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Azole resistance in *Candida albicans* from animals: Highlights on efflux pump activity and gene overexpression

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Summary

This study investigated potential mechanisms of azole resistance among *Candida albicans* from animals, including efflux pump activity, ergosterol content and gene expression. For this purpose, 30 azole-resistant *C. albicans* strains from animals were tested for their antifungal susceptibility, according to document M27-A3, efflux pump activity by rhodamine 6G test, ergosterol content and expression of the genes *CDR1*, *CDR2*, *MDR1*, *ERG11* by RT-qPCR. These strains were resistant to at least one azole derivative. Resistance to fluconazole and itraconazole was detected in 23 and 26 strains respectively. Rhodamine 6G tests showed increased activity of efflux pumps in the resistant strains, showing a possible resistance mechanism. There was no difference in ergosterol content between resistant and susceptible strains, even after fluconazole exposure. From 30 strains, 22 (73.3%) resistant animal strains overexpressed one or more genes. From this group, 40.9% (9/22) overexpressed *CDR1*, 18.2% (4/22) overexpressed *CDR2*, 59.1% (13/22) overexpressed *MDR1* and 54.5% (12/22) overexpressed *ERG11*. Concerning gene expression, a positive correlation was observed only between *CDR1* and *CDR2*. Thus, azole resistance in *C. albicans* strains from animals is a multifactorial process that involves increased efflux pump activity and the overexpression of different genes.

KEYWORDS
animal, antifungal, gene expression, resistance, yeast

1 | INTRODUCTION

The genus *Candida* gathers yeast that compose animal and human microbiota that may cause opportunistic infections.[1–4] Many of these yeast are potentially pathogenic, of which *C. albicans* is the most important in human and veterinary medicine.[5,6] Azole-resistant *C. albicans* strains have been isolated from healthy and diseased animal hosts with no history of prior antifungal treatment.[1,2,4]

In strains from humans, it is known that azole resistance is a multifactorial phenomenon. Resistance commonly emerges from long periods of antifungal treatment/prophylaxis, as a result of genetic mutations or increased expression of genes involved in the biosynthesis of ergosterol (eg *ERG3* and *ERG11*) and/or in the activity of efflux pumps of the Major facilitator superfamily (MFS) and the ATP-binding cassette (ABC).[7,8] ABC pumps are widely described as the main cause of azole resistance in *C. albicans* from humans.[9,10] These strains present higher mRNA levels of the efflux pump genes for *Candida* drug resistance 1 (*CDR1*) and 2 (*CDR2*), even after short exposure to antifungal drugs.[8,10–12] Furthermore, the increased expression of the multidrug resistant 1 (*MDR1*) gene of MFS, which encodes proton-dependent

efflux pumps, may be associated with specific fluconazole resistance.[13] Finally, the increased expression and/or polymorphism/mutation of *ERG11* gene may lead to increased amount of the enzyme Erg11p or decreased affinity between azoles and this enzyme, respectively, resulting in azole resistance.[11,14,15]

The emergence of resistance among *C. albicans* from animals is not well understood. Despite reports of resistance to azoles in these strains,[1,2,16–18] the contribution of the efflux pump encoding genes *CDR* and *MDR* and the gene involved in ergosterol biosynthesis *ERG11* had never been assessed in animal strains. It is hypothesised that the development of resistance in these fungi may be a way to adapt to stress in the environment and/or within animal hosts.[19] Thus, this study aimed to investigate the role of efflux pumps, ergosterol content and expression of the genes *CDR1*, *CDR2*, *MDR1* and *ERG11* in the development of azole resistance among *C. albicans* from animals.

2 | MATERIALS AND METHODS

2.1 | Assessed strains and antifungal susceptibility testing

Thirty azole-resistant *C. albicans* strains from animals were analysed in this study. The animal strains were recovered from rheas (*Rhea americana*, n=9), porcupine (*Coendou prehensilis*, n=1), sheep (*Ovis aries*, n=6), cockatiels (*Nymphicus hollandicus*, n=2), dogs (*Canis lupus familiaris*, n=3) and manatees (*Trichechus manatus*, n=9). One strain recovered from rhea was susceptible to all tested azoles and was chosen as the control strain for gene expression in this study. Almost all tested *C. albicans* strains were colonising different mucosal sites of healthy animals, except for that from porcupine, which was isolated from an invasive fungal infection. In addition, it is important to highlight that none of the animal hosts had a history of antifungal treatment. These strains belong to the fungal collection of the Specialized Medical Mycology Center of the Federal University of Ceará and are stored at −20°C.

