

# Correlation between genetic variability and virulence factors in clinical strains of *Malassezia pachydermatis* of animal origin

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## SUMMARY

*Malassezia pachydermatis* is a yeast belonging to the microbiota of the skin and mucous membranes of dog and cat, but it can also act as pathogen, causing dermatitis. The aim of this work was to evaluate the genetic variability of *M. pachydermatis* strains isolated from symptomatic dogs and cats and determine a correlation between genotype and phenotype. For this purpose eleven strains of *M. pachydermatis* were molecularly classified by nested-polymerase chain reaction (nested-PCR) based on ITS-1 and ITS-2 regions, specific for fungal rRNA genes. Furthermore, random amplification of polymorphic DNA (RAPD) was applied for genetic typing of *M. pachydermatis* isolates identifying four different genotypes. Strains belonging to genotype 1 produced the highest amount of biofilm and phospholipase activity. The inflammatory response induced by *M. pachydermatis* strains in immortalized human keratinocytes (HaCat cells) was significantly different when we compared the results obtained from each strain. In particular, HaCat cells infected with the strains belonging to genotypes 1 and 2 triggered the highest levels of increase in TLR-2, IL-1 $\beta$ , IL-6, IL-8, COX-2 and MMP-9 expression. By contrast, cells infected with the strains of genotype 3 and those of genotype 4 did not significantly induce TLR-2 and cytokines. The results obtained might suggest a possible association between genotype and virulence factors expressed by *M. pachydermatis* strains. This highlights the need for a more accurate identification of the yeast to improve the therapeutic approach and to monitor the onset of human infections caused by this emergent zoonotic pathogen.

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## INTRODUCTION

*Malassezia* yeasts are commensal microorganisms among which *M. pachydermatis* is frequently isolated from the skin of normal animals. When the physical, chemical or immunological mechanisms of the skin are altered, this yeast can become pathogenic (Ashbee, 2007; Bond, 2010; Gaitanis *et al.*, 2012; Nardoni *et al.*, 2005; Shokri *et al.*, 2010). Although rare, cases of life-threatening fungemia in humans have been attributed to *M. pachydermatis* for which dogs and cats are natural hosts (Guillot and Bond, 1999; Morris *et al.*, 2005; Prohic and Kasumagic-Halilovic, 2009). Risk factors for infants with *M. pachydermatis* fungemia were described to be similar to those of *M. furfur* sepsis (Mickelsen *et al.*, 1988). Moreover, *M. pachydermatis* can be transmitted to pet animal owners who can become reservoirs for mechanical transfer of the yeast (Fan *et al.*, 2006). Currently there is

a dispute regarding the pathogenic role and its interaction with the human immune system. In fact, the exact mechanism by which *M. pachydermatis* causes inflammation, as well as the factors promoting proliferation and its transition from a commensal organism to an apparent pathogen on host skin are poorly understood.

*M. pachydermatis* produces several virulence factors to colonize the host, such as the enzymes esterase, lipase, lipoxygenase, protease, chondroitin sulphatase and hyaluronidase (Ortiz *et al.*, 2013). *Malassezia* species also produce phospholipase activity that triggers the release of arachidonic acid from Hep-2 cell lines (Plotkin *et al.*, 1998; Riciputo *et al.*, 1996). Since arachidonic acid metabolites are involved in skin inflammation, this has been suggested as a mechanism by which *Malassezia* species may trigger inflammation. Recent data suggested that phospholipase activity may play an important role in the colonization of host tissues in chronic canine otitis cases (Ortiz *et al.*, 2013). Moreover, it has been demonstrated that phospholipase activity may play a pathogenic role in the occurrence of skin lesions caused by *Malassezia* spp., thus contributing to its virulence (Cafarchia and Otranto, 2004). Other authors showed that *M. pachydermatis* strains isolated from injured dogs' skin produced high amounts of phospholipase A2, supporting the hypothesis that the patho-

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genicity of the yeast is associated with the production of high levels of phospholipase A2 (Kobayashi *et al.*, 2011). Biofilm formation in fungi is a well-organized process that begins with attachment of a microorganism to a surface, followed by a cascade of differential gene expression resulting in biofilm formation (Martinez and Fries, 2010). *M. pachydermatis* biofilm formation has been studied *in vitro*, and its structure consists of clusters of blastoconidia, organized in mono or multilayers with variable production of extracellular matrices. Although biofilm production is not associated with the strain origin (e.g., from lesioned or healthy dog skin), it can act in synergy with phospholipase production, thus inducing or exacerbating skin lesions in dogs (Figuredo *et al.*, 2013).

Recently, we demonstrated that *M. pachydermatis* is able to stimulate the innate immune response in infected human keratinocytes, indicating a possible role of this yeast as a human opportunistic pathogen (Buommino *et al.*, 2013). Toll-like receptors (TLRs), among which TLR-2, are crucial players in the innate immune response to microbial invaders like *Malassezia* (Baroni *et al.*, 2006). One of the consequences of the activation of the TLRs is the release of antimicrobial peptides of the human  $\beta$ -defensin (HBD) family, crucial in mucosal and skin defence (Donnarumma *et al.*, 2004; Hazlett and Wu, 2011). Several studies have shown that *Malassezia* species may induce human keratinocytes to produce cytokines (Baroni *et al.*, 2001; Watanabe *et al.*, 2001).

