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Evaluation of Different Preservation and Storage Methods for *Malassezia* spp.

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Freezing at −80°C, lyophilization, preservation in distilled water, and storage in different culture media were performed in order to find a suitable method that allowed a prolonged storage of *Malassezia* spp. Freezing at −80°C was the only method successful at maintaining all species.

The genus *Malassezia* comprises lipophilic yeasts that belong to the normal cutaneous microbiota of humans and warm-blooded animals (10), but these yeasts are also associated with a variety of diseases in humans and animals (4, 9, 11).

The taxonomy of this genus has been a matter of controversy since its creation by Baillon in 1889. The great micromorphological polymorphism and the lack of suitable methods for isolation and preservation of these yeasts were the main reasons that made their study difficult. The genus has recently been revised by means of morphology, ultrastructure, physiology, and molecular biology and enlarged to seven species: *M. pachydermatis*, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* (5, 6). Except for *M. pachydermatis*, the remaining six species are lipid-dependent organisms, because they require long-chain fatty acids for in vitro growth. The isolation and identification of *Malassezia* spp., mainly lipid-dependent species, continue to be difficult due to low viability, especially with some of the species.

The purpose of this study was to assay different methods for preservation of microorganisms in order to find a suitable method that allowed a proper and long maintenance of *Malassezia* spp.

Twenty-four strains of *M. pachydermatis* and 11 lipid-dependent strains were used in this study. All strains were isolated from different animals except for six lipid-dependent type strains (kindly provided by E. Gricho and J. Guillot) (Table 1).

The strains of *M. pachydermatis* were grown on Sabouraud glucose agar (SGA) for 3 days at 35°C and the lipid-dependent strains on modified Dixon agar (MD) (5) for 5 days at 32°C.

After incubation, cells were harvested and suspended in 3 ml of 10% skim milk. The number of *Malassezia* cells was determined microscopically in a Bürker chamber, and serial dilutions in skim milk were performed to obtain final suspensions of approximately 10^7 and 10^4 cells per ml. Preservation of lipid-dependent strains was assayed only for the $\sim 10^7$-cells/ml suspension.

Four different preservation methods with some variables included were studied: freezing at −80°C, freeze-drying and storage at room temperature and at −80°C, preservation in distilled water, and use of different culture media stored at 4°C, room temperature, and 28°C. All of these assays were performed in duplicate. In freezing at −80°C, glycerol and di-

TABLE 1. *Malassezia* strains used in this study

Species and isolate[a]	Source
M. pachydermatis	
CCFVB 8	Dog otitis externa
CCFVB 9	Cat otitis externa
CCFVB 10	Dog otitis externa
CCFVB 12	Dog ear (normal)
CCFVB 14	Dog ear (normal)
CCFVB 17	Dog ear (normal)
CCFVB 19	Dog otitis externa
CCFVB 20	Dog otitis externa
CCFVB 22	Dog otitis externa
CCFVB 24	Dog otitis externa
CCFVB 26[b]	Dog otitis externa
CCFVB 27[b]	Dog otitis externa
CCFVB 29	Dog otitis externa
CCFVB 32[b]	Dog ear (normal)
CCFVB 34[b]	Dog otitis externa
CCFVB 35[b]	Dog ear (normal)
CCFVB 36[b]	Dog otitis externa
CCFVB 39[b]	Dog otitis externa
CCFVB 41[b]	Dog otitis externa
CCFVB 97[b]	Horse skin (normal)
CCFVB 107[b]	Goat ear (normal)
CCFVB 140[b]	Cat ear (normal)
CCFVB 145[b]	Dog skin (normal)
CCFVB 158[b]	Cat ear (normal)
M. furfur	
CBS 1878^{NT}[b]	Human pytiriasis capitis
CBS 7019^{NT}[b]	Human pytiriasis versicolor
CCFVB 157[b]	Cat ear (normal)
M. sympodialis	
CBS 7222^{T}[b]	Human skin (normal)
CCFVB 88[b]	Cow skin (normal)
M. slooffiae	
CBS 7956^{T}[b]	Pig skin (normal)
CCFVB 250[b]	Horse skin (normal)
M. globosa	
CBS 7966^{T}[b]	Human pytiriasis versicolor
CCFVB 76[b]	Goat skin (normal)
M. obtusa CBS 7876^{T}[b]	Human skin (normal)
M. restricta CCFVB 79[b]	Goat skin (normal)

[a] Abbreviations: CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra, Spain; CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[b] Strain used in freezing at −80°C, distilled water method, and preservation on different culture media.

