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Analysis of the culturable gut yeast microbiota of dogs with digestive disorders

Sergio Álvarez-Pérez [a,b,*], Fernando Rodríguez-Franco [b,c], Mercedes García-Sancho [b,c], Daniela Tercero-Guerrero [a], Ángel Sainz [b,c], Marta E. García [a,b], José L. Blanco [a,b]

[a] Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro s/n, Madrid 28040, Spain
[b] Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro s/n, Madrid 28040, Spain
[c] Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro s/n, Madrid 28040, Spain

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ABSTRACT

Despite the increasing interest in studying the gut mycobiota of dogs, the association between fungal colonization and the development of digestive disorders in this species remains largely understudied. On the other hand, the high prevalence of antifungal-resistant yeasts detected in previous studies in samples from animals represents a major threat to public health. We analyzed the presence of culturable yeasts in 112 rectal swab samples obtained from dogs with digestive disorders attended in a veterinary teaching hospital. Our results revealed that *Malassezia pachydermatis* was frequently isolated from the studied dog population (33.9% of samples), and that the isolation of this yeast was significantly associated to the age of animals, but not to their sex, disease group, or the presence of vomits and/or diarrhea. In contrast, other yeast species were less prevalent (17.9% of samples in total), and their isolation was not significantly associated to any variable included in the analysis. Additionally, we observed that 97.5% of the studied *M. pachydermatis* isolates ($n = 158$, 1–6 per positive episode) displayed a minimum inhibitory concentration (MIC) value >4 μg/ml to nystatin, 31.6% had a MIC ≥32 μg/ml to fluconazole, and 27.2% had a MIC >4 μg/ml to amphotericin B. The antifungal susceptibility profiles of non-*Malassezia* ($n = 43$, 1–7 per episode) were more variable and included elevated MIC values for some antifungal-species combinations. These results confirm that the intestine of dogs is a reservoir of opportunistic pathogenic yeasts and suggest that the prevalence of *M. pachydermatis* colonization depends more on the age of animals than on any specific digestive disorder.

1. Introduction

There is an increasing interest in studying the fungal component of the gut microbiota of animals (also known as 'mycobiota') and its relationship with diverse health and disease conditions (Harrison et al., 2021; Lv et al., 2023; Meili et al., 2023; Prisnee et al., 2022). In particular, although gut fungi typically follow a saprophytic lifestyle in which they take advantage of the nutrients and other environmental features provided by the host, under certain conditions (e.g., alteration of the systemic and/or local immune barriers of the digestive mucosa, dysbiosis caused by prolonged antibiotic treatments, etc.) some fungi such as the yeast genera *Candida* (Ascomycota) and *Malassezia* (Basidiomycota) can behave as opportunistic pathogens (LeibundGut-Landmann and Dawson Jr., 2021; Proctor et al., 2023). Furthermore, it has been suggested that gut fungi could be involved in diverse inflammatory digestive disorders, neoplasia, and other diseases, and that some alterations in the gut mycobiota could even have effects on other body locations (Kreulen et al., 2023; Lopez et al., 2017; Richard and Sokol, 2019; Smet et al., 2022; Szóstak et al., 2023; Wang et al., 2014; Wang et al., 2023). However, it is still unknown whether fungal dysbiosis is a causal factor or a consequence of the development of these diseases. Additionally, most evidence on the relationship between the gut mycobiome and disease comes from human medicine, whereas the link between fungal presence/absence and digestive tract disease in animals remains vastly understudied (but see Kathrani et al. (2023) and Suchodolski et al. (2008) for some studies in dogs).

On the other hand, antifungal resistance has become a major threat to public health (Fisher et al., 2022). As many fungal species are

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* Corresponding author at: Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro s/n, Madrid 28040, Spain.
E-mail address: sergioaperez@ucm.es (S. Álvarez-Pérez).

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regarded as 'One Health' pathogens that are widely present in the environment and can affect both humans and animals (Woods et al., 2023), the results of previous studies reporting a high prevalence of antifungal-resistant yeasts in samples from dogs and other animal species are worrisome (Álvarez-Pérez et al., 2021; Castelo-Branco et al., 2020; Lima et al., 2022).

In this study, we analyzed the presence of culturable yeasts and yeast-like fungi in rectal samples obtained from dogs with diverse digestive disorders attended in a veterinary teaching hospital and determined the antifungal susceptibility recovered isolates.