The inclusion criterion of the strains was the presence of resistance to at least one azole derivative, but all tested strains were susceptible to amphotericin B and caspofungin. This inclusion criterion was based on the frequent isolation of azole-resistant *Candida* strains from animals. As previously stated, one azole-susceptible strain from a rhea was used as control strain (calibrator for qPCR). Four human strains of *C. albicans* (two azole resistant—C1 and C2, and two azole susceptible—C3 and C4) were added for comparative purposes in different tests of this study. Antifungal susceptibility was reassessed using broth microdilution, following the M27-A3 document of CLSI,[20] as previously reported.[1,2] The assay was performed in duplicate and the strain *C. parapsilosis* ATCC 22019 was used as quality control.[20]

2.2 | Efflux of rhodamine 6G

Rhodamine 6G (R6G) acts as a substrate for CDR pumps.[21] This fluorescent dye is used as a tool for the evaluation of the efflux activity of these pumps. In this step, the efflux of rhodamine 6G was tested by the methodology described by Rocha et al., [22] with modifications. Eleven randomly selected azole-resistant *C. albicans* strains (numbers 3, 9, 10, 11, 18, 19, 21, 25, 27, 28 and 30) were grown in duplicate in 2 mL of YEPD broth, at 37°C, for 24 h. Afterwards, fungal inocula containing 2×10^7 cells mL^{-1} were prepared according to standard 2 on McFarland's scale, after prior validation by cell counting in Neubauer chamber and pour plate technique. Cells were centrifuged at 4500 *g* for 5 min, the supernatant was discarded, and the pellet was washed twice in 2 mL of PBS (pH 7.0). Subsequently, cells were deprived of energy by incubation under constant agitation in 2 mL of PBS at 37°C, for 1 h. Then, R6G was added to obtain a final concentration of 15 μmol L^{-1} and the suspension was incubated at 37°C, for 1 h, in the dark, under constant agitation, to allow the influx of R6G into the yeast cells. After incubation, the cells were washed twice in 2 mL of PBS and suspended in 2 mL of PBS at 4°C. Then, 50 μL of each sample was distributed in a 96-well plate previously filled with 150 μL PBS with or without 1% glucose, at 4°C. Cells were left to sediment for 5 min and the temperature was adjusted to 37°C. Then 50 μL of the supernatant was transferred to a new 96-well plate (Hard-Shell Low-Profile Thin-Wall) after 1, 15, 30 and 60 min. Relative fluorescence units (RFU) were measured at 37°C, using a CFX96 TouchTM Real-Time PCR Detection System (Bio Rad, Hercules, CA, USA), applying 560-580 nm filter. Wells containing only PBS were considered negative control for efflux activity, as they represent the basal fluorescence of each isolate. As previously reported by Rocha et al., [22] after time 15 min, fluorescence reading stabilises tending to a plateau, so this time was used to compare the samples. For comparative purposes, two susceptible *C. albicans* strains from humans (C3 and C4) were added in this step of the research. The obtained data are expressed as relative fluorescence unit of the supernatant of each fungal culture, which is defined as the difference between the fluorescence obtained for glucose-exposed and glucose-free (negative control for efflux activity) replicates of each strain. Therefore, higher RFU values indicate increased efflux activity.