The aim of this study was to evaluate the genetic diversity of eleven clinical strains of *M. pachydermatis* isolated from pets, and to investigate some virulence factors produced. In addition, the relationship between different genotypes and virulence factors as well as the innate immune response was determined.

## MATERIALS AND METHODS

Approvals for privately owned animal use were obtained from the University of Naples Federico II Institutional Animal Care and informed consent was obtained from participants.

### Fungal strains

*M. pachydermatis* isolates, five from cats and six from dogs, were isolated from auricular swabs of pets with otitis externa at the Department of Veterinary Medicine and Animal Production, "Federico II" University of Naples, Naples (Italy). The swabs were processed for cytologic examination and culturing, as previously described (Buommino *et al.*, 2013).

### Extraction of *Malassezia* DNA

*M. pachydermatis* strains were collected after Sabouraud's dextrose agar plating, placed in 2 mL screw-cap tubes containing 0.3 mL of 1% sodium dodecyl sulphate (Sigma, Milan, Italy) and vortexed at high speed for 30 s. Half of the tubes were filled with 425-600 mm glass beads (Sigma, Milan, Italy) and the samples were vortexed at maximum speed for 30 s. The samples were allowed to cool for 30 s. This vortexing and cooling procedure was repeated four times for a total of five times to break open the cell walls. DNA extraction was performed by phenol-chloroform procedure as previously reported (Buommino *et al.*, 2013). DNA was suspended in 50  $\mu$ L sterile ddH<sub>2</sub>O and stored at 4°C.

### Nested amplification of DNA

The first PCR was performed using the primer set of the internal transcribed spacer (ITS) located between ITS1 and ITS2, specific for fungal rRNA genes. The second two sets of PCR primers were designed to amplify either the ITS1 region (ITS1 forward and middle reverse primers) or the ITS2 region (middle forward and ITS2 reverse primers) in which differences in length among the *Malassezia spp* have been observed (Gemmer *et al.*, 2002). One  $\mu$ L of DNA was amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10  $\mu$ M dNTP, 10  $\mu$ M ITS1 forward, 10  $\mu$ M ITS2 reverse and 2.5 U of Taq DNA polymerase (Roche Diagnostics) in a final volume of 25  $\mu$ L. For nested PCRs, 1  $\mu$ L of the first round amplification product was added to 24  $\mu$ L of a new reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10  $\mu$ M dNTP, 10  $\mu$ M of the ITS1 forward sense and 10  $\mu$ M of the middle reverse primer, or 10  $\mu$ M of the

**Table 1** - Sense and antisense primers sequences and expected PCR products (bp).

Gene	Sense and antisense sequences	Conditions	bp
TLR2	5'-GCCAAAGTCAAAGCATGATCCG-3' 5'-TTGAAGTTCTCCAGCTCCTG-3'	95°C 5 s, 54°C 7 s, 72°C 14 s for 40 cycles	347
IL-1 $\beta$	5'-GTCTCTGAATCAGAAATCCTTCTATC-3' 5'-CATGTCAAATTTCACTGCTTCATCC-3'	95°C 5 s, 58°C 14 s, 72°C 28 s for 45 cycles	421
IL-6	5'-ATGAACTCCTCCTCCACAAGCGC-3' 5'-GAAGAGCCCTCAGGCTGGACTG-3'	95°C 5 s, 58°C 13 s, 72°C 25 s for 40 cycles	628
IL-8	5'-ATGACTTCCAAGCTGGCCGTG-3' 5'TGAATTCTCAGCCCTCTCAAAAACCTTCTC3'	95°C 5 s, 55°C 6 s, 72°C 12 s for 40 cycles	297
HBD-2	5'-GGAGCCCTTTCTGAATCCGCA-3' 5'-CCAGCCATCAGCCATGAGGGT-3'	94°C 5 s, 63°C 5 s, 72°C 11 s for 50 cycles	254
COX-2	5'-GCCTTCTCTAACCTCTCC-3' 5'-CTGATGCGTGAAGTGC-3'	94°C 5 s, 55°C 4 s, 72°C 8 s for 40 cycles	203
MMP-9	5'-GCCAACTACGACACCGACGA-3' 5'-CGCTGGTACAGGTCGAGTAC-3'	95°C 5 s, 56°C 6 s, 72°C 12 s for 40 cycles	310
ITS1-F Middle-R	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TTCGCTGCGTTCTTCATCGA-3'	94°C 1 min, 55°C 1 min, 72°C 1 min for 30 cycles	269
Middle-F ITS2-R	5'-TCGATGAAGAACGCAGCGAA-3' 5'-TCCTCCGCTTATTGATATGC-3'	94°C 1 min, 55°C 1 min, 72°C 1 min for 30 cycles	531

middle forward primer and 10  $\mu\text{M}$  of the ITS2 reverse primer, 2.5 U of Taq DNA polymerase (Roche Diagnostics) and deionised water. The conditions are shown in *Table 1*. The expected PCR products were 269 bp for ITS1-F/Middle-R primers and 569 bp for Middle-F/ITS2-R primers. The reaction was carried out in a DNA thermal cycler (Mastercycler Gradient; Eppendorf, Milan, Italy). The PCR products were analysed by electrophoresis on 1.8% agarose gel in TBE. The PCR products were analysed by PRIMM srl, using DNA Analyser 3730XL (Applied Biosystems, Carlsbad, CA, USA). The DNA alignments were performed submitting the gene sequences obtained to GenBank.

