

Wild boar (*Sus scrofa*) as reservoir of zoonotic yeasts: bioindicator of environmental quality

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Research Article

Keywords: Wild boar, *Candida* spp., antifungal susceptibility, South Italy

Posted Date: October 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-982703/v1>

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Version of Record: A version of this preprint was published at Mycopathologia on January 24th, 2022. See the published version at <https://doi.org/10.1007/s11046-021-00613-6>.

Abstract

Wildlife animals are recognized as reservoirs for zoonotic fungi and their faeces might play an important role in introducing pathogens into the environment. Thought wild boar (*Sus scrofa*) population has dramatically increased across Europe, information about their possible role in dissemination of zoonotic pathogenic yeasts in the environment is scant. Therefore, fecal samples (n=124) from wild boars from Campania region (Southern Italy) were collected and yeasts identified biochemically and molecularly by sequencing of the internal transcribed spacer region (ITS) and their phylogenetical relationship assessed. The antifungal susceptibility profiles of yeasts were also investigated using AFST-EUCAST method. Yeasts were isolated from 50.1% of the samples with the highest occurrence in samples from the province of Salerno (61.1%). A total of 368 *Candida* strains belonging to nine species were identified, with *Candida albicans* (45.7%), followed by *Candida krusei* (15.2%), *Candida slooffiae* (9.8%) and *Candida parapsilosis* (7.6%) as the most prevalent identified species. Among *C. albicans* four sequence types (i.e., ST1-ST4) were identified with an intraspecific nucleotide difference up to 0.21%. The ML tree grouped all representative sequence types as paraphyletic clades with those of the references yeast species, respectively and supported by high bootstrap values. Fluconazole was the less active drug whereas, posaconazole, voriconazole, and isavuconazole the most active one. No resistance phenomena were observed for *C. albicans* and high MICs values for 5FC, azoles and echinocandines were registered in non-*albicans Candida* spp. This study showed, for the first time, the important role of wild boars in dissemination of pathogenic fungi in the environment. The absence of resistance phenomena in the *Candida* spp. might reflect environmental free from residues of azoles antifungals pollution or chemicals and suggests the role of wild boar as bio indicators of environment quality.

Introduction

Fungal infections are considered a public health concern of emerging importance, due to the increased number of human and animal infections [1]. In particular, yeasts of both endogenous (e.g., *Candida albicans*, *Candida krusei*, *Candida parapsilosis*), or exogenous origin (e.g., *Candida guilliermondii*, *Candida fermentati*, *Candida lusitanae* and *Pichia fermentans*) may induce cutaneous and systemic diseases in humans and animals [2, 3]. For example, *Candida* spp., *Cryptococcus* spp., *Trichosporon* spp., *Rhodotorula* spp., *Malassezia* spp., *Sporobolomyces* spp. and *Saccharomyces* spp. are frequently responsible for animal and human infections, especially in immune-compromised individuals [1]. In the last years, an increasing number of non-*albicans Candida* species (*Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida auris*) associated with exogenously acquired infections has been recorded in humans and domestic animals and their low antifungal drug susceptibility has been considered, in some cases, as the major cause of outbreaks [4]. In particular, the role of wild animals in the dissemination of zoonotic pathogens, including fungi, as well as their utility as bio indicators of environmental quality has spurred the interest of the scientific community [5, 6, 7]. Among wild animals, birds have been considered the main global source of pathogenic fungi having an important role in the spreading these organisms through their faeces [5, 8]. For example, *C. albicans* has been isolated from gut, cloaca and bird droppings of domestic (i.e., laying hens, broiler chickens, pigeons) and wild birds (rhea, psittacinae, raptors, cockatiels) [8]. Besides *Candida*, other yeast genera such as *Cryptococcus*, *Geotrichum*, *Rhodotorula* and *Trichosporon* were also isolated from environments, avian sources, and eggs [5, 9], with a high number of yeasts species showing azole resistance phenomena, mainly from wild and domestic birds [9, 10]. However, it has been shown that the occurrence of different fungal species and their antifungal susceptibility profiles varied accordingly with animal species and geographical area [8, 11]. Among wild animals, wild boars (*Sus scrofa*) are considered one of the most extensively distributed mammals of the world, colonizing and occupying a variety of environments, from natural habitats to urban areas, exhibiting high tolerance to human disturbance, while also exploring anthropogenic food available in the environments [12]. The wild boar has dramatically increased in number and distribution, becoming one of the most numerous and hunted ungulate species in Europe [13]. In addition, they are considered model species to unveil the emergence, spread and persistence of antibacterial resistance in the wildlife-livestock interface [14]. Accordingly, wild boar was identified as hosts for filamentous fungi (i.e., *Aspergillus fumigatus* and *Penicillium verrucosum*) but their role in spreading pathogenic yeasts has not been well investigated [15]. Therefore, the aims of this study were to i) evaluate the presence of yeasts in the faeces of wild boars and ii) determine the antifungal profile of the isolated strains.