2.3 | Ergosterol content

The ergosterol content of six randomly selected strains was determined, according to Cordeiro et al., [23] with few adaptations. These azole-resistant *C. albicans* strains were cultured in the presence (pre-exposure) or absence (negative control) of MIC/4 fluconazole, in duplicate. The isolates were exposed to MIC/4 fluconazole in 5 mL of YEPD, based on the MIC values obtained for each strain. The initial inoculum was standardised to 0.5 on McFarland's scale and cultures were incubated at 37°C for 24 h. Then, strains were incubated at 37°C, overnight, under constant agitation in 2 mL RPMI 1640 broth, allowing growth to achieve a concentration of 2×10^7 cells mL^{-1}, according to standard 2 on McFarland's scale. Then replicates were centrifuged at 3500 *g* for 5 min. The pellets were washed in PBS and adjusted to the turbidity 2 of McFarland standard. Then 1 mL of the suspension was centrifuged at 3500 *g* for 5 min and the pellet resuspended in 0.5 mL of alcohol-KOH solution (0.7 mol L^{-1} KOH in 60% ethanol) and incubated for 1 h at 95°C in a waterbath. After cooling, 1 mL of

n-hexane was added and the tubes were vigorously shaken for 5 min. Subsequently, the tubes were centrifuged at 3500 *g* for 5 min and the upper organic layer transferred to a new tube and mixed with 1 mL of n-hexane. Then, absorbance of this solution was measured in duplicate at 261, 273, 282 and 294 nm in a spectrophotometer. For comparative purposes, two susceptible *C. albicans* strains from humans (C3 and C4) were added in this step of the research.

2.4 | Total RNA extraction and gene expression analysis

Total RNA was prepared in triplicate from three-pooled sample groups using the RNeasy mini kit (Qiagen Sci., Germantown, MD, USA) according to the manufacturer's instructions. Briefly, the strains were grown in YEPD broth (0.5% yeast extract, 1% peptone, 2% dextrose) at 30°C for 24 h, until reaching the mid-log growth phase. Then, 1 mL of each sample containing approximately 5×10^7 cells, according to McFarland 5. It was centrifuged at 4500 *g* and the pellet was lysed with RLT buffer and 0.45-0.55 mm glass beads using Precellys 24 disrupter (Bertin Technologies, Rockville, MD, USA). The lysate was diluted 1:1 with 70% ethanol and transferred to a spin column. Genomic DNA was degraded using RNase-free DNase for 15 min at room temperature. After three washes, the RNA was eluted in RNase-free water and the concentration was measured using Qubit fluorometer and RNA Assay Kit (Invitrogen, Paisley, UK). The reverse transcription was performed with 1 μg of RNA combined with 1 μL of Improm II (Promega, Madison, WI, USA), 0.5 mM of each dNTP (Promega), 40 U of RNaseOUT (Invitrogen, Grand Island, NY, USA), 0.5 μg of oligo-dT primers (Promega) and RNase-free water to make a final reaction volume of 20 μL. Reverse transcription was performed at 42°C for 60 min, followed by 70°C for 15 min. The cDNA products were stored at −80°C for later use as templates for quantitative real-time PCR (qPCR). Negative controls or RT blanks were prepared under the same conditions, but without inclusion of reverse transcriptase.

Expression levels of the target genes (*CDR1*, *CDR2*, *MDR1* and *ERG11*) and reference endogenous gene (*ACT1*) were assessed by qPCR. The primers used were *CDR1* (F: 5′-TGCCAAACAATCCAACAA-3′ and R: 5′-CGACGGATCACCTTTCATACGA-3′), *CDR2* (F: 5′-AAGGTTTTGATGCTACTGC-3′ and R: 5′-GTCGGACATGTGGCTCAAA-3′), *MDR1* (F: 5′-GTGTTGGCCCATTGGTTTTCAGTC-3′ and R: 5′-CCAAAGCAGTGGGGATTTGTAG-3′), *ERG11* (F: 5′-GGTGGTCAACATACTTCTGCTTC-3′ and R: 5′-GTCAAATCATTCAAATCACCACCT-3′) and *ACT1* (F: 5′-AAGAATTGATTTGGCTGGTAGAGA-3′ and R: 5′-TGGCAGAAGATTGAGAAGAAGTTT-3′).[24] Reaction mix contained 0.5 μL of cDNA, 1.5 μL of 2 μM primer and 7.5 μL of 2 × FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA) in a final volume of 15 μL. An EP Realplex Mastercycler (Eppendorf, HH, Hamburg, Germany) was set up with the following amplification conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 50°C for 15 s for *CDR1*, *CDR2*, *ERG11* and *ACT1*, or 55°C for 15 s for *MDR1*, and 60°C for 30 s. Fluorescence data were acquired during the 60°C extension step. To determine the linearity (R^2) and the efficiency (E) of the PCR amplifications, standard curves were generated for each gene using serial dilutions of cDNA preparations. Specificity of each reaction was ascertained after finishing the amplification protocol. This was achieved by performing the melting procedure (55-95°C, starting fluorescence acquisition at 55°C and taking measurements at 10 s intervals until the temperature reached 95°C). As negative controls, samples with RNA but without reverse transcriptase were used. The relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method.[25] Target gene expression was normalised against *ACT1* transcript levels. Overexpression was considered when the expression level of target genes of the resistant strains was statistically higher than that of the susceptible calibrator strain. Threshold and Cq (threshold cycle) values were automatically determined by the REALPLEX 2.2 software (Eppendorf), using default parameters. As previously stated, one azole-susceptible *C. albicans* strain from a rhea was used as control strain (calibrator for qPCR). For comparative purposes, two resistant *C. albicans* strains (C1 and C2) from humans were added in this step.