#### *Random amplification of polymorphic DNA (RAPD)*

The primers used in this study to amplify RAPD sequences were OPT20 (5'-GACCAATGCC-3'), M13 (5'-GAGGTGGCGTTCT-3'), OPA-2 (5'-TGCCGAGCTG-3') and FM-1 (5'-AGCCGCCTCCATGGCCCCAGG-3'). Preliminary experiments were performed to establish the concentration of DNA (0.1, 0.5 and 1  $\mu\text{l}$ , corresponding to 50, 100 and 200 ng of genomic DNA respectively) and PCR cycling conditions to improve RAPD results (data not shown). However, among the primers used FM1 presented the best result. It was selected based on high-intensity bands, hypervariability and good definition of DNA fragments. The final RAPD conditions for FM1 primer were: 100 ng of genomic DNA, 10  $\mu\text{M}$  FM1 primer, 10  $\mu\text{M}$  dNTP, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, and 2.5 U of Taq DNA polymerase (Roche Diagnostics) in a final volume of 25  $\mu\text{l}$ . The amplification program included an initial step at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 2 min at 40°C and 2 min at 72°C, with a final extension cycle at 72°C for 7 min. Reactions were performed using a DNA thermal cycler (Mastercycler Gradient; Eppendorf, Milan, Italy). The PCR products were analysed by electrophoresis on 1.8% agarose gel in TBE and stained with ethidium bromide. The gels were photographed under UV light to record the results. *Malassezia* isolates were considered with similar genetic types varying up to two DNA bands of electrophoresis on polyacrylamide gel (Duarte and Hamdam, 2009).

#### *Phospholipase activity*

The test medium consisted of SDA containing 1M sodium chloride 0.005M calcium chloride and 8% sterile egg yolk. The egg yolk (Difco laboratories, Detroit) was first centrifuged at 500 g for 15 min. The sterile supernatant was made up to its original volume in sterile distilled water and incorporated into the sterilized medium. Test isolates were grown on SDA for 24 h then adjusted to an optical density of 0.1 at 590 nm, in sterile saline. Each isolate was tested in triplicate. The plates were incubated at 37°C for 15 days. The dense white zone of precipitation around the colonies of phospholipase-positive isolates was distinctive and well defined. Phospholipase activity was measured in terms of the ratio of the diameter of the colony to the total diameter of the colony plus zone of precipitation. A phospholipase zone (Pz) of 1.00 means that the test strain is negative for phospholipase, while a Pz value of 0.63 means that the test strain is releasing large amounts of phospholipase (Price *et al.*, 1982).

#### *Biofilm production*

Biofilm formation was assayed by measuring the ability of cells to adhere to sterile 96-well polystyrene flat-bottom

microtiter plate (BD Falcon, Mississauga, Ontario, Canada) (Figueredo *et al.*, 2012). One hundred  $\mu\text{l}$  *M. pachydermatis* cell suspension ( $1.0 \times 10^6$  CFU/mL) were transferred to a 96-well microplate to allow biofilm formation and incubated at 37°C for 24 h. After incubation, the wells were rinsed with phosphate-buffered saline (PBS) and air dried at room temperature for 45 min. Two hundred  $\mu\text{l}$  of crystal violet (0.4%) solution were added to each well, and the dishes were incubated for 30 min. Then the wells were washed four times with distilled water and immediately discoloured with 200  $\mu\text{l}$  of 95% ethanol. Elapsed 45 min after the last procedure, 100  $\mu\text{l}$  of discolored solution was transferred to a well of a new plate and the crystal violet measured at 570 nm in an ELISA reader (MICROPLATES Reader, Biorad, Milan, Italy). The strains were tested in triplicate and the average of the absorbance value was measured in optical density (OD).

#### *Real-time PCR analysis*

Semi-confluent keratinocytes (10<sup>6</sup>/well) were infected with *M. pachydermatis* strains for 24 h at a yeast cell to human keratinocyte ratio of 40:1. The ratio yeast/keratinocytes of 40:1 and 24 h incubation were considered the best for the induction of TLR-2 mRNA expression. Total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from human keratinocytes infected and non-infected with *M. pachydermatis*. Three hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase, Roche Diagnostics) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche Diagnostics), at 42°C for 45 min according to the manufacturer's instructions. Real-time PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Diagnostics) (LightCycler 2.0 Instrument) using 2 mL of cDNA, corresponding to 10 ng of total RNA in a 20 mL final volume, 3 mM MgCl<sub>2</sub> and 0.5 mM each primer (final concentration). Primer sequences and annealing temperatures are shown in *Table 1*. A melting curve was made at the end of each amplification step to ensure the absence of non-specific reaction products. The accuracy of mRNA quantification depends on the linearity and efficiency of the PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Quantification was based on the threshold cycle values, measured in the early stage of the exponential phase of the reaction, and by normalisation to the internal standard curve obtained with the housekeeping *b*-actin, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes to avoid discrepancies in input RNA or in reverse transcription efficiency.