Materials And Methods

Study area and sampling

From October and December 2019, wild boar carcasses (n=124) were collected from Salerno (40°40'50"N 14°45'35"E, altitude:4 m), Benevento (41°08'N 14°47'E, altitude:135 m), Avellino (40°54'55"N 14°47'23"E, altitude: 348 m), and Caserta (41°04'N 14°20'E, altitude: 68 m) provinces in Campania region, southern Italy (41.488772° N, 15.558892° E), within a multi-regional health surveillance plan. For each animal, a specific form was filled including animal's age, gender and location of carcass retrieval. All carcasses were analysed within the field activities of the project 'Piano Emergenza Cinghiali in Campania - PECC 2016-2019' (protocol number: Decreto Dirigenziale no. 210-Piano B7 DPAR 2018).

Necropsy examination

Wild boar carcasses were delivered to the Department of Veterinary Medicine and Animal Productions of the University of Naples (Italy), within 24 hours post-mortem. In order to perform a complete necropsy examination, all organs and viscera were removed from abdominal and thoracic cavities and carefully inspected to detect any sign/lesion of traceable pathology.

Faecal samples were collected in sterile conditions from wild boar guts, stored at 4°C in labelled 50ml plastic tubes and delivered to the Mycology Unit of Department of Veterinary Medicine, University of Bari (Italy).

Mycological culture and identification procedures

One gram from each faecal sample was suspended in 9 ml sterile saline solution (NaCl 0.9%) containing 1000 mg/ml streptomycin and 500 UI penicillin/ml. Then, samples were serially diluted in sterile saline solution until reaching a 10^{-4} dilution and one hundred microliters of each dilution were cultured onto Sabouraud dextrose agar with chloramphenicol (0.5 gr/l) (SAB, BioLife ®), incubated at 32°C for 7 days and daily observed. Cultures were defined “positive” when fungal colonies were confirmed microscopically by Gram staining. Colonies were counted, and the yeast population size was expressed as colony forming units (CFU)/gr. Four colonies, for each positive sample were sub-cultured in SAB agar slants for yeast identification at species level. The strains were isolated and identified based on colonial morphology, microscopic and biochemical features as previously reported [11], and by matrix-assisted laser desorption/ ionisation time of flight mass spectrometry (MALDI-TOF MS).

Molecular identification

All yeast strains were molecularly confirmed by amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region. In particular, genomic DNA was isolated from each sample using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following manufacturer's instructions. The nuclear ribosomal ITS region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (REF). The PCR reaction consisted of 4 µl genomic DNA 100 ng and 46 µl of PCR mix containing 2.5 mM MgCl₂, 10 mM Tris-HCl (pH8.3) and 50 mM KCl, 250 µM of each dNTP, 100 pmol of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), and run PCR protocol previously described [9]. PCR products were examined on a 2% agarose gel stained with GelRed (VWR International PBI, Milano, Italy) and visualised on a Gel Logic 100 gel documentation system (Kodak, New York, USA). The PCR products were purified and sequenced in both directions using the same primers, employing the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic analyzer (Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377). Nucleotide sequences were edited, aligned and analyzed using Bioedit sequence Alignment Editor 7.0.5.3 [16], and compared with available sequences in the GenBank data base by Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The percentage of inter and intra-specific ITS nucleotide variation of yeast was calculated by pairwise comparison (Kimura 2-Parameter model) [17] using MEGA5 software [18].