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TABLE 2. Effect of freezing and storage at −80°C with glycerol and DMSO on viability of *Malassezia* spp.[a]

No. of mo.	% Recovery (no. of viable strains) for indicated inoculum on indicated medium							
	M. pachydermatis (n = 13)				Lipid-dependent species (n = 11) (10^7 cells/ml)			
	10^7 cells/ml		10^4 cells/ml					
	Glycerol	DMSO	Glycerol	DMSO	Glycerol		DMSO	
					MD	LM	MD	LM
1	118.4 (13)	87.2 (13)	163.0 (13)	121.7 (13)	74.2 (11)	227.0 (11)	82.7 (11)	174.9 (11)
2	105.8 (13)	102.5 (13)	147.4 (13)	98.4 (13)	92.8 (11)	191.1 (11)	89.3 (11)	119.3 (11)
3	126.4 (13)	90.4 (13)	175.6 (13)	98.7 (13)	76.2 (11)	185.0 (11)	70.1 (11)	122.3 (11)
4	131.3 (13)	108.2 (13)	170.2 (13)	95.9 (13)	100.9 (11)	381.0 (11)	86.6 (11)	228.4 (11)
5	176.3 (13)	165.0 (13)	171.5 (13)	105.1 (13)	111.4 (11)	531.2 (11)	148.2 (11)	294.7 (11)
6	112.4 (13)	97.0 (13)	207.1 (13)	100.4 (13)	75.8 (11)	245.9 (11)	78.4 (11)	151.8 (11)
7	144.1 (13)	101.9 (13)	195.8 (13)	108.1 (13)	96.8 (11)	518.6 (11)	86.3 (11)	258.8 (11)
8	95.7 (13)	108.5 (13)	204.1 (13)	100.6 (13)	238.6 (11)	400.7 (11)	110.6 (11)	280.5 (11)
9	148.7 (13)	138.9 (13)	189.6 (13)	121.9 (13)	104.3 (11)	505.7 (11)	66.8 (11)	227.6 (11)
10	103.1 (13)	89.5 (13)	193.9 (13)	116.4 (13)	104.4 (11)	669.5 (11)	79.3 (11)	342.2 (11)
11	108.1 (13)	90.5 (13)	169.2 (13)	129.2 (13)	161.0 (11)	209.4 (11)	120.3 (11)	89.9 (11)
12	102.1 (13)	93.2 (13)	184.8 (13)	174.8 (13)	99.7 (11)	159.0 (11)	39.9 (11)	60.0 (11)
18	121.4 (13)	76.6 (13)	218.8 (13)	93.7 (13)	54.4 (11)	141.2 (11)	69.0 (11)	90.6 (11)
Total	122.6	103.8	183.9[b]	112.7	107.0	335.8[b]	86.7	187.8[b]

[a] Results are expressed as mean percentages of recovery from mean colony counts obtained before freezing; n, number of strains tested. Mean colony counts obtained before lyophilization were as follows: for *M. pachydermatis* (10^7 cells/ml) with glycerol and DMSO, 0.32×10^6 and 0.30×10^6, respectively; for *M. pachydermatis* (10^4 cells/ml) with glycerol and DMSO, 0.19×10^4 and 0.46×10^4, respectively; for lipid-dependent species (10^7 cells/ml for all) with glycerol plus MD and LM, 0.43×10^6 and 0.30×10^6, respectively; and for lipid dependent species with DMSO plus MD and LM, 0.43×10^6 and 0.35×10^6, respectively.

[b] $P < 0.01$.

methyl sulfoxide (DMSO) were employed as cryoprotective agents at concentrations of 10% (vol/vol) and 5% (vol/vol), respectively (2). The solid media used to maintain *M. pachydermatis* strains were SGA and a medium described by Lorenzini and de Bernardis (LDB) (8). For lipid-dependent strains, the media tested were MD, Leeming's medium (LM) (7), and SGA supplemented with olive oil (10 ml per liter). The preparation of inoculum was performed in distilled water to obtain final suspensions of approximately 10^7 cells per ml.

Colony counts before freezing at −80°C, lyophilization, and preservation in distilled water were determined on solid media using the surface-spread method. Aliquots of 0.1 ml of the appropriate dilutions were inoculated on three plates of SGA for *M. pachydermatis* strains and MD and LM for lipid-dependent strains. SGA plates were incubated at 35°C for 5 days, whereas both MD and LM plates were incubated at 32°C for 7 days. In the lyophilization method, colony counts were also determined immediately after the end of the process. Colony counts were determined monthly for a year and a final check at the 18th month when the methods of freezing at −80°C, lyophilization, and preservation in distilled water were used. During the preservation of different culture media stored, the viability was first checked at day 15 and then was checked monthly for a year.