#### *Statistical analysis*

Each experiment was performed at least five times. The results are expressed as mean  $\pm$  standard deviation (SD). Student's t test was used to determine statistical differences between the means, and P<0.05 was considered a significant difference.

## RESULTS

#### *Biochemical identification of Malassezia pachydermatis isolates*

We isolated eleven *Malassezia* strains that were identified by their microscopic morphology and physiologically by

**Table 2** - Molecular analysis of *M. pachydermatis* strains isolated from auricular swabs of cats or dogs with otitis externa.

Animal	<i>M. pachydermatis</i> strains	Accession number	% identity
cat	1	AY743637.1	99%
cat	3	AB118940.1	96%
cat	4	AB724301.1	99%
cat	27	AB118939.1	96%
cat	47	EU915453.1	99%
dog	50	AB724303.1	99%
dog	89	EU915453.1	99%
dog	105	AB724312.1	99%
dog	106	AB118937.1	99%
dog	115	AB118941.1	99%
dog	116	EU915447.1	99%

their ability to grow on Sabouraud's dextrose agar without olive oil supplementation and by a positive urease test on Christensen's urea slants after 48 h incubation at 37°C.

#### Molecular identification and genetic typing of *Malassezia pachydermatis* isolates

To confirm the biochemical identification of the yeasts isolated, nested PCR of the ITS1 and ITS2 regions of the ribosomal gene cluster was performed as previously reported (Buommino *et al.*, 2013). Isolation of fungal DNA from the *M. pachydermatis* samples resulted in amplicons of the expected sizes (data not shown). In addition, the molecular analysis by PRIMM s.r.l. reported that the DNA of the strains isolated was closely related to the sequences of *M. pachydermatis* available in the GenBank database, as shown in Table 2.

All the strains were subjected to RAPD analysis using two primers OPA2 and FM1. FM1 displayed the overall highest grade of discrimination and was selected for studying DNA polymorphism-based variability among the selected isolated (data not shown). The reproducibility of RAPD patterns was assessed by carrying out a number of experiments in triplicate. No significant differences in the triplicate were observed indicating the robustness of the condition used (data not shown). As illustrated in Figure 1, the strains of *M. pachydermatis* presented different genotypes. Isolates with identical

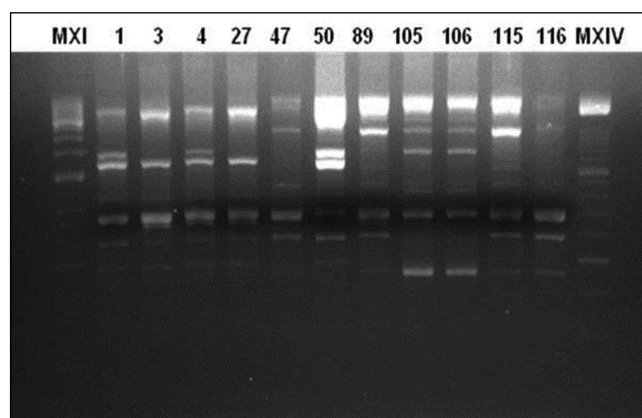
banding profiles were regrouped in four different genotypic clusters as follows: cluster 1 for the strains *M.p.* 3 and 27; cluster 2 for the strains *M.p.* 1, 4 and 50; cluster 3 for the strains *M.p.* 47, 89, 115 and 116; cluster 4 for the strains *M.p.* 105 and 106.

#### Phospholipase activity

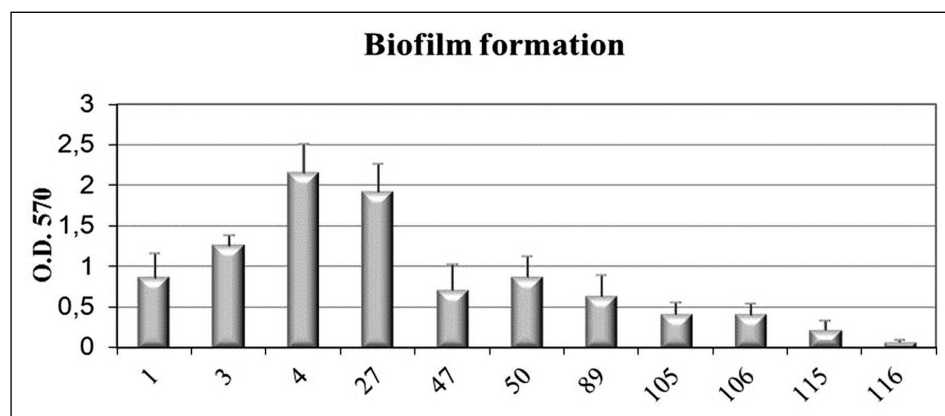
Strains positive for phospholipase activity produced a separate zone of precipitation around the colony. This is due to the formation of a complex between calcium and fatty acids released by the activity of the enzyme on the phospholipids present in the egg yolk. The zone of precipitation varied depending on the isolate under test. However, in all cases the triplicate plates showed that the system gave a constant zone width for any given positive isolate. Of the eleven strains isolated only three were positive to the phospholipase test (strains 3 and 27, belonging to cluster 1; strain 1, belonging to cluster 2). Phospholipase activity was measured in terms of ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation (Pz value).