Phylogenetic analysis

To assess the phylogenetic relationship for each yeast species herein identified, all ITS sequences were analysed along with the reference sequences of *Candida* spp. isolated from different animal species, from environment and from different geographical areas available in the GenBank database. Phylogenetic tree was inferred using the Maximum Likelihood (ML) method based on the Tamura 3-parameter [19], with Gamma distribution (+G) of evolutionary rate differences among sites and on Hasegawa-Kishino-Yano models, respectively, selected by best-fit model [20]. Evolutionary analysis was conducted on 8000 bootstrap replications using the MEGA6 software [21].

Antifungal susceptibility testing

The antifungal susceptibility profile of *Candida* spp. strains was evaluated using the reference microdilution method (AFST-EUCAST, definitive document 7.3.2) [22]. The antifungal agents used were itraconazole (ITZ), posaconazole (POS), amphotericin B (AmB), fluconazole (FLZ), isavuconazole (ISA), caspofungin (CP), micafungin (MCF), 5-flucytosine (5FC), anidulafungin (ANI) and voriconazole (VOR) (Sigma-Aldrich, Madrid, Spain). All plates were incubated for 24 hr at 35°C. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as control strains.

Data obtained were reported as MIC ranges, MIC mean value (MIC_m), and MIC at which 50% (MIC₅₀) and 90% (MIC₉₀) of the strains were inhibited.

Isolates were classified as susceptible or resistant according to former and updated EUCAST breakpoints and current EUCAST ECOFFs [23]. The following MIC values for ISA (MIC > 0.5 µg/mL), for CP (MIC > 1 µg/mL) and for 5FC (MIC > 0.5 µg/mL) were considered to indicate probable resistance [24–26].

Candida spp. with no available breakpoints and EUCAST ECOFFs were classified as strains with high MICs values (low susceptible) when their MIC values were equal or higher than those registered for resistant *C. albicans* strains.

Statistical analysis

The distribution of positive/negative animals in the study areas was obtained with ArcGIS (version 10.3, ESRI, Redlands, CA, USA) and associated with the administrative boundaries of provinces, regional and national parks.

A Chi-squared test was used to assess statistical differences among wild boar's age, gender and location. A P value of <0.05 was considered significant.

Results

Necropsy examination and culture yeast identification

At necropsy, all animals showed the absence of clinical signs of localized or systemic infections. Yeasts were isolated from 50.1% of the wild boar faecal samples with the highest occurrence in Salerno (61.1%) and the lowest in Avellino (34.5%) provinces (Table 1, Fig. 1). A greater population size of yeasts (1856.9±5073.3) was recorded and the highest values from samples collected in Caserta (3750.8±9292.7) and in Salerno (1380.8±3334.7). A total of eleven yeasts species belonging to *Candida*, *Geotrichum* and *Rodotorula* genera were isolated with the highest species diversity in samples collected from Salerno. No significant differences in yeasts occurrence were found according to gender or sex of tested animals (Table 1) but the highest diversity of yeast species was recorded in adult animals (Table 1).

Table 1

Number and percentage of faeces from wild boar positive for yeasts divided according to sex, age, and origin. Population size expressed as colony forming unit/gr (CFU/gr) and number of isolated yeasts species were also reported. The statistically significant differences were reported with the same superscript letters

Wild boars		Pos/Total (%)	Means CFU \pm sd	Number of Yeast spp isolated
Sex	Male	36/68(52.9)	1799.9 \pm 4310.7	11/11 (100)
	Female	27/56(48.2)	1941.1 \pm 6030.8	9/11 (81.8)
Age	Young	14/24(58.3)	827.9 \pm 1166.8 ^b	6/11 (72.7)
	Adults	49/100(49)	2195.5 \pm 5753.2 ^b	11/11 (100)
Origin	Salerno	33/54 (61.1) ^a	1380.8 \pm 3334.7	10/11 (90.9) ^c
	Benevento	8/21(38.1)	401.9 \pm 505.9	7/11(63.6)
	Avellino	10/29 (34.5) ^a	873.5 \pm 1062	7/11(63.6)
	Caserta	12/20 (60)	3750.8 \pm 9292.7	5/11(45.4) ^c
Total		63/124 (50.1)	1856.9 \pm 5073.3	11/11 (100)