2. Materials and methods

2.1. *Ethical issues*

This study was approved by the Ethics Committee on Animal Experimentation of Complutense Veterinary Teaching Hospital (HCVC, Madrid, Spain; protocol number 24/2021). Animals were always handled by experienced veterinary practitioners in strict accordance with good animal practice and national legislation and all samples were collected and analyzed (see below) with written owner consent. Furthermore, the owners were aware that samples were taken for research purposes only and no treatment decisions were made based on the study results.

2.2. *Samples and isolates*

A total of 109 dogs with diverse digestive disorders (Fig. 1) and attended between 14 Oct 2021 and 29 Nov 2022 at the Digestive Medicine Unit of HCVC were analyzed upon admission for presence of culturable yeasts in fecal samples. Sampled animals were of both sexes (50.5% males, 49.5% females), 38 different breeds (9.2% of dogs were German Shepherd, 7.3% Yorkshire Terrier, 6.4% Labrador Retriever, and other breeds included <5% of dogs each), and age (between 0.6 and 16.2 years; see details in Table S1). Three animals (two females and one male) were sampled twice during the study period, but these were nevertheless regarded as nonrelated episodes because they occurred with a > 2-month difference and in two of the three cases a different diagnosis was reached (Table S1). Therefore, the study comprised a total of 112 clinical episodes and samples.

A rectal swab sample was taken from each sampled animal and immediately streaked on the surface of the following two media: (i) Sabouraud dextrose agar with chloramphenicol (SDA; Merck Millipore, Madrid, Spain), which is a general mycological medium; and (ii) CHROM agar *Malassezia* (CHROMagar, Paris, France), a chromogenic medium that facilitates the detection and differentiation of the most common species of genus *Malassezia*. Inoculated plates were incubated at 35 °C for 7 days. Multiple colonies (between 1 and 6, to account for possible intra-species variability in antifungal susceptibility) of each different colony type identified as yeasts by macroscopic and microscopic criteria (de Hoog et al., 2020) were picked up, subcultured on