Data obtained were analyzed statistically by means of one-way analysis of variance test, Student's t test, and chi-square test. All statistical analyses were performed using SPSS software (version 8.0).

The effect of freezing at −80°C with glycerol and DMSO on the viability of *Malassezia* spp. is detailed in Table 2. Results are expressed as mean percentages of recovery from mean colony counts obtained before freezing for all strains tested. Mean percentages of recovery of viable *M. pachydermatis* cells from the suspensions of inocula estimated in a Bürker chamber of approximately 10^7 and 10^4 cells per ml, were 3.2 and 19.5%, respectively, for glycerol and 3 and 46.5%, respectively, for DMSO. Mean percentages of recovery of viable lipid-dependent species from the approximately 10^7-cells/ml suspension were 4.3% on MD and 3% on LM for glycerol and 4.3% on MD and 3.5% on LM for DMSO.

The effect of lyophilization and storage at −80°C and at room temperature on the viability of *Malassezia* spp. is detailed in Table 3. Results are expressed as mean percentages of recovery from mean colony counts obtained immediately after lyophilization for all strains tested. Mean percentages of recovery of viable *M. pachydermatis* cells from the suspensions of inocula estimated in a Bürker chamber of approximately 10^7 and 10^4 cells per ml were 8.8 and 37%, respectively. On the other hand, mean percentages of recovery of these yeasts immediately after the lyophilization process were 43.2% ($\sim 10^7$-cells/ml suspension) and 64.9% ($\sim 10^4$-cells/ml suspension). Mean percentages of recovery of viable lipid-dependent species from the $\sim 10^7$-cells/ml suspension were 4.8% on MD and 4% on LM. Mean percentages of recovery of these yeasts immediately after the lyophilization process were 31.2% on MD and 27.5% on LM from the mean counts obtained before lyophilization.

The effect of preservation in distilled water on the viability of *Malassezia* spp. is shown in Table 4. Results are expressed as mean percentages of recovery from mean counts obtained at time zero. Mean percentages of recovery of viable *M. pachydermatis* cells from the suspensions of inocula estimated in a Bürker chamber of $\sim 10^7$ and 10^4 cells per ml were 3.6 and 23%, respectively. Mean percentages of recovery of viable lipid-dependent species from the $\sim 10^7$-cells/ml suspension were 0.9% on MD and 1% on LM.

The number of viable *M. pachydermatis* strains stored in different solid media at 4°C, room temperature, and 28°C are detailed in Table 5. This method of preservation was also unsuccessful for maintaining viable *M. pachydermatis*. Although SGA and LDB were very inefficient for maintaining *M.*

TABLE 3. Effect of lyophilization and storage at −80°C and at room temperature on the viability of *Malassezia* spp.[a]

No. of mo.	% Recovery (no. of viable strains) for indicated inoculum under indicated condition(s)							
	M. pachydermatis (n = 24)				Lipid-dependent species (n = 11)			
	−80°C		Room temperature		−80°C		Room temperature	
	10^7 cells/ml	10^4 cells/ml	10^7 cells/ml	10^4 cells/ml	MD	LM	MD	LM
1	86.8 (24)	106.6 (24)	4.9 (18)	4.7 (13)	121.7 (10)	120.2 (10)	0	0
2	70.0 (24)	114.2 (24)	2.5 (11)	0.6 (7)	96.5 (10)	87.7 (10)	0	0
3	63.5 (24)	109.5 (24)	0.01 (8)	0.01 (5)	58.7 (9)	73.7 (9)	0	0
4	64.8 (24)	100.1 (24)	0.0002 (3)	0.001 (1)	166.8 (10)	86.7 (10)	0	0
5	84.7 (24)	116.4 (24)	0	0	240.0 (10)	120.3 (10)	0	0
6	90.8 (24)	111.2 (24)	0	0	132.1 (10)	100.2 (10)	0	0
7	96.5 (24)	93.1 (24)	0	0	104.1 (10)	139.3 (10)	0	0
8	59.2 (24)	128.9 (24)	0	0	134.8 (9)	65.8 (10)	0	0
9	94.7 (24)	125.5 (24)	0	0	63.2 (9)	88.0 (10)	0	0
10	78.0 (24)	114.9 (24)	0	0	136.0 (10)	135.6 (10)	0	0
11	88.1 (24)	129.0 (24)	0	0	281.4 (10)	226.9 (10)	0	0
12	85.8 (24)	111.8 (24)	0	0	103.7 (10)	75.7 (10)	0	0
18	52.3 (24)	125.7 (24)	0	0	125.4 (9)	139.1 (10)	0	0
Total	78.1	114.4[b]			135.7	112.2		

