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Vital growth factors of *Malassezia* species on modified CHROMagar Candida

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A comparison of several media, *i.e.,* potato dextrose agar with olive oil (Oil-PDA), modified Dixon agar (mDIX) and variations of Leeming and Notman agar (LNA) for the isolation and growth of *Malassezia* and *Candida* species was examined. Since LNA supported the highest growth of *Malassezia* species its key components, i.e., ox bile, glycerol monostearate, glycerol and Tween 60, were added to CHROMagar Candida. All 7 species of *Malassezia* grew well on this modified medium (LN-CHROM) after incubation for 4 days at 30°C and development was equal to that observed on LNA. Colonies on LN-CHROM were smooth and from pink to dark purple in color. Furthermore, the use of LN-CHROM did not alter the colony characteristics of *Candida* species as compared to that found on CHROMagar Candida. The results of the present investigation indicate that the use of LN-CHROM would make possible the simultaneous isolation and identification of *Malassezia* and *Candida* species.

Keywords *Malassezia* spp., *Candida* spp., essential ingredients

Introduction

The members of the genera *Malassezia* and *Candida* are known to be components of the microbial flora of the skin of warm-blooded animals. Most *Malassezia* species are lipophilic yeasts which colonize the sebaceous glands and are known to be the causative agents of pityriasis versicolor and contribute to the inflammation of seborrheic dermatitis. Reports of the relationship between atopic dermatitis and *Malassezia* yeasts have also appeared in the literature [1–12].

To date, *Malassezia* has been classified into 7 species by molecular analysis of nuclear ribosomal DNA/RNA [13,14]. Two additional species were reported last year [15,16]. Unfortunately, it is difficult to cultivate some of the new species of *Malassezia* on potato dextrose agar with olive oil (Oil-PDA). Therefore, the development of an optimized culture medium capable of supporting the growth of all *Malassezia* species would be of great assistance in the diagnostic laboratory. *Candida* species have also been associated with atopic dermatitis [17] and consequently, a medium suitable for the simultaneous separation and identification of *Malassezia* and *Candida* species would facilitate the study of this disease. Although CHROMagar Candida is widely used as an isolation medium on which colonies of *Candida albicans, C. glabrata, C. tropicalis* and *C. krusei* can be distinguished by their color, it is not suitable for the growth of lipid dependent species of *Malassezia.*

The aims of this study were: i) selection of essential ingredients in *Malassezia* culture media and ii) the evaluation of the use of these components to support the growth of *Malassezia* species on CHROMagar Candida.

Materials and methods

Organisms

The following *Malassezia* and *Candida* species were used in this study: *Malasseszia furfur* CBS 1878,

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M. sympodialis CBS 7222, *M. globosa* CBS 7966, *M. obtusa* CBS 7876, *M. slooffiae* CBS 7956, *M. restricta* CBS 7877, *M. pachydermatis* CBS 1879, *C. albicans* ATCC 90029, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019.

Culture media

Strains of *Malassezia* were maintained on Leeming and Notman Agar (LNA), comprised (per liter) of 10 g peptone (Oxoid), 5 g glucose, 0.1 g yeast extract (Oxoid), 8 g ox bile (Oxoid), 1 mL glycerol, 0.5 g glycerol monostearate, 0.5 mL Tween 60, 10 mL milk and 12 g agar (Oxoid) and sterilized by autoclaving.

Growth of the *Malassezia* species was studied on several different variations of LNA, potato dextrose agar (Oxoid) with olive oil (Oil-PDA) and modified Dixon agar (mDIX). The latter contained, per liter; 6 g peptone (Oxoid), 36 g malt extract, 20 g ox bile (Oxoid), 2 mL glycerol, 2 mL oreic acid and 12 g agar (see Tables 1 and 2). In addition, the development of these same species was investigated on a modified CHROMagar Candida (LN-CHROM) which was comprised (per liter) of 47.5 g CHROMagar Candida (10 g peptone, 22 g special chromgen mixture, 0.5 g chloramphenicol, 15 g agar), 8 g ox bile (Oxoid), 1 mL glycerol, 0.5 g glycerol monostearate and 0.5 mL Tween 60.