2.5 | Statistical analysis

Efflux pump activity, ergosterol content and expression levels of *CDR1*, *CDR2*, *MDR1* and *ERG11* were subjected to Log(Y) transformation, assuming a Gaussian distribution, and expressed as mean ± standard error (SE) of three measurements. These data were then compared using a two-way analysis of variance (ANOVA), followed by Tukey's method for multiple comparison for efflux pump activity and ergosterol content and Fisher's Least Significant Difference post hoc test for gene expression analysis. Spearman's rho (r_s) correlation test was performed with gene expression data. In all tests, $P<.05$ was considered statistically significant. Correlation tests were performed by Spearman method.

3 | RESULTS

The poststorage reevaluation of the antifungal susceptibility demonstrated stability of the azole-resistant phenotype of these animal *C. albicans* strains (Table 1). Resistance to fluconazole and itraconazole was observed in 23 and 26 isolates respectively.

Rhodamine 6G (R6G) test was used to detect efflux pump activity. Figure 1 shows the Relative Fluorescence Unit (RFU) values, which represent the difference between the fluorescence obtained for glucose-exposed and glucose-free (negative control) replicates of each tested strain. Even though azole-resistant strains presented great variability in efflux activity, it was observed that the supernatant obtained from these strains in the presence of glucose showed significantly higher RFU values ($P<.05$) than that obtained from susceptible strains, suggesting enhanced activity of ATP-dependent efflux pumps in the azole-resistant *C. albicans*. Although this significant difference was observed, significant correlations between efflux activity, azole MIC values and gene expression were not found by Spearman's test.

Ergosterol content was determined for eight randomly selected strains at four different wavelengths (261, 273, 282, 294 nm). There were no differences between azole-resistant or susceptible strains,

TABLE 1 Antifungal susceptibility of *Candida albicans* strains from different animal species

Source	Strains	Minimum inhibitory concentration (μg mL^{-1})	
		Fluconazole	Itraconazole
Rhea	1[a]	1[b]	0.5[b]
	2	64	16
	3	64	16
	4	64	16
	5	64	16
	6	64	0.25[b]
	7	1[b]	16
	8	1[b]	4
	9	1[b]	>16
	10	32	0.125[b]
Porcupine	11	64	16
Cockatiel	12	64	16
	13	64	16
Sheep	14	64	16
	15	64	0.5[b]
	16	64	16
	17	64	0.5[b]
	18	>64	>16
	19	>64	>16
Dog	20	64	16
	21	64	16
	22	2[b]	2
	23	8	>16
	24	4[b]	>16
	25	0.125[b]	>16
Manatee	26	32	>16
	27	16	>16
	28	32	>16
	29	4[b]	>16
	30	16	>16
	31	64	>16

[a]Number 1 strain is a susceptible *C. albicans* strain used as control for gene expression tests.
[b]Susceptible.