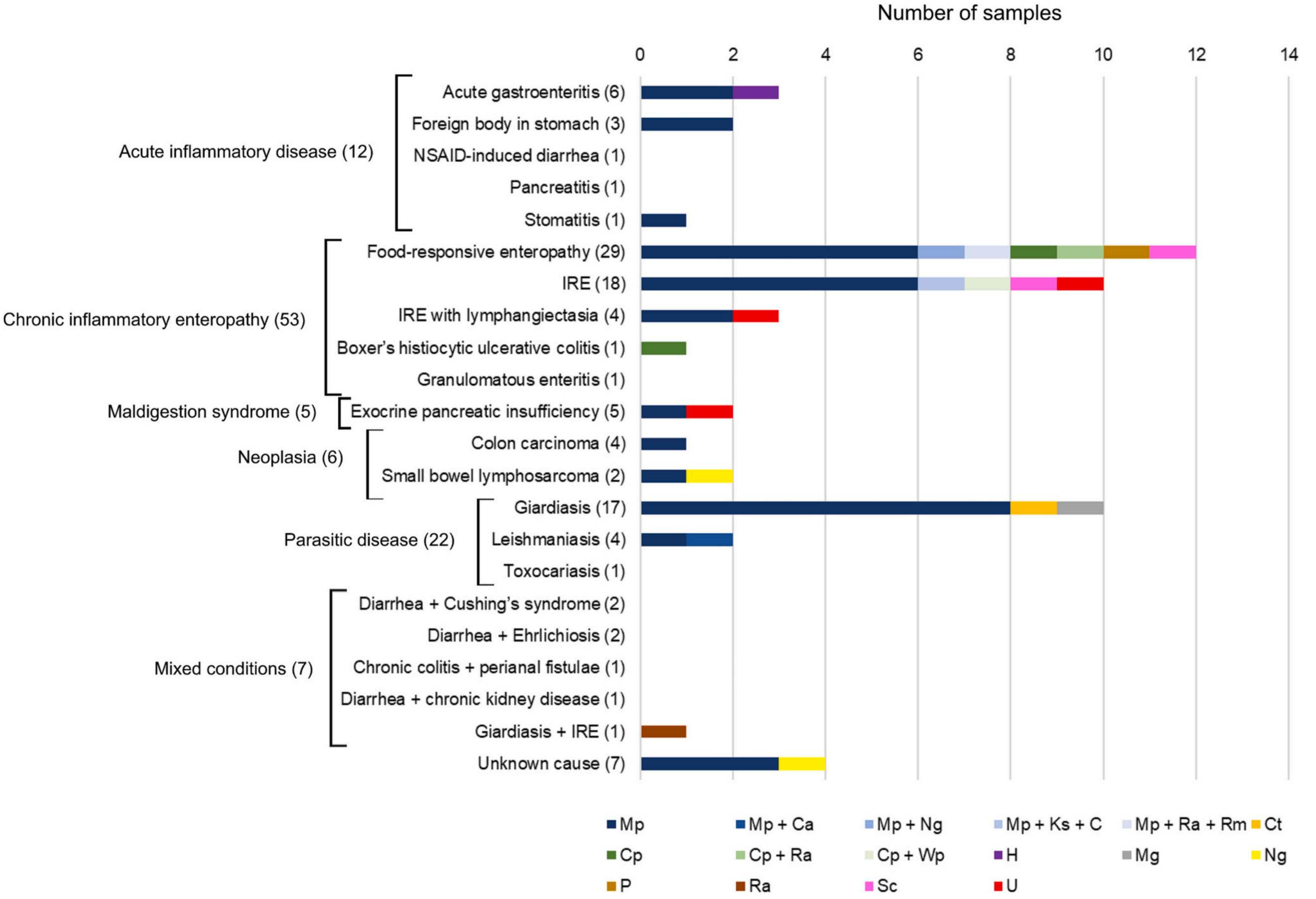

Fig. 1. Overview of the culturable yeasts obtained from rectal samples of the dogs with digestive disorders analyzed in this study (see details in Table S1). Disease groups and diagnoses are presented in rows, and the number of animals belonging to each group is indicated between parentheses; abbreviations of disease conditions: IRE, immunosuppressant-responsive enteropathy; NSAID, nonsteroidal anti-inflammatory drug. Abbreviations of species names: C, *Coniochaeta* sp.; Ca, *Candida albicans*; Cp, *Candida parapsilosis*; Ct, *Coniochaeta taeniospora*; H, *Hyphozyma* sp.; Ks, *Kazachstania slooffiae*; Mg, *Meyerozyma guilliermondii*; Mp, *Malassezia pachydermatis*; Ng, *Nakaseomyces glabratus*; P, *Pseudozyma* sp.; Ra, *Rhodotorula alborubescens*; Rm, *Rhodotorula mucilaginosa*; Sc, *Saccharomyces cerevisiae*; Wp, *Wickerhamiella pararugosa*; U, unknown species.

SDA, and then stored at −80 °C in Sabouraud dextrose broth (SDB; Merck Millipore) containing 25% glycerol (Merck Millipore) until further characterization (see below).

2.3. Species-level identification of isolates

Identification of yeast isolates as *M. pachydermatis* was based on the aspect of their colonies, their microscopic morphology, and their ability to grow on SDA without lipid supplementation at 32 °C (Guillot et al., 1996). Additionally, for a selection of *M. pachydermatis* isolates (>20% of total), the phenotype-based identification was confirmed by sequencing the D1/D2 domains of the large subunit (LSU) rRNA gene using primers NL-1/F63 and NL-4/LR3 (Fell et al., 2000; Kurtzman and Robnett, 1998). Genomic DNA was isolated using the protocol detailed in previous publications (Álvarez-Pérez et al., 2010), and PCR amplification and Sanger sequencing were performed as in Klaps et al. (2020). On the other hand, species-level identification of non-*Malassezia* yeasts was achieved by LSU rRNA sequencing. The nucleotide sequences determined in this study have been deposited in the GenBank database under the accession numbers OR336352–OR336383 and OR338201–OR338244 (for *M. pachydermatis* and non-*Malassezia* isolates, respectively).