#### Biofilm production

The strains isolates were analyzed to assess their ability to produce biofilm on abiotic surfaces. Biofilm formation showed variability among the different strains: almost all the strains tested produced biofilm. In particular, strains *M.p.* 3, 4 and 27 produced the highest amount of biofilm (Figure 2).



**Figure 1** - Electrophoretic profiles of *M. pachydermatis* strains generated by RAPD with FM1 primer. Markers XIV and XVI (Roche Diagnostics).



**Figure 2** - Evaluation of biofilms formed by *M. pachydermatis* strains after 24 h of growth and stained by crystal violet. Bars represent one standard deviation from the mean of five experiments. The number of the strains is reported in the x-axis.

### Modulation of TLR expression in *M. pachydermatis*-infected keratinocytes

To investigate if *M. pachydermatis* strains induced a pro-inflammatory response in human keratinocytes gene expression of TLR-2,  $\beta$ -defensin 2, IL-1 $\beta$ , IL-6, IL-8, as well as COX-2 and MMP-9 were analyzed by real-time PCR. HaCaT cells were, thus, infected for 24 h to the ratio yeast/keratinocyte of 40:1. The results show that almost all *M. pachydermatis* strains were able to modulate the inflammatory response in HaCaT cells, although to a different extent. In particular, as shown in Figure 3, a significant increase in TLR-2 gene expression was observed in keratinocytes infected with strains *M.p.* 1, 3, 4, 27, 50 and 89. The other strains determined a scant increase in TLR-2, considered not to be significant.

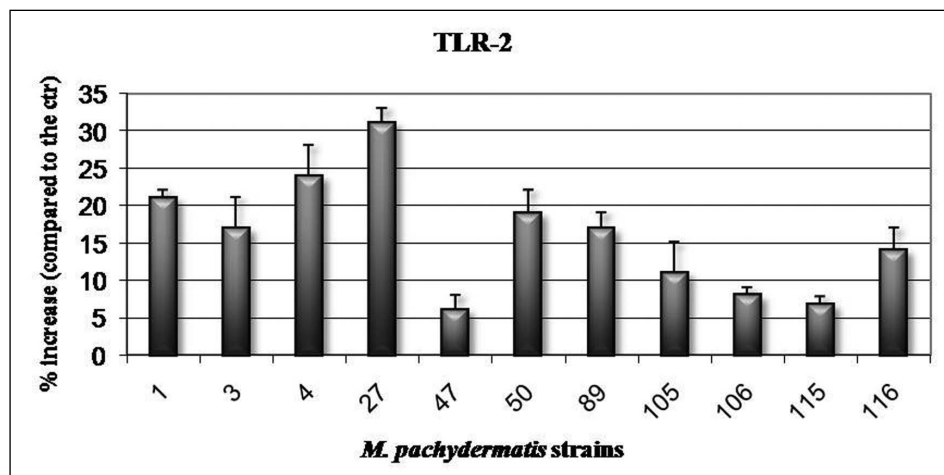
It is known that the activation of the TLRs induces the release of antimicrobial peptides of the  $\beta$ -defensin family. To investigate if this activation also occurred in *M. pachydermatis*-infected keratinocytes the HBD-2 gene expression was analyzed. As shown in Figure 4, HBD2 gene expression was upregulated after 24 h only when *M. pachydermatis* strains 1, 3, 4 and 27 were used to infect HaCat cells at the ratio 40:1. Subsequently, the induction IL-1 $\beta$ , IL-6 and IL-8 gene expression was investigated. As shown in Figure 5A, IL-1 $\beta$  was strongly induced in keratinocytes infected with strains *M.p.* 1, 3, 27 and 50. The other strains induced lower levels of IL-1 $\beta$  gene expression (*M.p.* 4, 47, 105 and

106), whereas the remaining strains (*M.p.* 89, 115 and 116) induced values that were not significant. IL-6 was induced in keratinocytes infected with almost all strains (Figure 5B). However, the strongest induction was observed for strains *M.p.* 1, 3, 4 and 27 with a gene expression increase superior to 30%. Finally, almost all the strains induced a significant IL-8 gene expression, except strains *M.p.* 115 and 116 (Figure 5C). The highest values were observed when HaCat cells were infected with strains *M.p.* 3 and 27 (gene expression increase  $\geq 60\%$ ).