Molecular identification and phylogenetic analysis

Only *Candida* species were employed for identification purposes. A total of 368 *Candida* spp. strains were isolated and molecularly identified as belonging to nine species (Table 2). *Candida albicans* was the most frequently isolated species (45.7%), followed by *Candida krusei* (15.2%), while *C. lusitanae* was the less frequent one (1.1%) (Table 2). Sequences of amplicons representing the strains of *Candida* spp. showed the nucleotide identity of 100% with reference strains from GenBank (Table 2, Fig. 2). Only one sequence type was identified for non-*Candida albicans* spp. (Table 2, Fig. 2), while four sequence types, named from ST1 to ST4 were identified for *C. albicans* with an overall mean intraspecific variation ranging from 0.21–0.64%. The ITS interspecific nucleotide variation among *Candida* species ranged from 0.70–65.6% (Table 3). The phylogenetic analyses confirm the molecular identification in clustering each yeast species within the clade of the same species as a paraphyletic group, being supported by good bootstrap values (i.e., up to 99%) (Fig. 2). In particular, each *Candida* spp. or ST clustered in the clade including *Candida* strains from different geographic areas and causing human and animal infections (Fig. 2). All representative sequence types were deposited in the NCBI Sequence Read Archive under accession numbers MW279244– MW279253.

Table 2

Candida species from wild boars: Frequencies, internal transcribed spacer (ITS) sequence types (ST) and percentage of sequence nucleotide identity with GenBank accession number

<i>Candida</i> spp.	Pos/Tot (%)	Sequence types (%)	Nucleotide identity	GenBank accession number
<i>Candida albicans</i>	168/368 (45.7)	ST1 (7.1)	100%	LT577608
		ST2 (14.3)	100%	LT577608
		ST3 (7.1)	100%	MN398198
		ST4 (71.5)	100%	MT131348
<i>Candida krusei</i>	56/368 (15.2)	ST1 (100)	100%	KX218263
<i>Candida slooffiae</i>	36/368 (9.8)	ST1 (100)	100%	KY103671
<i>Candida parapsilosis</i>	28/368 (7.6)	ST1 (100)	100%	KY075672
<i>Candida fermentati</i>	24/368 (6.5)	ST1 (100)	100%	NR 149348
<i>Candida guilliermondii</i>	20/368 (5.4)	ST1 (100)	100%	KF746422
<i>Candida lambica</i> (<i>Pichia fermentans</i>)	20/368 (5.4)	ST1 (100)	100%	MT64542
<i>Candida metapsilosis</i>	12/368 (3.3)	ST1 (100)	100%	MF940132
<i>Candida lusitanae</i>	4/368(1.1)	ST1 (100)	100%	MT534186

Table 3

Pairwise comparisons (Pwc) of sequence differences (%) in ITS among all *Candida* spp strains cultured from wild boar faeces

<i>Candida</i> spp	<i>C. albicans</i>	<i>C. metapsilosis</i>	<i>C. parapsilosis</i>	<i>C. guilliermondii</i>	<i>C. fermentati</i>	<i>C. slooffiae</i>	<i>C. lusitaniae</i>	<i>C. lambica</i>
<i>C. albicans</i>								
<i>C. metapsilosis</i>	17.55%							
<i>C. parapsilosis</i>	18.68%	1.41%						
<i>C. guilliermondii</i>	21.82%	21.89%	20.19%					
<i>C. fermentati</i>	21.81%	21.84%	20.14%	0.70%				
<i>C. slooffiae</i>	40.86%	49.31%	49.70%	40.98%	40.75%			
<i>C. lusitaniae</i>	60.51%	65.62%	63.04%	60.82%	59.62%	65.15%		
<i>C. lambica</i>	54.76%	55.77%	57.01%	53.84%	53.39%	58.55%	48.97%	
<i>C. krusei</i>	57.56%	61.25%	61.25%	57.85%	57.28%	56.02%	42.26%	18.68%

Antifungal susceptibility testing

The MICs for the quality control strain *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were all within the reference ranges.

FLZ was the less active drug whereas, POS, VOR and ISA were the most active azoles, regardless the species. The antifungal profile varied accordingly to *Candida* species (Table 4).

Table 4

Minimum inhibitory concentration (MIC, µg /mL) data of Itraconazole (ITZ), Posaconazole (POS), Amphotericin (AmB), Voriconazole (VOR), Fluconazole (FLZ), Isavuconazole (ISA), Caspofungin (CP), Micafungin (MCF), Flucytosine (5FC), anidulafungin (ANI), of *Candida* spp. from wild boars.