2.4. Antifungal susceptibility testing

In vitro antifungal susceptibility of *M. pachydermatis* isolates was determined by the modified Clinical and Laboratory Standards Institute (CLSI) broth microdilution procedure described in previous studies (Álvarez-Pérez et al., 2014; Álvarez-Pérez et al., 2016; Cafarchia et al., 2012a, 2012b, 2012c; Cafarchia et al., 2015), which uses SDB supplemented with 1% *v*/v Tween 80 (SDB-T_{80}) instead of Roswell Park Memorial Institute (RPMI) 1640 as the test medium. The antifungal agents (all purchased from Sigma-Aldrich, Madrid, Spain) and concentrations tested were as follows: amphotericin B, nystatin, itraconazole, ketoconazole, posaconazole, ravuconazole, and voriconazole (0.032 to 16 μg/ml), fluconazole (0.125 to 64 μg/ml), and terbinafine (0.008 to 4 μg/ml). Assay plates were incubated at 32 °C and read macroscopically at 72 h. For polyenes (amphotericin B and nystatin), the endpoint for minimum inhibitory concentration (MIC) was the antifungal concentration that produced a complete (i.e., 100%) inhibition of visual growth, whereas for the other antifungals tested the MIC endpoint was the lowest concentration that produced prominent (≥90%) inhibition of growth (Álvarez-Pérez et al., 2014; Álvarez-Pérez et al., 2016). All isolates were tested at least twice on different days.

Antifungal susceptibility of non-*Malassezia* isolates to nystatin, terbinafine, ketoconazole, and ravuconazole was determined by the reference CLSI broth microdilution procedure (CLSI, 2017). Assay plates were incubated at 35 °C or 32 °C (depending on the optimal growth temperature of the yeast species tested) and read macroscopically after 24–96 h. The MIC endpoint was the lowest antifungal concentration that produced 100% (for nystatin) or ≥ 50% inhibition (for the other antifungal agents) of visual growth. *Pichia kudriavzevii* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains and all isolates were tested at least twice on different days. Additionally, susceptibility of non-*Malassezia* isolates to amphotericin B, fluconazole, itraconazole, posaconazole, voriconazole, 5-flucytosine, and the echinocandins anidulafungin, caspofungin, and micafungin was determined by the Sensititre YeastOne colorimetric antifungal panel (Thermo Scientific, Madrid, Spain), following the manufacturer's instructions. The assay plates were covered with adhesive seals and incubated at 35 °C or 32 °C for 24–96 h. The MIC endpoints were defined as the lowest concentration of antifungal drug preventing the development of a red color (i.e., first blue or purple well).

2.5. Statistical analysis

Data analysis was performed using EpiInfo v.7.2.5.0 (Dean et al., 2011). Logistic regression was used to model the effect of disease group, sex, age of animals, and the presence of diarrhea and/or vomits on yeast colonization, whereas the association between categorical variables was evaluated by the Fisher's exact test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Prevalence and diversity of culturable yeasts

Yeasts were recovered from 54 out of the 112 clinical episodes analyzed in this study (i.e., 48.2%). The clinical characteristic of the dogs from which yeasts were isolated are detailed in Table S1. Yeasts were found in samples from animals of all disease groups, but its prevalence ranged from 14.3% in animals with mixed conditions to 54.6% in animals with parasitic disease (Fig. 1). Furthermore, four out of seven animals (i.e., 57.1%) for which the diagnosis was unclear also yielded yeast growth.

Malassezia pachydermatis was found in 38 episodes (33.9% of total and 70.4% of the episodes yielding yeast growth), either alone (34 episodes) or with other yeasts (*Candida albicans*, *Kazachstania slooffiae* + *Coniochaeta* sp., *Nakaseomyces glabratus*, and *Rhodotorula alborubescens* + *Rhodotorula mucilaginosa*, one episode each combination; Fig. 1 and Table S1). Non-*Malassezia* yeasts were isolated from 16 additional episodes and included the following taxa or combinations of taxa: *Candida parapsilosis*, *N. glabratus*, and *Saccharomyces cerevisiae* (2 episodes each), *Coniochaeta taeniospora*, *Hyphozyma* sp., *Meyerozyma guilliermondii*, *Pseudozyma* sp., *R. alborubescens*, *C. parapsilosis* + *R. alborubescens*, and *C. parapsilosis* + *Wickerhamiella pararugosa* (one episode each), and unknown yeast species that could not be identified because the isolates failed to grow on subculture (three episodes in total; Fig. 1 and Table S1).