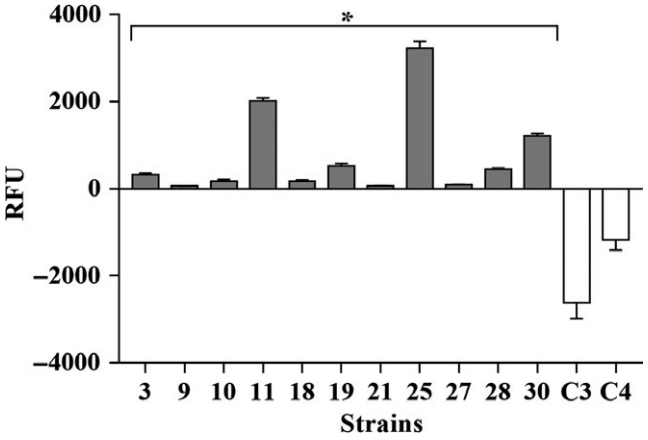

FIGURE 1 Efflux of Rhodamine 6G in azole-resistant *Candida albicans* strains. Data are expressed in Relative Fluorescence Unit (RFU) values, which represent the difference between the fluorescence obtained for glucose-exposed and glucose-free (negative control for efflux activity) replicates of each tested strain. RFU at 15 min after exposure to glucose. White, susceptible strains. Grey, resistant strains. Statistically significant differences were observed between the RFU values of susceptible and resistant strains. Values are expressed as mean ± standard error (SE) of three measurements. *P<.05

when they were exposed or not to fluconazole. These findings suggest that there were no changes in the final amounts of ergosterol or in the sterol profile extracted from the strains, even under the stress of exposure to subinibitory doses of fluconazole.

CDR1, *CDR2*, *MDR1* and *ERG11* expression profiles for all strains are presented in Figure 2. Twenty-two out of thirty (22/30, 73.3%) analysed resistant strains presented overexpression of one or more genes. Of these samples, 40.9% (9/22) overexpressed *CDR1*, 18.2% (4/22) overexpressed *CDR2*, 59.1% (13/22) overexpressed *MDR1* and 54.5% (12/22) overexpressed *ERG11*. Eleven strains (11/22, 50%) overexpressed only one gene alone: *CDR1* (strains 5, 11, 16), *MDR1* (strains 7, 19, 20) and *ERG11* (strains 10, 24, 25, 27, 28). None overexpressed only *CDR2*. Moreover, 27.3% simultaneously overexpressed two genes (6/22; strains 6, 14, 22, 26, 29 and 30), 22.7% overexpressed three genes (5/22, strains 2, 15, 17, 21, 31). None overexpressed the four tested genes. Positive correlations in gene expression were observed between *CDR1* and *CDR2* (r_s=0.787, P<.0001). Spearman's correlation test was performed, but no significant correlations were detected between gene expression, rhodamine 6G efflux and azole MIC values.

4 | DISCUSSION

This study investigated *CDR1*, *CDR2*, *MDR1* and *ERG11* gene expression in *C. albicans* strains from animals with antifungal drug resistance. Previous works of our group indicated the involvement of ATP-dependent efflux pumps in the phenotype of azole resistance, as exposure of azole-resistant strains to an efflux pump inhibitor (promethazine) reversed the antifungal resistance.[1,26] In addition, some of the strains tested in this study showed cross-resistance to different azole drugs, which also suggests the involvement of ATP-dependent efflux pumps, since all azole drugs can act as substrates for these pumps. Antifungal resistance in veterinary strains has been reported in several studies,[1,2,17,18] but little is known about the molecular mechanisms that lead to azole resistance among these strains.

In an attempt to assess possible resistance mechanisms in the studied strains, we performed the rhodamine 6G efflux assay. This substance acts as substrate for ATP-dependent efflux pumps. In