Inoculation to plates

Lipid dependent *Malassezia* species were initially cultured on LNA. All strains were transferred onto fresh LNA either at monthly (*M. furfur, M. sympodialis, M. obtusa, M. slooffiae*) or weekly (*M. restricta, M. globosa*) intervals and then washed once with sterilized saline. The cells were suspended in 1 mL saline, then centrifuged at 1,000 × g for 5min. The pellets were resuspended in 1 mL saline, then left for 1 minute to precipitate agglutinating cells out of the suspension. Supernatant was transferred to a new tube and the turbidity of it was adjusted to McFarland 0.5 standard with saline which was used as the original suspension (10^0). Cell number was not counted because the members of genus *Malassezia* tend to self agglutinate. The original suspension was diluted 10 or 100 times with 1 mL saline (10^{-1} and 10^{-2}). Plates (90 mm) containing 20 mL of Oil-PDA, mDIX, LNA and variations of LNA were streaked with a 100 μL of each cell suspensions (10^0, 10^{-1} and 10^{-2}). Four days after incubation at 30C, growth was assessed as follows:

Score A: visible growth at 10^{-2} inoculation levels;
Score B: visible growth at 10^{-1} inoculation levels;
Score C: visible growth at 10^0 inoculation levels;
Score D: cells suspensions form the maintenance medium; and
Score E: no growth

For lipid independent *M.pachydermatis*, PDA was used in place of LNA to establish the inoculum suspension. *M. pachydermatis* and *Candida* species were transferred onto fresh PDA, weekly. Single colonies from 7 days old PDA cultures were directly streaked on to LN-CHROM and CHROMagar Candida then incubated for 4 days at 30°C.

Saccharolytic activity supplemented with lipids

There was a little change in the color tone or colony form of the *Candida* species in LN-CHROM compared with the CHROMagar Candida. To evaluate possible metabolic changes caused by the use of the *Malassezia* essential ingredients, we studied the saccharolytic activity of the *Candida* species through the use of the API ZYM (BioMerieux). All *Candida* species were cultured at 30°C for 2 days and 7 days on PDA and PDA

Table 1 The composition of culture media to examine essential ingredients

	LNA	LNA (−)	LNA S (−)	LNA GT (−)	LNA GT (10)
Peptone (Oxoid)	10 g	10 g	10 g	10 g	10 g
Glucose	5 g	5 g	5 g	5 g	5 g
Yeast extract (Oxoid)	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g
Ox bile (Oxoid)	8 g	8 g	8 g	8 g	8 g
Glycerol	1 mL	1 mL	1 mL		10 mL
Glycerol monostearate	0.5 g	0.5 g		0.5 g	0.5 g
Tween 60	0.5 mL	0.5 mL	0.5 mL		5 mL
Milk	10 mL				
Agar (Oxoid)	12 g	12 g	12 g	12 g	12 g

LNA = Leeming and Notman agar.

Table 2 The composition of culture media to examine substitutes of ox bile

	LNA a	LNA b	LNA c	LNA d	LNA e	LNA f	LNA g
Peptone (Oxoid)	10 g	10 g	10 g	10 g	10 g	10 g	10 g
Glucose	5 g	5 g	5 g	5 g	5 g	5 g	5 g
Yeast extract (Oxoid)	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g
Bile salts (Oxoid)	0 g	4 g	8 g	12 g			
Bile salts No.3 (Oxoid)					4 g	8 g	12 g
Glycerol	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Glycerol monostearate	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
Tween 60	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Agar (Oxoid)	12 g	12 g	12 g	12 g	12 g	12 g	12 g

containing essential ingredients, the cells were suspended 2 mL saline (McFarland No.5–6) and 65 μL of each cell suspension was added to the API ZYM kit wells. Following incubation at 37°C for 4 h, ZYM A and ZYM B reagents were added to the each well and the color generated was assessed 5 min after the addition of the reagents. This kit can be used to detect the following enzymes: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alpha-chymotrypsin, acid phosphatase, naphtol phosphohydrolase, alpha-galactosidase, beta-galactosidase, beta-glucuronidase, alpha-glucosidase, beta-glucosidase, N-acetyl-beta-glucosaminidase, alpha-mannosidase and alpha-fucosidase. Since we thought that CHROMagar Candida containing chloramphenicol and supecial chromogen mixture was not appropriate for this study, we chose to use PDA.