[a] Results are expressed as mean percentages of recovery from mean colony counts obtained after lyophilization; n, number of strains tested. Mean colony counts obtained before lyophilization were as follows: for *M. pachydermatis* (10^7 and 10^4 cells/ml), 0.88×10^6 and 0.37×10^4, respectively; for lipid-dependent species (10^7 cells/ml) grown on MD and LM, 0.48×10^6 and 0.40×10^6, respectively. Mean colony counts obtained after lyophilization were as follows: for *M. pachydermatis* (10^7 and 10^4 cells/ml), 0.38×10^6 and 0.24×10^4, respectively; for lipid-dependent species (10^7 cells/ml) grown on MD and LM, 0.15×10^6 and 0.11×10^6, respectively. Mean percentages of recovery immediately after the lyophilization process from the mean colony counts obtained before lyophilization for *M. pachydermatis* (10^7 and 10^4 cells/ml) were 43.2 and 64.9, respectively. Mean percentages of recovery immediately after the lyophilization process from the mean colony counts obtained before lyophilization for lipid-dependent species (10^7 cells/ml) grown on MD and LM were 31.2 and 27.5, respectively.

[b] $P < 0.01$.

pachydermatis strains, SGA recovered a higher number of strains than LDB and particularly in the first months at 28°C.

The number of viable lipid-dependent strains stored in different solid media at 4°C, room temperature, and 28°C are detailed in Table 6. *M. globosa* CBS 7966^T and *M. globosa* CCFVB 76 did not grow on MD at the concentration of inoculum assayed in this method. Therefore, the maintenance on this culture medium was not performed for these strains. Maintenance of lipid-dependent species on different solid media was not a good method of preservation. Similar results were obtained for the different culture media assayed and for both media utilized for the recovery of viable yeasts.

The low viability of the yeasts of the genus *Malassezia* and their difficult maintenance in vitro, especially with some species, constitute the main difficulties that affect the study of this genus (1, 5, 8, 10). In all cases a sharp decrease resulted from the suspensions of inocula estimated in the Bürker chamber in our study.

The methods of preservation assayed in this study are classic methods for maintenance of bacteria and fungi (2, 12, 13). The majority of yeasts can be stored at temperatures between 4 and

TABLE 4. Effect of preservation in distilled water on the viability of *Malassezia* spp.[a]

No. of mo.	% Recovery (no. of viable strains) for indicated inoculum under indicated condition			
	M. pachydermatis (n = 13)		Lipid-dependent species (n = 11) (10^7 cells/ml)	
	10^7 cells/ml	10^4 cells/ml	MD	LM
1	0.004 (6)	0.3 (1)	0.05 (3)	0.003 (3)
2	0.0003 (2)	0	0.004 (2)	0.02 (3)
3	0.0003 (1)	0.07 (1)	0.003 (2)	0.01 (2)
4	0.00007 (1)	0.1 (1)	0.001 (1)	0.0001 (1)
5	0.00007 (1)	0	0	0
6	0	0	0	0.0008 (1)
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
18	0	0	0	0

[a] Results are expressed as mean percentages of recovery from mean colony counts obtained at time zero; n, number of strains tested. Mean colony counts obtained at time zero were as follows: for *M. pachydermatis* (10^7 and 10^4 cells/ml), 0.36×10^6 and 0.23×10^4, respectively; for lipid-dependent species (10^7 cells/ml) grown on MD and LM, 0.95×10^5 and 0.10×10^6, respectively.

TABLE 5. Number of viable *M. pachydermatis* strains stored in different solid media at various temperatures (n = 13)

No. of mo.	No. of viable strains on indicated medium at temp					
	SGA			LDB		
	4°C	RT[a]	28°C	4°C	RT	28°C
0.5	13	13	13	11	3	7
1	8	11	13	5	0	5
2	1	5	13	0	0	6
3	2	5	11	0	0	6
4	0	5	13	0	0	2
5	0	3	12	0	0	1
6	0	3	6	0	0	1
7	0	1	4	0	0	0
8	0	0	1	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0

[a] RT, room temperature.