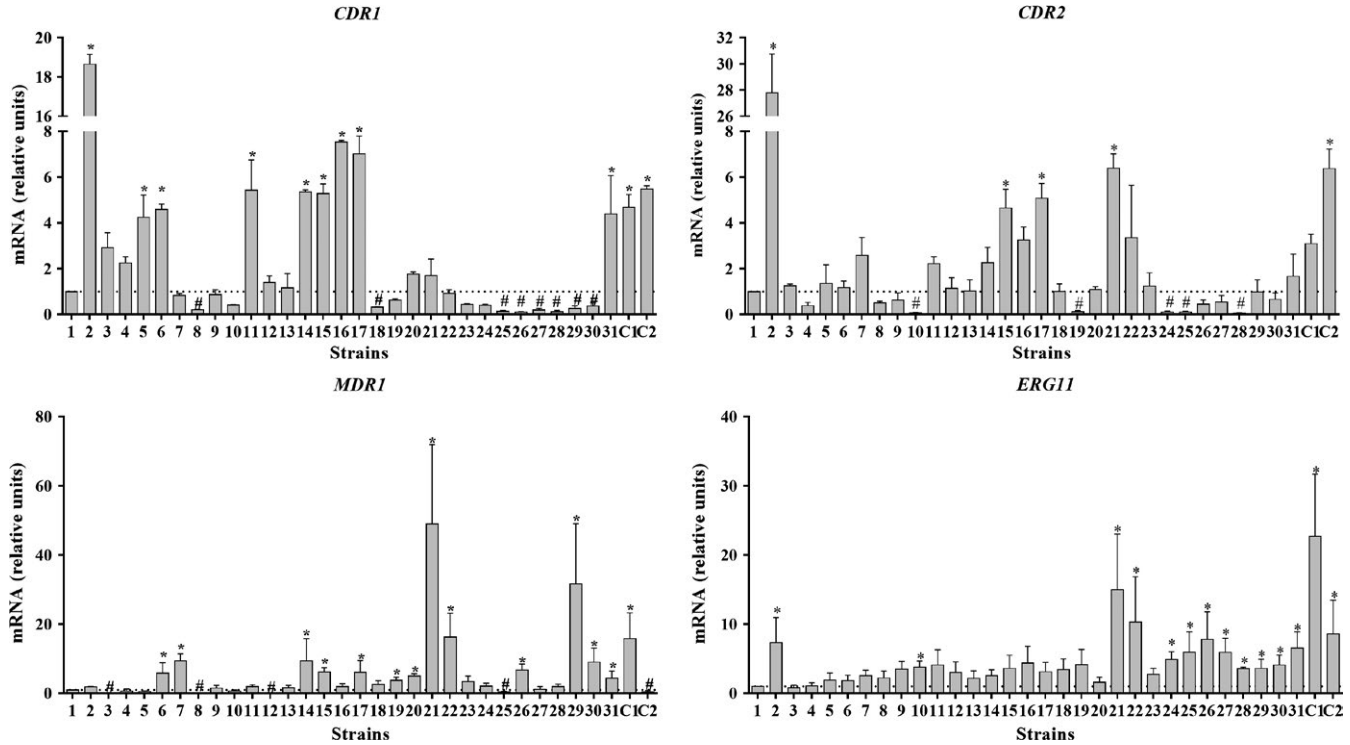

FIGURE 2 Expression of *CDR1*, *CDR2*, *MDR1* and *ERG11*genes in *Candida albicans* strains. Overexpressed genes are detected in several strains. Data were normalised to *ACT1* signal. A susceptible strain (strain 1) was used as calibrator and its transcription level was set as "1 relative unit" (dotted line). Values are expressed as mean ± standard error (SE) of three measurements. * overexpressed gene: $P<.05$; # down regulated gene expression strain

this study, although a large variability in relative fluorescence unit (RFU) values was observed in azole-resistant strains, these strains presented statistically significant higher efflux of rhodamine 6G than the azole-susceptible strains of *C. albicans*, emphasising the contribution of these pumps for the development of azole resistance. Moreover, this variability in RFU values is expected between isolates of the same species and even between clones of the same isolate, as demonstrated by Rocha et al., [22] who reported that replicates of the same azole-resistant isolate showed variable fluorescence values that were compatible with enhanced efflux activity. Even though no significant correlations between efflux activity and gene expression were found in this study, Bhattacharya et al. [27] demonstrated a good correlation between rhodamine 6G efflux assay and gene expression, by RT-qPCR, among *C. albicans* from vaginal candidiasis.

