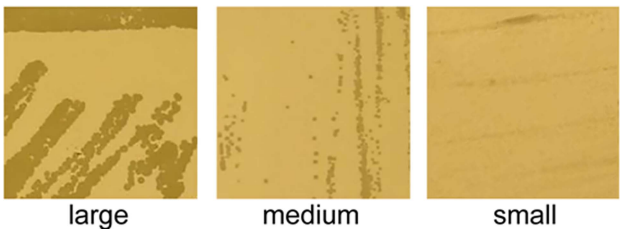

Fig 1. Colony morphology of *Malassezia pachydermatis* each representative three types of colony; Large (M121L), Medium (M27M), Small (M72S) in SDA medium cultured after 3 days at 37°C.

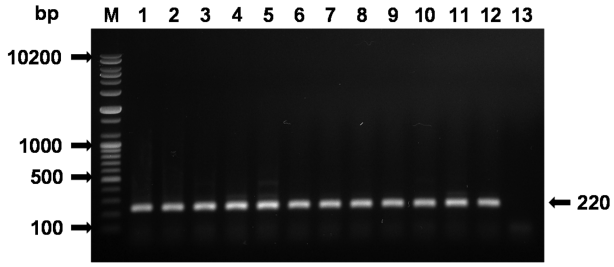

Fig 2. Detection of DNA specific to *Malassezia pachydermatis* (220 bp) in representative isolates in four types of colonies; typical amplicons of LC type isolates (lanes 1 to 3), of MC type isolates (lanes 4-6), SC type (lanes 7-9), and Lipo types (lanes 10-12); lane M, 100 bp plus DNA ladder (Bioneer Co., Daejeon, South Korea); lane 13, negative control without the template DNA.

Table 2. The number of *Malassezia pachydermatis* isolated from the ear wax of dog using SDA, SDAO and LM media as primary isolation medium, respectively

Samples	Biotypes with				No. of isolates/ No. of samples positive
	LC	MC	SC	Lipo	
SDA	4	2	1	0	7/6
SDAO	11	3	2	3	19/13
LM	11	1	1[a)]	3	16/12
Total	12	5	3[a)]	4[b)]	23/14

Abbreviation: SDA, Sabouraud dextrose agar; SDAO, Sabouraud dextrose agar with olive oil; LM, Leeming's medium; LC, large colony type; MC, medium colony type; SC, small colony type; Lipo, Lipophilic yeast. a) Isolated one organism was dead before colony typing. b) All isolated lipophilic yeast showed SC type, except with one isolate (SC) from both SDAO and LM media.

Antifungal effect of essential oils

Anti-*M. pachydermatis* activity testing was done using disc-diffusion assays and well diffusion tests. Thyme and eucalyptus oils were found to be highly effective to inhibit *Malassezia* growth. The inhibition zone was larger in SC types than in LC types of *Malassezia* strains, thereby suggesting that LC type strains had a higher ability to grow and survive oil contact (Table 3 and Fig 3).

With the well diffusion test, almost all 6 essential oils inhibited the growth of *M. pachydermatis* in the range from 0.5% to 1.0% of essential oils. MIC90 and MIC50 varied between essential oils. Thyme oil was found to be the most

Table 3. Means of inhibitory disc diameter zone (mm) of 6 essential oils

Biotypes	Th	E	G	M	L	T
SC type	CI	52	CI	12	11	10
MC type	CI	40	21	13	11	10
LC type	50	33	33	11	9	10

Th, Thyme; E, Eucalyptus; G, Geranium; M, Marjoram; T, L, Lavender; Tea tree; SC, Small Colony; MC, Medium Colony; LC, Large Colony; CI, Complete inhibition

Table 4. MIC90 and MIC50 values of essential oils on *Malassezia pachydermatis*

Essential oil	Faster grower (n = 22)		Slow grower (n = 16)	
	MIC90	MIC50	MIC90	MIC50
Th	0.125%	0.0625%	0.125%	< 0.0625%[a)]
M	0.5%	0.25%	0.5%	0.25%
T	0.5%	< 0.5%[c)]	0.5%	< 0.5%[c)]
L	1%	0.5%	1%	0.5%
E	1%	0.5%	1%	0.5%
G	> 1.0%[b)]	> 1.0%[b)]	> 1.0%[b)]	> 1.0%[b)]

MIC90 and MIC50 mean the concentration of essential oils inhibiting 90% and 50% of the organisms tested, respectively. T, Tea tree; L, Lavender; G, Geranium; Th, Thyme; E, Eucalyptus; M, Marjoram; a) MIC50 at 0.065% and 0.032% were difficult to assess; b) All isolates could grow in media containing 1% geranium oil. c) MIC50 at 0.5% and 0.25% could not be determined.

effective in inhibiting *M. pachydermatis* at a concentration ranging from 0.125% to 0.0625% (Table 4). Marjoram and tea tree oil exhibited also some antifungal effect (Table 4). Interestingly, Eucalyptus and Geranium exhibited low inhibitory effect in this test, as opposed to what had been shown in the in disk diffusion test (Table 4). The MIC values did not appear to be related to either colony types or growth rates of the isolates.