Logistic regression analysis identified age as the only factor significantly associated with *M. pachydermatis* colonization (odds ratio: 0.894, 95% CI: 0.803–0.995; $P = 0.04$; Fig. S1), whereas the sex, disease group, and occurrence of vomits and/or diarrhea did not have any significant effect on the response variable (Table S2). In particular, *M. pachydermatis* isolation from fecal samples was significantly more frequent in dogs with <6 years than in dogs of an older age (21/47 vs. 17/65; $P = 0.046$). In contrast, isolation of all yeast species (i.e., *M. pachydermatis* and non-*Malassezia*) was not associated to any analyzed factor (Table S2).

3.2. Antifungal susceptibility

All *M. pachydermatis* isolates analyzed in this study (158 in total, 1–6 per positive episode) displayed low MICs to terbinafine (0.063 μg/ml to 1 μg/ml), itraconazole (≤0.032 μg/ml to 0.25 μg/ml), ketoconazole (0.125 μg/ml to 2 μg/ml), posaconazole (0.125 μg/ml to 1 μg/ml), ravuconazole (0.125 μg/ml to 2 μg/ml), and voriconazole (0.063 μg/ml to 4 μg/ml) (Fig. 2 and Table S3). In contrast, all isolates of this species showed reduced susceptibility to fluconazole (8 μg/ml to 64 μg/ml), and some of them also had an elevated MIC to amphotericin B (range: 0.25–8 μg/ml, with 27.2% of isolates showing a MIC >4 μg/ml) and/or nystatin (range: 2–8 μg/ml, with 97.5% of isolates showing a MIC >4 μg/ml) (Fig. 2). The antifungal susceptibility profiles of the other yeast species isolated from rectal swabs (43 isolates, 1–7 per episode) were more variable between (and sometimes within) species (Fig. 2 and Table S4). In general, MICs to amphotericin B were low for most isolates (≤2 μg/ml), whereas the MICs obtained for other antifungals covered a broader range and included elevated values for some species (Fig. 2 and Table S4). For example, all *Rhodotorula* isolates displayed elevated MICs to terbinafine (>4 μg/ml), fluconazole (≥128 μg/ml), and