Sterol content and profile of resistant strains were also studied. The ergosterol content showed no significant differences between resistant and susceptible strains, even after preexposure to MIC/4 of fluconazole, as an attempt to activate resistance mechanisms. Preexposure to fluconazole (MIC/4) did not trigger alterations in cellular sterols, possibly, due to overproduction of ergosterol by gene overexpression to antagonise the action of antifungal drugs, stabilising the final content of ergosterol and consequent fungal cell viability. This hypothesis seems reasonable, but other studies are necessary to better understand the dynamics of ergosterol biosynthesis and adaptation in *C. albicans* isolates, as suggested by Lv et al. [28].

In human strains, the emergence of resistance is attributed to the misuse or chronic use of azoles in specific patient groups[29] and these strains have frequently shown stable phenotypes of azole resistance. Considering the resistance phenotype in the analysed strains was stable, even after storage, and the animals had no history of prior antifungal exposure, it is suggested that other processes triggered resistance in these strains. Thus, we hypothesise that strains from veterinary sources may acquire azole resistance after exposure to environmental stressors.[19] Even though non-human *Candida* strains are not commonly submitted to selective pressures by azoles of clinical use, this pressure still occurs through the environmental exposure to this class of antifungals, especially in agricultural practices, and cross-resistance between these drugs and those azoles used in medical and veterinary practice has been detected.[22,30,31] Chowdhary et al., [32] for instance reported the occurrence of azole-resistant *Aspergillus fumigatus* infections in treatment-naive patients. These isolates were possibly exposed to azoles of agricultural use, in the environment, leading to the emergence of antifungal resistance. Although it is a filamentous fungus, this *Aspergillus* species is also of clinical importance and can cause pathological conditions in immunocompetent and immunocompromised individuals. Moreover, the mutations found in these *A. fumigatus* strains may be similar to those found in azole-resistant in *Candida* strains.

The co-regulation of gene expression has also been reported in human strains, especially between *CDR1* and *CDR2*,[7] which may be

similar in animal strains, leading to the development of azole resistance. In these strains, probably both *CDR* genes are regulated by a common mechanism. The overexpression of the *CDR* genes and the subsequent increase in efflux pump activity are widely associated with azole resistance in *C. albicans* strains from humans.[24,33,34] Regarding the correlation between the expression of *CDR* genes and *MDR1* or *ERG11* genes, it has been described that some *Candida* strains, under selective pressure, can generate a resistant phenotype through different unrelated genetic mechanisms, resulting in cross-resistance to different azole antifungals.[35]

In human strains, CDR1 protein contributes more than CDR2 protein for fluconazole resistance,[10,36] when compared with itraconazole resistance.[10] In the present investigation, no alterations in gene expression were observed in eight strains (3, 4, 8, 9, 12, 13, 18, 23), hence, it is likely that other molecular mechanisms are involved in the development of azole resistance by these isolates, such as *ERG11* polymorphisms/mutations, as previously reported for some human strains,[29,37–39] even for other *Candida* species, such as *C. parapsilosis*.[40] Thus, further investigations are required to elucidate the occurrence of *ERG11* polymorphisms/mutations in azole resistance among animal strains of *C. albicans*.

We observed in this work that some strains are sensitive to fluconazole and resistant to itraconazole or otherwise. This unusual pattern of antifungal susceptibility supports the involvement of other molecular mechanisms. As shown by other authors, the development of azole resistance in *Candida* spp. classically involves different mechanisms, such as the activation of transcription factors, up-regulation or mutation of genes associated with ergosterol biosynthesis (ERG3 and ERG11), and up-regulation of genes encoding efflux pumps (CDR1, CDR2 and MDR1).[10,41–43] To fully elucidate the mechanisms underlying azole resistance in animal strains of *C. albicans*, further investigations are required to assess the role of ERG11 polymorphisms/mutations and transcription factors (UPC2, TAC1 and MRR1) in this phenomenon.

On the basis of this study, we conclude that resistance to azoles in *C. albicans* strains from animals mainly involves the enhanced activity of efflux pumps and *CDR1* and *CDR2* gene overexpression. Furthermore, the other classically studied genes *MDR1* and *ERG11* were also overexpressed in the tested strains, suggesting that azole resistance in *C. albicans* strains from healthy and diseased animal hosts with no history of antifungal treatment is likely a result of multifactorial mechanisms, including increased efflux pump activity and the overexpression of different genes.

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CONFLICT OF INTEREST

No conflicts of interest to declare.

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