TABLE 6. Number of viable lipid-dependent strains stored in different solid media at various temperatures ($n = 11$)[a]

No. of mo.	No. of viable lipid-dependent strains stored in indicated medium at indicated temp																	
	MD[b]						LM						SGA + olive oil					
	4°C		RT[c]		28°C		4°C		RT		28°C		4°C		RT		28°C	
	MD	LM	MD	LM	MD	LM	MD	LM	MD	LM	MD	LM	MD	LM	MD	LM	MD	LM
0.5	8	7	9	9	9	9	9	8	8	7	11	11	9	8	10	8	10	9
1	9	7	9	8	8	8	6	5	9	8	11	11	10	6	10	9	9	9
2	7	7	8	6	8	8	6	5	8	7	10	10	4	4	8	7	8	7
3	2	2	5	5	7	7	1	0	6	5	10	10	3	3	6	6	8	7
4	1	1	4	4	7	6	1	0	4	4	11	11	4	4	5	6	8	7
5	1	2	3	3	4	4	0	0	4	4	8	8	3	2	5	5	7	7
6	1	0	3	3	5	5	0	0	3	3	9	8	2	3	5	4	7	7
7	1	0	3	3	5	6	0	0	4	4	8	8	2	3	4	4	8	7
8	0	0	3	3	4	5	0	0	3	3	7	7	2	2	3	3	5	5
9	0	0	2	2	1	1	0	0	3	3	6	7	0	1	4	4	5	5
10	0	0	1	1	2	2	0	0	3	3	5	4	2	2	4	4	6	6
11	0	0	2	2	1	1	0	0	2	2	3	2	1	1	2	2	4	4
12	0	0	1	1	1	1	0	0	2	2	3	3	1	0	2	2	3	3

[a] Each strain was originally grown on MD or LM.
[b] The two strains of *M. globosa* (CBS 7966^T and CCFVB 76) did not grow on MD at the concentration of inoculum assayed.
[c] RT, room temperature.

12°C and subcultured at intervals of 6 to 8 months (13). However, *Malassezia* spp. do not fit this pattern.

In our study, freezing at −80°C was the only successful method to maintain viable all *Malassezia* spp., particularly *M. globosa*, *M. restricta*, and *M. obtusa*, which were reported as difficult species to maintain in vitro (5). However, the best results were obtained when glycerol was used as the cryoprotective agent. It has been reported that the use of skim milk in combination with glycerol is an excellent cryoprotective agent for the preservation of bacteria (3). In this method, mean percentages of recovery were >100% in many cases, especially with the lipid-dependent yeasts.

Homogeneous suspensions are very difficult to obtain with these yeasts and in particular with the lipid-dependent species due to clustering. The cryoprotective agents would facilitate the dispersal of the yeasts, yielding mean recoveries of >100%. The highest mean percentages of recovery were obtained with the lowest suspension of inoculum assayed in *M. pachydermatis* freezing. In this method, LM improved the mean percentages of recovery of lipid-dependent species. LM was described (7) as the best culture medium for isolation and enumeration of *M. furfur* in comparison to MD and Faergemann and Fredriksson's medium.

In our study, lyophilization and storage of the freeze-dried cultures at room temperature was an unsuitable method for preservation of *Malassezia* spp. However, when these freeze-dried cultures were stored at −80°C, all strains tested were recovered except for *M. globosa* CCFVB 76. Other authors observed that this species does not survive lyophilization (5, 10). Some authors recommend the storage of the freeze-dried cultures between 15 and 18°C, and others recommend storage between 4 and 18°C (12). However, *Malassezia* spp. only survived when the freeze-dried cultures were stored at −80°C. The best results for the lyophilization and storage at −80°C of *M. pachydermatis* were obtained with the suspension of approximately of 10^4 cells per ml.

Among the different methods used to preserve fungi, the distilled water method and preservation on different culture media are two of the more-utilized methods, because they are technically simple and easy to perform. Nevertheless, in our study they were unsuccessful methods for maintaining the yeasts of the genus *Malassezia*.

As has been mentioned previously, the low viability of *Malassezia* spp. constitutes one of the main difficulties in the study this genus. Due to the reclassification of the *Malassezia* genus into seven species and the isolation of new strains from different origins, these results might be considered as preliminary in nature and should be substantiated with further studies on the preservation of *Malassezia* isolates. According to our findings, we recommend the method of freezing at −80°C immediately after the first isolation in order to avoid the loss of isolates.

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