<i>Candida</i> spp.	MIC data	ITZ	POS	AMB	VOR	FLZ	ISA	CP	MICA	5FC	ANI
<i>Candida albicans</i> (n =168)	RANGE	0.015-0.06	0.03-0.06	0.03-0.5	0.015-0.25	0.12-4	0.015-0.25	0.25-0.5	0.004-0.06	0.12-4	0.007-0.03
	MIC50	0.015	0.03	0.25	0.015	0.12	0.015	0.25	0.008	0.25	0.007
	MIC90	0.03	0.06	0.5	0.12	0.25	0.12	0.5	0.008	0.5	0.03
	MICm (dev.st)	0.03(0.04)	0.04(0.02)	0.25(0.1)	0.04 (0.05)	0.32 (0.83)	0.06 (0.09)	0.38 (0.3)	0.01(0.02)	0.34 (0.59)	0.02(0.03)
<i>Candida krusei</i> (n =56)	RANGE	0.06-0.5	0.06-0.5	0.03-1	0.12-0.5	16-64	0.12-0.25	0.25-1	0.015-0.25	1-4	0.007-0.06
	MIC50	0.25	0.12	0.5	0.25	32	0.12	1	0.125	2	0.007
	MIC90	0.5	0.25	1	0.25	32	0.25	1	0.25	4	0.06
	MICm (dev.st)	0.25(0.16)	0.23 (0.13)	0.64(0.35)	0.24(0.13)	30.9(11.68)	0.14(0.06)	0.86(0.29)	0.17(0.08)	2.6(1.08)	0.04(0.02)
<i>Candida slooffiae</i> (n =36)	RANGE	0.015-0.25	0.015-0.25	0.25-1	0.015-0.25	0.25-32	0.015-0.25	0.25-1	0.008-0.25	0.12-2	0.007-0.06
	MIC50	0.015	0.015	0.25	0.015	0.25	0.015	0.5	0.015	0.5	0.007
	MIC90	0.25	0.25	0.5	0.25	32	0.12	1	0.25	2	0.06
	MICm (dev.st)	0.10 (0.11)	0.10 (0.10)	0.39 (0.27)	0.13 (0.11)	10.88(13.66)	0.08 (0.08)	0.5(0.31)	0.089(0.1)	1.0(0.96)	0.02(0.023)
<i>Candida parapsilosis</i> (n =28)	RANGE	0.06-0.12	0.015-0.06	0.25-1	0.015-0.25	0.12-4	0.015-0.12	0.5-2	0.5-1	0.12-0.25	1-2
	MIC50	0.12	0.06	0.5	0.12	1	0.12	2	0.5	0.12	1
	MIC90	0.12	0.06	1	0.25	4	0.12	2	1	0.25	2
	MICm (dev.st)	0.10(0.09)	0.05(0.02)	0.68 (0.3)	0.13 (0.09)	1.62(1.76)	0.082(0.05)	1.5 (0.65)	0.57 (0.19)	0.16 (0.06)	1.28(0.48)
<i>Candida fermentati</i> (n =24)	RANGE	0.06-0.5	0.06-1	0.06-0.5	0.015-0.12	1-16	0.06-0.25	0.5-1	0.03-0.5	0.12-2	0.03-2
	MIC50	0.12	0.12	0.25	0.06	2	0.12	0.5	0.125	0.12	0.125
	MIC90	0.25	0.25	0.5	0.12	8	0.12	1	0.50	0.12	2
	MICm (dev.st)	0.22(0.2)	0.3(0.35)	0.34(0.19)	0.07(0.04)	5.5(5.7)	0.13(0.06)	0.75(0.3)	0.3(0.22)	0.43(0.77)	0.87(0.95)
<i>Candida lambica</i> (n =20)	RANGE	0.06-0.12	0.03-0.12	0.03-0.06	0.03-0.06	8	0.015-0.03	0.25-0.5	0.015-0.03	0.25	0.007-0.015
	MIC50	0.012	0.12	0.03	0.03	8	0.03	0.5	0.015	0.25	0.007
	MIC90	0.12	0.12	0.06	0.06	8	0.03	0.5	0.03	0.25	0.015
	MICm (dev.st)	0.1 (0.03)	0.08(0.05)	0.04(0.01)	0.05(0.02)	8(0)	0.02(0.01)	0.4(0.14)	0.02(0.01)	0.25(0)	0.01 (0.004)
<i>Candida guilliermondii</i> (n =20)	RANGE	0.12-0.25	0.06-0.25	0.25-1	0.06-0.5	4-32	0.12-0.25	0.5-1	0.008-0.25	0.12-1	0.03-1
	MIC50	0.25	0.12	0.25	0.25	16	0.12	1	0.125	0.12	0.03
	MIC90	0.25	0.12	0.5	0.5	32	0.25	1	0.125	0.25	0.03
	MICm (dev.st)	0.22(0.06)	0.14(0.1)	0.45(0.32)	0.32(0.17)	26.4 (23.3)	0.2(0.07)	0.8(0.3)	0.11(0.1)	0.35(0.37)	0.22(0.44)
<i>Candida metapsilosis</i> (n =12)	RANGE	0.12-0.25	0.06-0.25	0.25	0.015-0.03	0.25-0.5	0.015	0.5-1	0.004-0.25	0.12	0.125-0.5
	MIC50	0.12	0.25	0.25	0.015	0.25	0.015	0.5	0.25	0.12	0.125
	MIC90	0.25	0.25	0.25	0.03	0.5	0.015	1	0.25	0.12	0.5