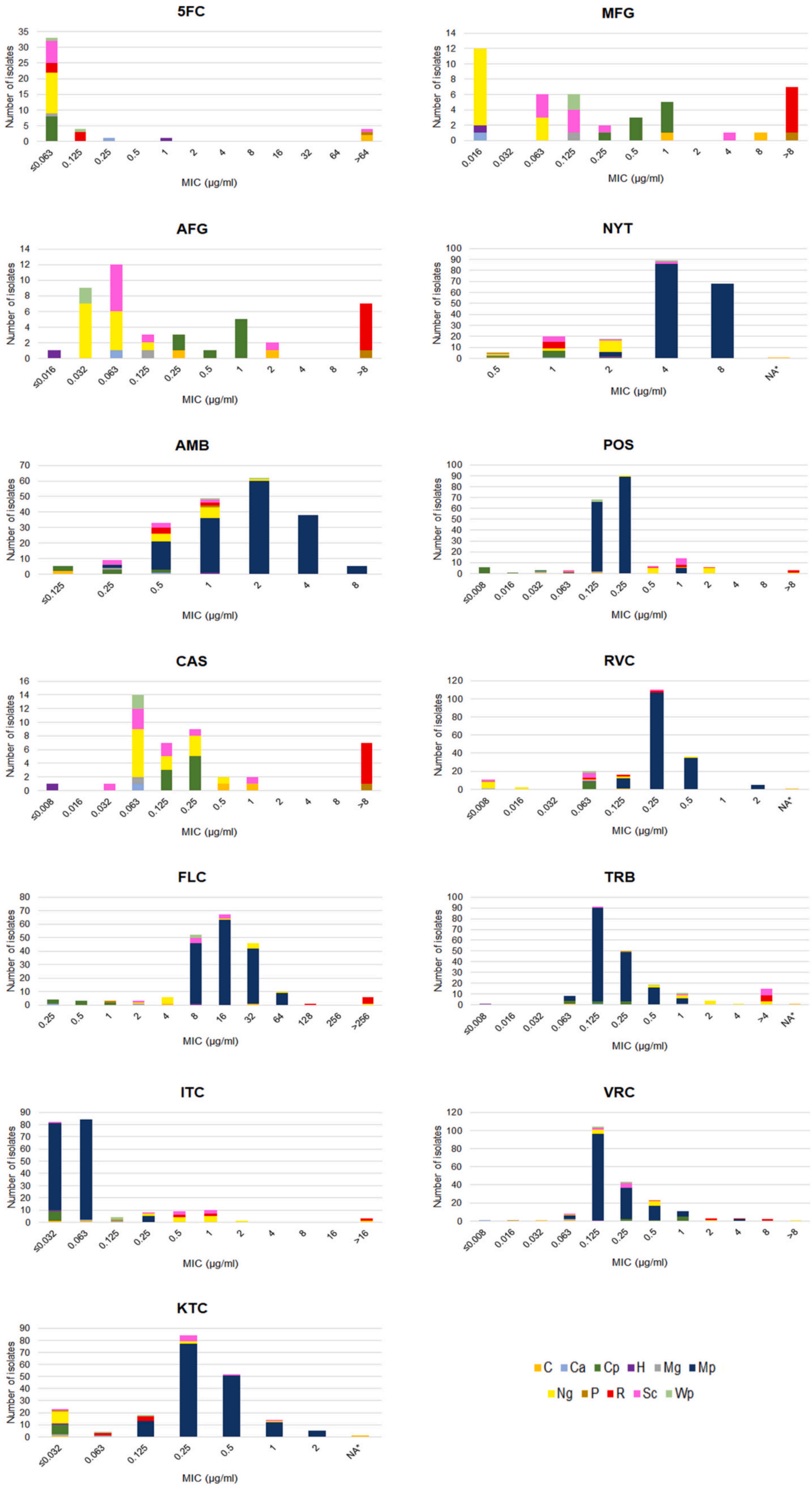

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Fig. 2. Overview of the antifungal susceptibility results obtained for the yeast isolates analyzed in this study. Minimum inhibitory concentration (MIC) values are expressed in μg/ml. Abbreviations of antifungal names: 5FC, flucytosine; AFG, anidulafungin; AMB, amphotericin B; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole; KTC, ketoconazole; MFG, micafungin; NYT, nystatin; POS, posaconazole; RVC, ravuconazole; TRB, terbinafine; and VRC, voriconazole. Abbreviations of species names (number of isolates): C, *Coniochaeta* spp. ($n = 2$, including *C. taeniospora* and *Coniochaeta* sp., 1 isolate each); Ca, *Candida albicans* ($n = 1$); Cp, *Candida parapsilosis* ($n = 8$); H, *Hyphozyma* sp. ($n = 1$); Mg, *Meyerozyma guilliermondii* ($n = 1$); Mp, *Malassezia pachydermatis* ($n = 158$); Ng, *Nakaseomyces glabratus* ($n = 13$); P, *Pseudozyma* sp. ($n = 1$); R, *Rhodotorula* spp. ($n = 6$, including *R. alborubescens* (5 isolates) and *R. mucilaginosa* (1 isolate)); Sc, *Saccharomyces cerevisiae* ($n = 8$); Wp, *Wickerhamiella pararugosa* ($n = 2$). Susceptibility of *Malassezia pachydermatis* isolates to 5FC, AFG, CAS, and MFG was not determined because of their intrinsic resistance to these antifungals. NA, MIC not available because the isolate(s) displayed very limited growth in the susceptibility assay. The detailed antifungal susceptibility results obtained for all *Malassezia pachydermatis* isolates and all non-*Malassezia* isolates characterized in this study are shown in Tables S3 and S4, respectively. *Kazachstania slooffiae* and other yeast isolates that failed to grow on subculture are not included in this figure.

anidulafungin, micafungin, and caspofungin (>8 μg/ml in all cases), and some of them also showed decreased susceptibility to itraconazole, posaconazole, and/or voriconazole (Fig. 2 and Table S4). Similarly, an isolate identified as member of genus *Pseudozyma* had elevated MIC values to flucytosine (>64 μg/ml) and all echinocandins (>8 μg/ml). Finally, a *N. glabratus* isolate was found to be resistant to fluconazole (>64 μg/ml) and caspofungin (0.5 μg/ml) according to the latest CLSI clinical breakpoint values (CLSI, 2022), and this isolate also showed elevated MICs to itraconazole (>16 μg/ml), posaconazole (>8 μg/ml), and voriconazole (>8 μg/ml).