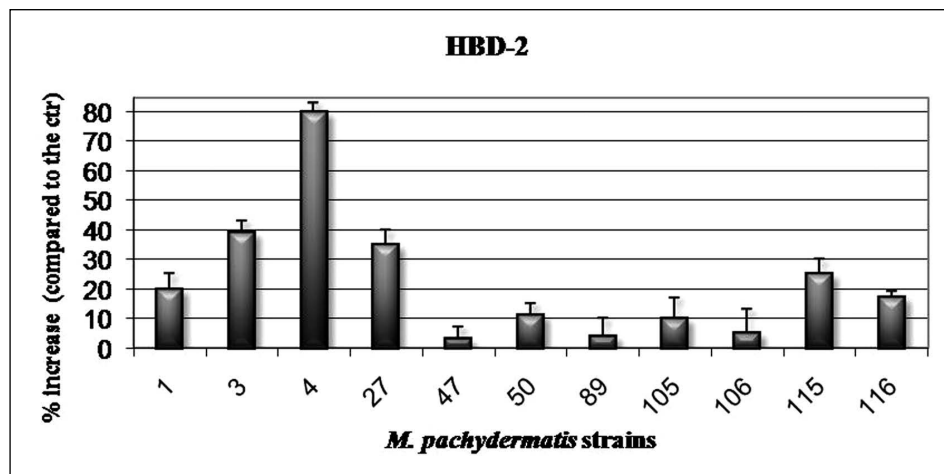
To further investigate the inflammatory response COX-2 and MMP-9 gene expression was analyzed. As reported in Figure 6A, COX-2 gene expression was induced in keratinocytes infected with strain *M.p.* 1 (30%), *M.p.* 3 (25%), *M.p.* 27 (24%) and *M.p.* 105 (22%). However, strain *M.p.* 1 induced the strongest increase. Strains *M.p.* 1, 3 and 27 induced also MMP-9 gene expression, with the strongest increase reached with strain *M.p.* 3 (51%). Strains *M.p.* 4 (31%) and *M.p.* 50 (30%) were also able to induce MMP-9 gene expression. The remaining strains did not induce MMP-9 gene expression (Figure 6B).

## DISCUSSION

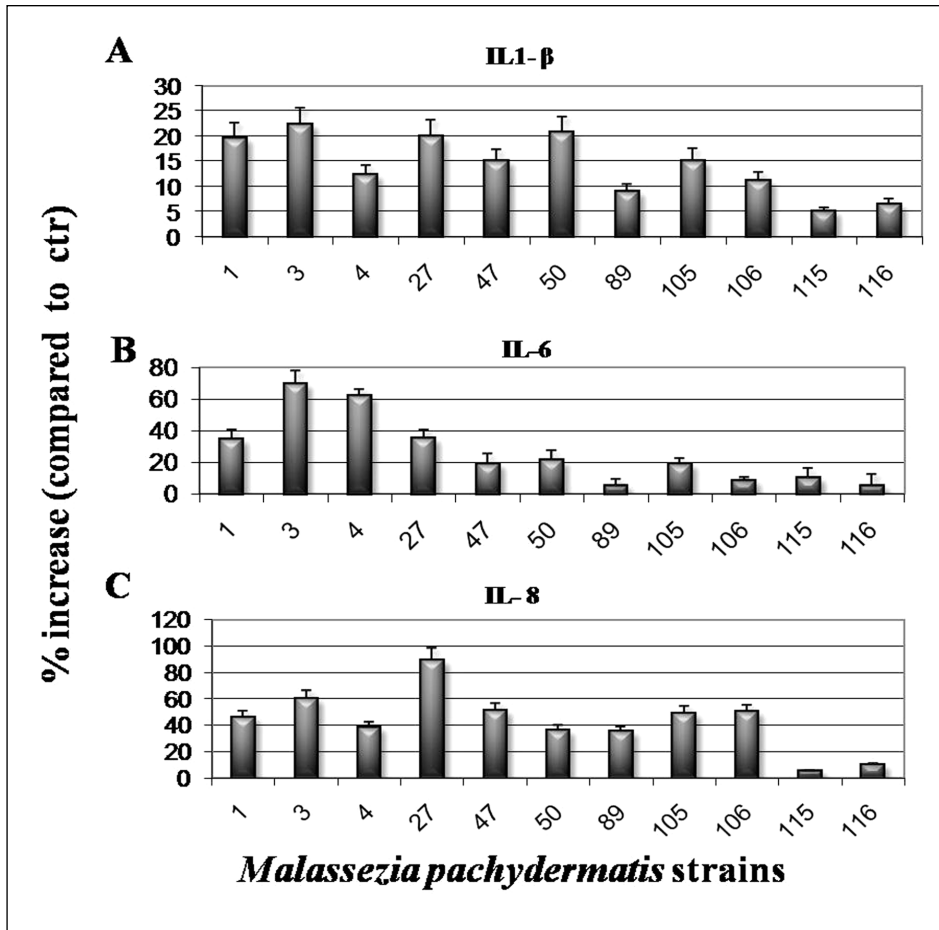
This study genetically characterized eleven strains of *M. pachydermatis* isolated from dogs and cats with otitis externa. In particular, the aim was to assess whether a cor-