<i>Candida</i> spp.	MIC data	ITZ	POS	AMB	VOR	FLZ	ISA	CP	MICA	5FC	ANI
	MIC _m (dev.st)	0.2 (0.08)	0.19(0.11)	0.25 (0)	0.02 (0.01)	0.33 (0.087)	0.015(0)	0.7(0.3)	0.17(0.14)	0.12 (0)	0.25(0.21)
<i>Candida lusitaniae</i> (n =4)	RANGE	0.25	0.06	0.03	0.12	4	0.015	0.5	0.015	2	0.004
	MIC50	0.25	0.06	0.03	0.12	4	0.015	0.5	0.015	2	0.004
	MIC90	0.25	0.06	0.03	0.12	4	0.015	0.5	0.015	2	0.004
	MIC _m (dev.st)	0.25 (0)	0.06(0)	0.25 (0)	0.12 (0)	4 (0)	0.015(0)	0.5 (0)	0.015(0)	2 (0)	0.004 (0)

ITZ, MICA and ANI were the most active drugs and CP the less active one (0.38 µg/ml) for *C. albicans*. For *C. krusei*, the highest sensitivity was recorded for ANI (MIC_m = 0.04 µg/ml) and lowest for FLZ (MIC_m 30.09 µg/ml). For *C. parapsilosis*, the lowest MIC_m value was recorded for VOR, POS, ISA and the highest MIC_m were registered for CP and ANI. *C. slooffiae*, *C. fermentati*, *C. lambica*, *C. guilliermondii* and *C. lusitaniae* showed low susceptibility to FLZ (Table 4).

Based on the clinical breakpoints and current EUCAST ECOFFs no resistance phenomena were observed for *C. albicans*. High MICs values (Table 5) were detected for 5FC in *C. krusei* and *C. lusitaniae*, for CP in *C. parapsilosis*, for ITZ, POS and FLZ in *C. slooffiae*, and *C. lambica* (from 33.3 % to 100 %), for ITZ and POS in *C. metapsilosis*. for MICA and AND in *C. slooffiae*, *C. fermentati* and *C. guilliermondii* (from 20 to 100%).

Table 5

Number and percentage of *Candida* spp. from wild boar resistant or low susceptible (MIC values higher that those registered for resistant *C. albicans*) to itraconazole (POS), amphotericin (AMB), voriconazole (VOR), fluconazole (FLZ), isavuconazole (ISA), caspofungin (CP), micafungin (MCF), 5-Flucytosine (5FC) and anidulafungin (ANI)