Discussion

Yeast organisms of the genus *Malassezia* are lipophilic fungi that are commensal inhabitants of the skin of mammals and birds in very low numbers (2). Ten distinct species are now recognized (19), and *M. pachydermatis, M. furfur, M. globosa,* and *M. sympodialis* are the best characterized in regard to their role in disease causation (24). *M. pachydermatis*, a unique non-lipophilic *Malassezia*, is a component of the normal cutaneous microflora of dogs and many other mammals (2), while *M. furfur, M. globosa, M. sympodialis,* and *M. restricta* reside naturally on the skin of human beings (4). Lipophilic organisms exhibit the unique capability of using lipid as a source of carbon. While *M. pachydermatis* does not exhibit an absolute requirement for lipid, its growth is still enhanced by the addition of lipid substrates to culture media (19).

In normal dogs with healthy skin, *M. pachydermatis* can routinely be isolated by fungal culture, but proving the presence of the organism by skin surface cytology can be difficult (26). In dogs with allergic or seborrheic dermatitis, the homeostasis of the local cutaneous microenvironment is disrupted by inflammation and increased levels of moisture or sebum (33). Under these conditions, the number of *M. pachydermatis* organisms on the skin and in the ear canals can increase dramatically, increasing the likelihood of readily identifying this organism with rapid screening of surface cytology specimens (32).

In this study, we found four distinct types of *M. pachydermatis* in 78% of 18 dogs. Four biogroups had been separated based on colony size and growth rates, while lipophilic strains were also found. Isolation rate of *M. pachydermatis* was the highest in SDAO (72%) and was lowest onto SDA (33%) due to a possible interference with rapidly growing high populations of *Staphylococcus* spp. A total of 23 isolates biogrouping were recovered from 14 samples with *M. pachydermatis,* indicating some samples included more than one *M. pachydermatis* biogroup. LC type isolates were most prominent (12 of 23 isolates) and 1 isolate classified as SC type might

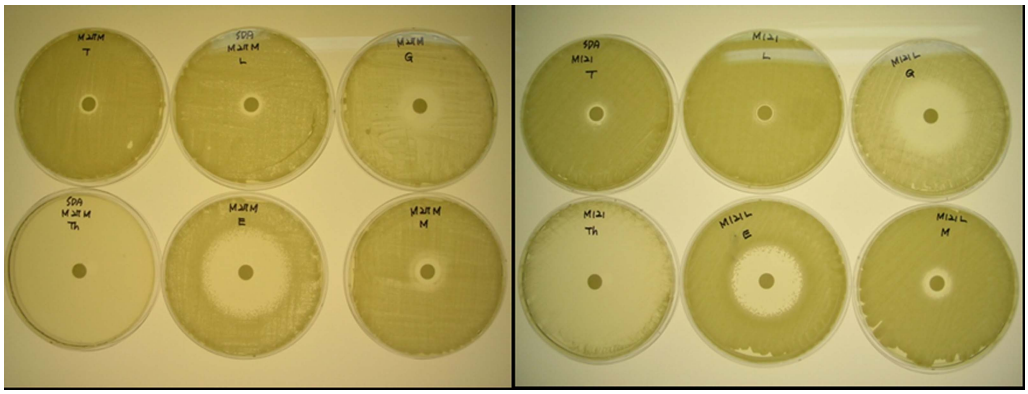

Fig 3. Inhibition zone diameters of representative *Malassezia pachydermatis* by essential oils by disc-diffusion test onto SDA (T, Tea tree; L, Lavender; G, Geranium; Th, Thyme; E, Eucalyptus; M, Marjoram). Left, MC type strain #27; Right, LC type strain # 121
Left and right upper lane, in order of T, L, G
Left and right lower lane, in order of Th, E, M

be Lipo type because it was not failed to subculture (Table 2), but the strain was also identified as *M. pachydermatis* by PCR amplification using colony itself (Data not shown).

Although *M. pachydermatis* is well-known as a non-lipophilic species, four lipophilic strains were found onto SDAO and LM in this work. As a result of this observation, some *M. pachydermatis* may be missed from clinical samples if cultured using routine methods, and lipid supplementation of the culture media may be needed to enhance the sensitivity of the culture method. One of the main causes for the difficulty of characterization of lipid-dependent species is the lack of suitable methods for the isolation and preservation of this yeast to allow them to be studied (19). Moreover, the isolation and identification of lipid-dependent species continues to be difficult due to the low viability associated with certain types of species.