Results

Six of the 7 *Malassezia* species grew better on LNA and LNA(–) than on Oil-PDA and mDIX (Table 3).

Table 3 Growth ability score on each medium after 4 days incubation at 30°

	Oil-PDA	mDIX	LNA	LNA (–)
M. furfur	A	A	A	A
M. sympodialis	C	A	A	A
M. globosa	E	E	B	B
M. obtusa	E	D	B	B
M. slooffiae	C	A	A	A
M. restricta	E	D	D	D
M. pachydermatis	A	A	A	A

Oil-PDA = potato dextrose agar with olive oil; mDIX = modified Dixon agar; LNA = Leeming and Notman agar; LNA (–) = Leeming and Notman agar (without milk); A = growth at 10^{-2} inoculation levels; B = growth at 10^{-1} inoculation levels; C = growth at 10^{0} inoculation levels; D = growth only in direct plating; E = no growth.

The one exception was *M. restricta* which grew very slowly and formed small colonies on these two media. The growth of these same species on LNA without lipids is shown in Table 4. While *M. globosa* and *M. restricta* would not grow in the absence of glycerol monosterate and developed poorly on media lacking Tween 60, no other differences were observed in the growth of the *Malassezia* species on the test media. Since ox bile could not be obtained, bile salts and bile salts No.3 were studied as substitutes, but these substances did not support the growth of the *Malassezia* species (Table 5). Six of the 7 *Malassezia* species grew well on LN-CHROM agar (Table 6) and all formed distinctive colonies with the following characteristics: *M. furfur* – pink, smooth with raised center; *M. sympodialis* – smooth and purple in color; *M. globosa* – light pink, smooth, and small; *M. obtusa*- dark purple, small, smooth; *M. slooffiae* – light pink, rough; *M. restricta* – dark purple, rough; and *M. pachydermatis* – light purple, smooth (Fig. 1).

Table 4 Growth ability score on LNA which excluded various lipid sources after 4 days incubation at 30°

	LNA S (–)	LNA GT (–)	LNA (–)	LNA GT (10)
M. furfur	A	A	A	A
M. sympodialis	A	A	A	A
M. globosa	E	D	C	C
M. obtusa	C	B	B	C
M. slooffiae	B	B	B	B
M. restricta	E	C	C	C
M. pachydermatis	A	A	A	A

LNA S (–) = Leeming and Notman agar (without milk and glycerol monostearate); LNA GT (–) = Leeming and Notman Agar (without milk, glycerol and Tween 60); LNA (–) = Leeming and Notman Agar (without milk); LNA GT (10) = Leeming and Notman Agar (without milk and with a 10 fold quantity of glycerol and Tween 60 added); A = growth at 10^{-2} inoculation levels; B = growth at 10^{-1} inoculation levels; C = growth at 10^{0} inoculation levels; D = growth only in direct plating; E = no growth.

Table 5 Influence of bile salts in Leeming and Notman Agar after 4 days incubation at 30°

	LNA (−)	LNA a	LNA b	LNA c	LNA d
M. furfur	A	A	A	A	A
M. sympodialis	A	D	B	C	C
M. globosa	C	E	C	C	E
M. obtusa	B	E	C	D	D
M. slooffiae	B	E	C	D	D
M. restricta	C	E	C	C	D
M. pachydermatis	A	A	A	A	A

LNA (−) = Leeming and Notman agar (without milk); LNA a = LNA (−) (without ox bile); LNA b = LNA (−) (bile salts with 4 g/L added instead of ox bile); LNA c = LNA (−) (bile salts with 8 g/L added instead of ox bile); LNA d = LNA (−) (bile salts with 12 g/L added instead of ox bile); A = growth at 10^{-2} inoculation levels; B = growth at 10^{-1} inoculation levels; C = growth at 10^{0} inoculation levels; D = growth only in direct plating; E = no growth.