4. Discussion

Despite recent advances in the study of the gut microbiome of dogs with diverse enteropathies, most published studies on this topic focus on bacteria (see, e.g., Doulidis et al. (2023), Galler et al. (2022), Nagahara et al. (2023), and Pilla and Suchodolski (2020)), whereas the association between fungal colonization of the digestive tract and the development of those conditions remains clearly understudied (but see some recent studies on this topic cited below).

In this study, we found that *M. pachydermatis* and other yeast species, including some that are often involved in different human and animal mycoses (e.g., *C. albicans*, *C. parapsilosis*, *M. guilliermondii*, *N. glabratus*, and *Rhodotorula* spp.), were isolated from dogs with diverse digestive disorders. The prevalence of *M. pachydermatis* colonization was particularly high (≥50%) in animals with parasitic and acute inflammatory diseases. These findings are in accordance with the results obtained by Kathrani et al. (2023), who recently documented the presence of yeasts from genera *Malassezia*, *Kazachstania*, and *Candida* in duodenal juice aspirates obtained from dogs with suspected enteropathy and identified *M. pachydermatis* as the most prevalent species. Moreover, in the present study, *M. pachydermatis* isolation was significantly associated to the age of animals, showing a higher prevalence in dogs of <6 years old, but not to the disease group or any other variable. In contrast, other culturable yeast species were less prevalent and were not significantly associated to any variable included in the analysis. Unfortunately, a more detailed examination of the relationship between clinical and microbiological variables was not possible, as that would require using data obtained from specifically designed case-control studies (which was not possible in our case, as healthy animals are normally not brought to the Digestive Medicine Unit of HCVC) and considering other variables that might alter the gut microbiome of dogs, such as the diet (Pilla and Suchodolski, 2021), pharmacological treatments (Bottero et al., 2022; Stavroulaki et al., 2023), and occurrence of other diseases (e.g., metabolic disorders such as diabetes mellitus (Jaffey et al., 2022)).

On the other hand, although antifungal susceptibility testing is still less commonly performed in the veterinary setting than in human clinical institutions, the results of some reports indicate that antifungal resistance is relatively common among yeast isolates from dogs and other animals (Brilhante et al., 2015; Brito et al., 2009; Castelo-Branco et al., 2020; de Cordeiro et al., 2015; Shirakata et al., 2022; Sidrim et al., 2016). Accordingly, in this study we found that most *M. pachydermatis* isolates displayed elevated MICs nystatin, and *c.*30% of the isolates of this species also had decreased susceptibility to fluconazole and/or amphotericin B. *Malassezia pachydermatis* isolates with high MIC to fluconazole have been found in diverse studies that analyzed isolates from ear and/or dermatological samples (Álvarez-Pérez et al., 2016; Cafarchia et al., 2012a, 2012c, 2015), whereas decreased susceptibility to the polyenes amphotericin B and nystatin has been less commonly reported (Bernardo et al., 1998; Brilhante et al., 2018). Furthermore, the *Rhodotorula* and *Pseudozyma* isolates found in the present study displayed elevated MICs to most of the antifungals tested, and a *N. glabratus* isolate was found to be resistant to both fluconazole and caspofungin. Given the role of animals as potential sources of fungal infection for humans (Blanco and García, 2010), continuous monitoring of antifungal susceptibility of clinical isolates is highly recommended.

The main limitation of the present study is that we focused on culturable yeasts, even when unculturable fungi likely comprise a significant part of the mycobiome of animals, as they do in humans (Belvoncikova et al., 2022; Cui et al., 2013). Additionally, we analyzed yeast presence in rectal swabs, rather than in various sites within the intestine, as this approach was less invasive and more practical (but it is prone to contamination by fungi inhabiting the perineal skin and/or the anal mucosa, such as *M. pachydermatis* (Bond et al., 1995; Bond and Lloyd, 1997)). Therefore, to better characterize the gut mycobiome of dogs, future studies should consider unculturable fungi and other locations within the intestinal tract (Kathrani et al., 2023; Suchodolski et al., 2008).

5. Conclusions

In conclusion, the results of this study confirm that the intestine of dogs is a reservoir of *M. pachydermatis* and other yeasts, some of which display elevated MICs to different antifungals. Furthermore, it seems that the prevalence of *M. pachydermatis* colonization depends more on the age of animals than on any specific digestive disorder, but this aspect deserves further epidemiological investigation using specifically designed case-control studies.

Declaration of competing interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105153.

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