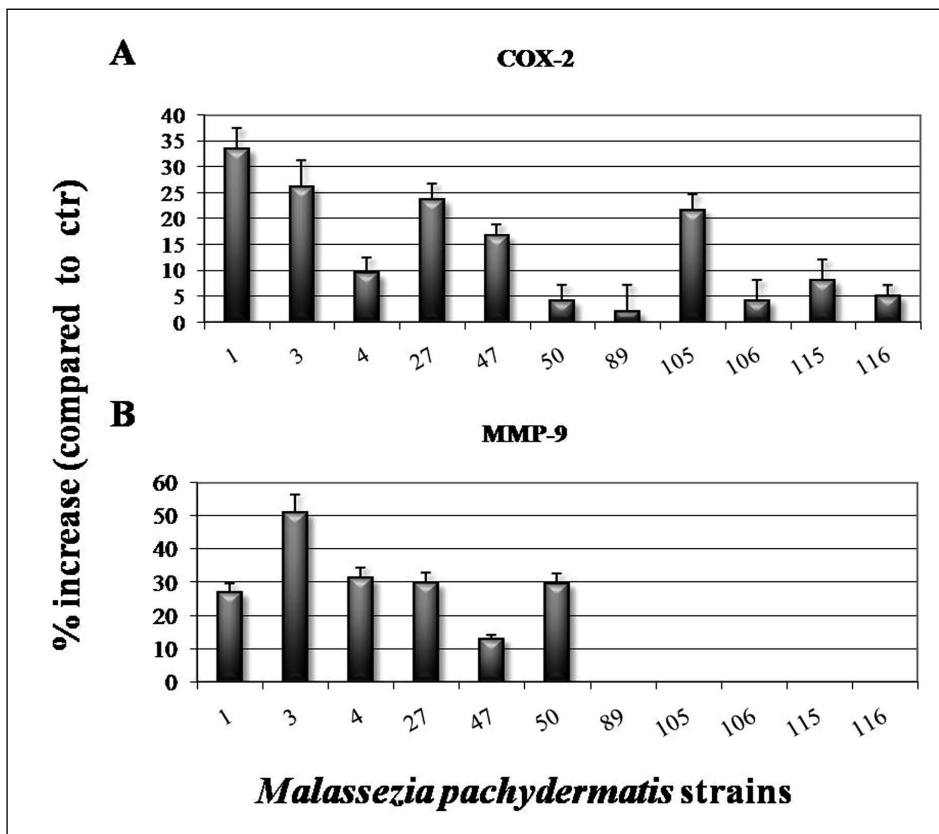
**Figure 3** - Real-time PCR analysis using specific primers for TLR-2. HaCat cells infected with *M. pachydermatis* strains for 24 h at the ratio of 40:1. The data shown are representative of five different experiments  $\pm$  SD ( $P < 0.05$ ).



**Figure 4** - Real-time PCR analysis using specific primers for HBD-2. HaCat cells infected with *M. pachydermatis* strains for 24 h at the ratio of 40:1. The data shown are representative of five different experiments  $\pm$  SD ( $P < 0.05$ ).



**Figure 5** - Real-time PCR analysis using specific primers for IL-1 $\beta$  (A), IL-6 (B) and IL-8 (C). HaCat cells infected with *M. pachydermatis* strains for 24 h at the ratio of 40:1. The data shown are representative of five different experiments  $\pm$  SD ( $P < 0.05$ ).



**Figure 6** - A) Real-time PCR analysis using specific primers for COX-2; B) Real-time PCR analysis using specific primers for MMP-9. HaCat cells infected with *M. pachydermatis* strains for 24 h at the ratio of 40:1. The data shown are representative of five different experiments  $\pm$  SD ( $P < 0.05$ ).

relation exists between different genotype strains and virulence factors and what role such variability could have in the early stages of colonization. Our previous study already demonstrated the ability of *M. pachydermatis* to induce an inflammatory response *in vitro* in human keratinocytes (Buommino *et al.*, 2013). Few studies have, however, attempted to establish a relationship between genotype and virulence of the strain in the host (Castellà *et al.*, 2004).

In this study, RAPD analysis demonstrated four different genotypes indicating a moderate genetic variability among isolates. On the basis of the genetic polymorphisms obtained it was interesting to determine whether particular RAPD patterns could be associated with different virulence factors expressed by the strains tested. The production of phospholipase has been demonstrated for different species of the genus *Malassezia* (Coutinho and Paula, 2000; Riciputo *et al.*, 1996). A previous study demonstrated that yeasts belonging to *M. pachydermatis* species isolated from dogs and cats showed a strong enzyme activity producing 15 different enzymes including phospholipase (Mancianti *et al.*, 2001). The ability to produce this enzyme might play a role in the pathogenicity of the yeast and in the formation of skin lesions thus contributing to the virulence of the yeast itself (Cafarchia and Otranto, 2004; Mancianti *et al.*, 2001). Here, we showed that strains *M.p.* 3 and 27 (genotypic cluster 1), and *M.p.* 1 (genotypic cluster 2) were positive to phospholipase assay, with a more marked production for *M.p.* 1. Although the production of phospholipase plays an important role in the exacerbation of skin diseases, it would not seem to be related to the severity of lesions (Cafarchia and Otranto, 2004).