Candida species on LN-CHROM formed colonies with the following characteristics: *C. albicans*-light green and smooth; *C. glabrata*-purple and smooth; *C. tropicalis* – smooth and light blue with a violet halo: *C. krusei* – smooth and pink; and *C. parapsilosis* – cream colored and smooth (Fig. 2). A culture containing a mixture of *C. albicans, C. tropicalis, C.parapsilosis* and *M. furfur* is shown in Fig. 3.

Discussion

In this investigation we found that LNA and LNA(−) best supported the growth of the *Malassezia* species. Furthermore, ox bile, glycerol monostearate, glycerol and Tween 60 would appear to be essential components of LNA required for the development of these yeastlike fungi. While we employed a small test population, we believe that our results may be of broad application in their isolation and identification.

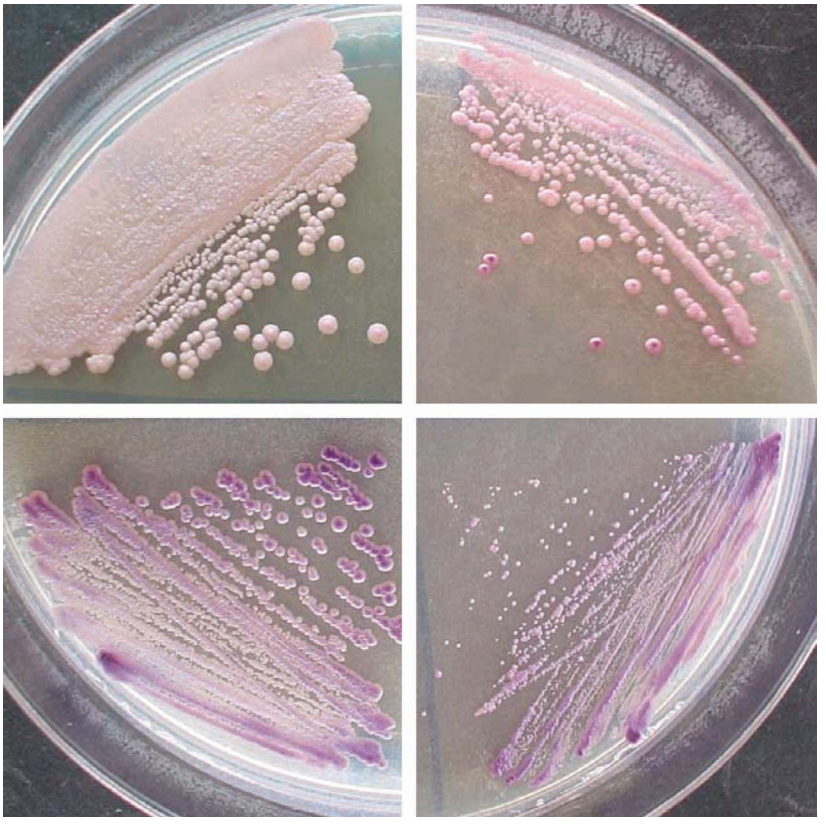

Fig. 1 Appearance of *Malassezia* species on LN-CHROM after 4 days incubation at 30°. Clockwise from 12 o'clock: *M. pachydermatis*, *M. slooffiae*, *M. sympodialis*, *M. furfur*.

Table 6 Growth ability score on LNA and LN-CHROM after 4 days incubation at 30°

	LNA	LN-CHROM
M. furfur	A	A
M. sympodialis	A	A
M. globosa	B	B
M. obtusa	B	B
M. slooffiae	A	A
M. restricta	D	D
M. pachydermatis	A	A

LNA = Leeming and Notman agar; LN-CHROM = CHROMagar Candida (containing ox bile, glycerol monostearate, glycerol and Tween 60); A = growth at 10^{-2} inoculation levels; B = growth at 10^{-1} inoculation levels; C = growth at 10^{0} inoculation levels; D = growth only in direct plating; E = no growth.