An estimated 3,000 essential oils are known, of which about 300 are commercially important. They are destined chiefly for the flavoring and fragrances market (36) and there are many pharmacological activities, such as antiseptic, antimicrobial, anti-infectious, detoxifying, revitalizing, regulators of the nervous system and hormonal glands (15). Many medicinal plants that contain essential oils have potential wound healing activity (21).

Two oil inhibition tests, disc and well diffusion tests, were used in this study. Small colony types of *Malassezia* strains showed larger inhibition zone than that in LC types. Moreover, some LC type yeast appeared able to regrow from the edge of the inhibition zone after 3 days. Thyme oil showed the highest anti-*Malassezia* effect in both test methods used in this study. It has been already demonstrated that essential oils of female and hermaphrodite, *Thymus bacticus* (thyme oil) showed strong activity against some Gram-positive and Gram-negative bacteria and yeast (10). However, eucalyptus and geranium oils were found to be highly effective in inhibiting the growth of *Malassezia* using the disc diffusion test while both oils showed low MIC90 and MIC50 using the well diffusion test (Table 3, Fig 2 and Table 4). This observation indicates that the antifungal effects of essential oils might be determined by the test method and their diluents as shown in other studies (5,21,23). The antimicrobial activity assays used currently were originally developed to determine the activity of non-volatile and water soluble compounds and their application to volatile and hydrophobic essential oils was unpractical unless assay conditions were adequately modified (22). To overcome this problem, some surface active agents such as Tween 80 were frequently used (7), but these studies have reported the observation that the bioactivity of tested essential oils had weakened (25). Edwards-Jones *et al* (13) have reported that geranium oil and tea tree oil showed the antibacterial effects against methicillin-resistant *S. aureus* (MRSA) in the vapor phase. Recently, essential oil vapors were effective at inhibiting plant fungal colony growth (35). These researches made reasonable results using self-devised oil vaporing apparatuses but it is still difficult to evaluate relatively the antimicrobial activity of oil because it is associated with the vapor concentrations of oils according to their volatility and/or vaporing temperature as mentioned as in the report by Szczerbanik, *et al*. (35).

Although essential oils are one of many candidates to be studied in alternative herbal medicine, their safety has not yet been established. As shown in this study, their estimated value depends on the antifungal testing method. Therefore, further studies on the antimicrobial properties of essential oils must await the development of standard methods to determine antimicrobial activity. Moreover, the toxicity of these oils should be investigated in the target species before recommending their use in clinical patients.

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개에서 분리된 *Malassezia pachydermatis*의 특성과 Essential Oil의 항진균 효과

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요 약 : 이 연구는 개의 귀 속에서 분리한 *Malassezia pachydermatis*의 특성과 이들 효모에 대한 essential oil들의 항진균효과를 측정한 결과로서 일반적인 *M. pachydermatis*를 분리하기위한 Sabouraud dextrose agar (SDA), olive oil이 첨가된 SDA (SDAO) 배지 및 지방 친화성 효모를 검출하기 위한 Leeming's medium (LM)으로 구성된 세종류의 분리배지가 사용되었다. *Malassezia* spp는 77.8%의 개로부터 분리되었으며 분리배지에 따라 분리율에 큰 차이를 나타내었으며 지방성분이 포함된 배지에서의 분리율이 일반적으로 사용하는 SDA에서 보다 높았다. 분리된 모든 *Malassezia* spp는 *M. pachydermatis*로 동정되었지만 육안적으로 보이는 집락의 모양과 SDA에서의 증식속도를 고려했을 때 4개의 아종으로 관찰되었다. 배양 72시간 후 집락의 직경이 3 mm 이상인 큰 집락(LC)과 2 mm 정도인 중간 집락(MC) 및 배양 5일 후에도 1 mm 이상으로 크지지 않는 작은 집락(SC), 그리고 SDA에서 증식하지 않는 지방의존 집락(Lipo)으로 분류되었다. SDA, SDAO 및 LM 배지에서 각 type의 효모가 분리된 정도를 보면, LC type 효모는 각각 4, 11, 11개 시료에서, MC type 효모는 각각 2, 3, 1개 시료에서, SC type 효모는 각각 1, 2, 1개시료에서 분리되었으며 Lipo type은 SDAO과 LM 배지 중 3 곳에서 분리되었다. *M. pachydermatis*에 대한 essential oil의 항진균효과 측정은 디스크 확산법과 well 확산법에 의해 이루어졌으며, 대부분의 essential oil은 0.5에서 1.0%의 농도에서 *M. pachydermatis* 의 증식을 억제하였다. MIC90과 MIC50은 essential oil의 성분에 따라 다양하였으나, Thyme oil이 가장 억제 효과가 좋았으며 marjoram과 tea tree oil은 비교적 낮은 억제 능력을 나타내었다.

주요어 : *Malassezia pachydermatis,* 개, 항진균효과, 이센셜오일