The strains analyzed here also showed a different ability to form biofilm, indicating a strong variability among the clinical isolates. In particular, strains *M.p.* 3, 4 and 27 produced the highest amount of biofilm. It has been reported that yeast strains isolated from animals with skin lesions produced a quantity of biofilm directly proportional to phospholipase activity (Figueredo *et al.*, 2012). *Malassezia* strains producing both virulence factors might thus induce or exacerbate skin lesions in the host (Figueredo *et al.*, 2013).

Pathogenic fungi interact with a variety of host cells and can penetrate a normally non-phagocytic host cell (Baroni *et al.*, 2001; Filler and Sheppard, 2006). The immune system is activated through a first phase of recognition of the pathogen, thanks to specific receptors on the host cell (TLR) and particular molecules recognized as non-self on the surface of the pathogen. The *M. pachydermatis* strains isolated in this study were differently able to induce TLR-2 and HBD-2 gene expression. A correspondence between Toll-2 and HBD-2 induction was evident for almost all the strains of genotypes 1 and 2, even though a strict correspondence between Toll-2 and HBD-2 was not evident for all the strains. Such result could be the consequence of the activation of different receptors or antimicrobial peptides not contemplated in the present study.

TLRs triggers the inflammatory cascade that leads to the production of interleukins (IL) among which IL-8 represents an important cytokine chemotactic for neutrophils and T lymphocytes (Kunkel *et al.*, 1991). We demonstrated that IL-1 $\beta$ , IL-6 and IL-8 gene expression was induced in keratinocytes infected with the strains belonging to genotype 1 (*M.p.* 3, 4 and 27) and genotype 2 (*M.p.* 1 and 50). In particular, *M.p.* 27 induced a strong increase in TLR-2, IL-1 $\beta$ , IL-6 and IL-8 gene expression. In contrast, cells infected with the strains of genotype 3 (*M.p.* 47 and 89) and

those of genotype 4 (*M.p.* 105 and 106) were not able to significantly induce TLR-2 and cytokines.

To corroborate the data obtained up to now, COX-2 gene expression, another marker of inflammation, was evaluated. In accordance with previous results, COX-2 induction occurred mainly in HaCat cells infected with the strains belonging to genotypes 1 and 2, with the exception of strain *M.p.* 50. This could be justified by the fact that *M.p.* 50, showed less virulence than the other strains of the same group. However, it is important to note that COX-2 mRNA and protein is often enhanced in various human cell types by inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Kuwano *et al.*, 2004). As reported by Watanabe *et al.* (2001), *M. pachydermatis* was able to induce a greater release of IL1 $\alpha$ , IL6, IL8 and TNF $\alpha$  by human keratinocytes than the other species. Consequently, the induction of a strong proinflammatory response by almost all the strains is not surprising, as well as the induction of COX-2 gene expression. The particular composition of the *M. pachydermatis* strain cell wall might be responsible for immune system activation, irrespective of genetic polymorphism.

Metalloproteinase 9 (MMP-9) can be produced by keratinocytes during the inflammatory response (Harvima, 2008). An increased production of MMP-9 by infected keratinocytes could facilitate the invasion of pathogenic microorganism in tissue and the migration of leukocytes into the site of infection, through the degradation of the extracellular matrix. In order to highlight differences in the ability of the strains to penetrate tissues, MMP-9 gene expression was evaluated in infected keratinocytes. The induction of matrix metalloproteinases occurred only in cells infected with strains belonging to genotypic cluster 1 (*M.p.* 3 and 27) and genotypic cluster 2 (*M.p.* 1, 4 and 50). The strains *M.p.* 3 and 27 induced the highest increase in MMP-9 gene expression.

In summary, the results reported here showed that a correlation between virulence factors and genetic profiles can be demonstrated mainly for strains belonging to cluster 1. Castellà *et al.* (2004) reported that different genotypes were associated with varying virulence factors, but the pathogenicity of the yeast could also depend on other factors such as yeast adaptation on skin with different lipid compositions. Even though all the strains were able to induce the inflammatory response, only the strains belonging to cluster genotype 1 were able to produce biofilm and phospholipase activity. Phospholipase production may be important in the first phase of colonization of host skin (adhesion), while the ability to form biofilms can lead to chronic infection. Biofilm formation might thus provide a safe environment for cells, protecting them from host immunity and reducing antibiotic efficacy (Donlan and Costerton, 2002). Both mechanisms might exacerbate lesions at topical and/or systemic level. Consequently, some strains of *M. pachydermatis*, by virtue of their virulence factors, could adapt to a new habitat better than others. The study of genetic variability could therefore provide useful information on the degree of virulence of the isolated yeast, helping healthcare workers in the identification of appropriate antifungal therapies, especially in critically ill patients. However, additional experimental studies *in vivo* are needed to confirm this hypothesis. More fungal isolates should be collected in order to provide other important information necessary for the understanding of the pathogenic role of *M. pachydermatis* in humans.

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## Conflict of interest

Authors declare no conflict of interest.

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