By adding the four essential ingredients to CHROMagar Candida (LN-CHROM), we found that it supported the growth of all 7 *Malassezia* species. In addition, the use of these exogenous lipid sources did not significantly affect the color and colony morphologies of the *Candida* when compared to their appearance on standard CHROMagar Candida medium.

Although an overlay of olive oil on a solid nutrient medium has been widely used for the growth of *Malassezia*, several of the recently recognized species do not develop well under these conditions. In contrast, LN-CHROM did support the growth of all 7 test *Malassezia* species.

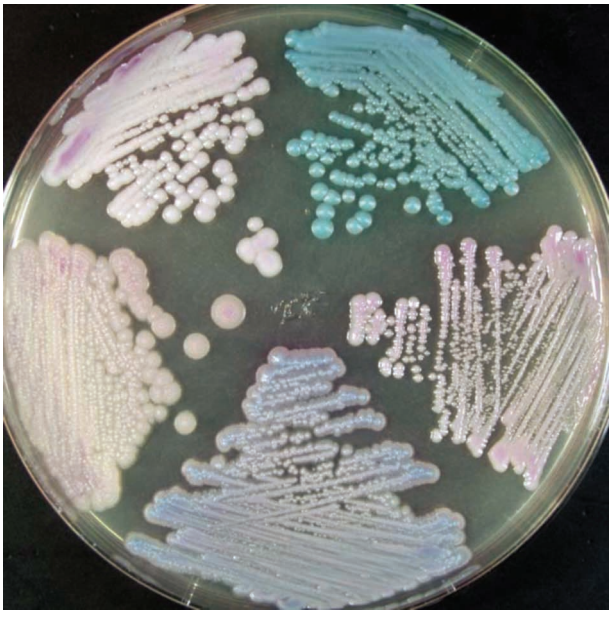

Fig. 2 Appearance of *Candida* species on LN-CHROM after 4 days incubation at 30°. Clockwise from 12 o'clock: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei, C. parapsilosis*.

Based upon these initial studies, it would appear that LN-CHROM can be used for the simultaneous initial isolation of clinical important species of *Candida* and *Malassezia.*

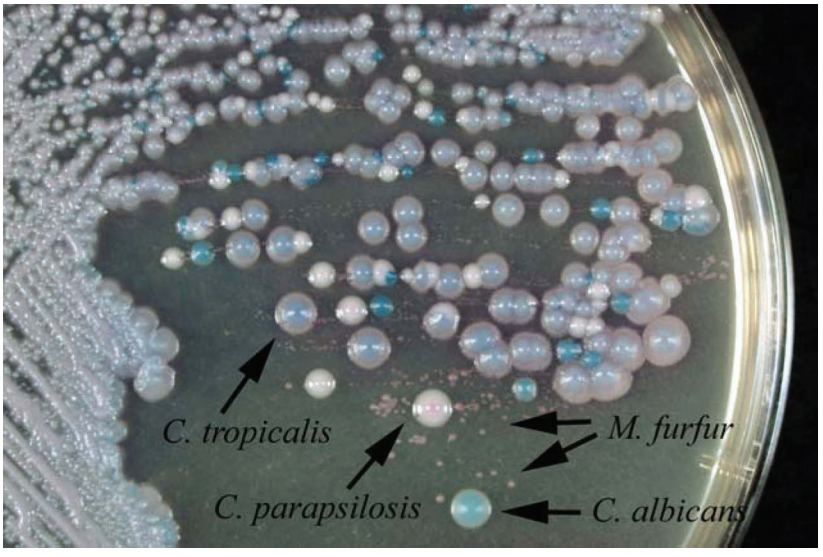

Fig. 3 Appearance of *Malassezia* and *Candida* species on LN-CHROM after 4 days incubation at 30°. Mixture of *C. albicans*, *C. tropicalis, C. parapsilosis* and *M. furfur*.

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