	ITZ	POS	AMB	VOR	FLZ	ISA	CP	MICA	5FC
<i>C. albicans</i> (n =168)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)	0/168(0%)
<i>C. krusei</i> (n =56)	0/56(0%)	0/56(0%)	0/56(0%)	0/56(0%)	0/56(0%)	0/56(0%)	0/56(0%)	0/56(0%)	56/56(100%)
<i>C. slooffiae</i> (n =36)	12/36 (33.3%)	20/36(55.6%)	0/36(0%)	0/36(0%)	16/36(44%)	0/36 (0%)	0/36 (0%)	16/36(44%)	16/36(44%)
<i>C. parapsilosis</i> (n =28)	0/28(0%)	0/28(0%)	0/28(0%)	0/28(0%)	0/28(0 %)	0/28(0%)	16/28(57.1%)	0/28(0%)	0/28(0%)
<i>C. fermentati</i> (n =24)	0/24(0%)	4/24(16.6%)	0/24(0%)	0/24(0%)	0/24(0%)	0/24(0%)	0/24(0%)	24/24(100%)	4/24(16.6%)
<i>C. guilliermondii</i> (n =20)	0/20(0%)	0/20(0%)	4/20(20%)	8/20(40%)	8/20(40%)	0/20 (0%)	0/20 (0%)	16/20(80%)	4/20(20%)
<i>C. metapsilosis</i> (n =12)	8/12(66.6%)	8/12(66.6%)	0/12(0%)	0/12(0%)	0/12(0%)	0/12 (0%)	0/12(0%)	0/12(0%)	0/12(0%)
<i>C. lambica</i> (n =20)	12/20(60%)	12/20(60 %)	0/20(0%)	0/20(0%)	20/20(100%)	0/20(0%)	0/20(0%)	0/20(0%)	0/20(0%)
<i>C. lusitaniae</i> (n =4)	0/4(0%)	0/4(0%)	0/4(0%)	0/4(0%)	0/4(0%)	0/4(0%)	0/4(0%)	0/4(0%)	4/4(100%)

Discussion

Data indicated that wild boar harbour, in their faeces many pathogenetic yeasts thus, suggesting their potential role in the spreading these organisms in the environment. The high density of yeasts in the faeces (i.e., CFU ≥ 10³) of a large population of wild boar (i.e., 50%) might indicate that the yeasts have also a role in causing infection in these animals. However, the absence of any signs of localized or systemic infections at the necropsy, confirm that these animals play a role in spreading pathogenic microorganisms in the environment. A similar picture was observed in others wild and domestic animals (i.e., rodents, cats), that are often asymptomatic carriers of pathogenic yeasts to humans [27]. In addition, the large range of wild boar movements (i.e., up to 16 Km) [28], as well as the changes of human habitation to suburban areas, the increased deforestation may increase the dissemination of fungal organisms of zoonotic concern [6, 29]. Similar prevalence of yeasts occurrence was previously observed in swine (44.2%), in cattle (46.8%), and in horses (56%) but it is lower than in wild birds (90%), thus confirming that host environment, as well as lifestyle-related factors of a host might play a primary role in shaping the fungal community [30]. In addition, the age of animals could be a factor affecting the diversity in the yeast species being lower in young animals compared to adults in which fungal flora showed the highest variety of yeast species. These finding might be due to the microbial community of adult animal is gradually formed based on exposure factors to different ecological and environmental conditions and immune system response [31].

In this study, *Candida* spp. was the most frequent isolated yeast genus with a mean population size $\geq 10^4$ CFU/gram of faeces. The occurrence of these fungi in the faeces of examined animals could be explained by the fact that most of these yeast species are commensal organisms of intestinal and cloacal tracts of animals, whereas the high population density could be the result of compromised status of hosts or environmental contamination and consumption of contaminated food. On the other hand, these findings also indicate that wild boars harbour many important infectious agents that could be transmissible to domestic pigs and other animal species, including humans [6, 32]. Overall, all the isolated *Candida* species are causative agents of candidiasis or candidemia with clinical manifestations in humans (e.g., fungaemia, endophthalmitis, arthritis and endocarditis) which may ultimately cause high mortality and life-threatening infections in immune-compromised patients [33]. *C. albicans* is the most common agent of life-threatening human candidemia and candidiasis [34] and it has also been recovered from both wild and domestic animals, such as birds and reptiles [35]. *Candida krusei* and *C. parapsilosis* were retrieved in wild birds, cockatiels, dogs, cows and pigs faecal samples [10] and were considered the most common non-*albicans* *Candida* species causing severe infection in immune-compromised human patients [36]. In addition, *C. krusei* strains were characterized by a high mortality rate (40-58%) and poor response to standard antifungal therapies [37].

C. slooffiae was never reported as causative agent of severe infection in animals and humans but it has been frequently found in different parts of the gastrointestinal tract of apparently healthy pigs and therefore considered as a natural inhabitant of the porcine intestinal environment [38]. As far as *C. guilliermondii* and *C. lusitanae*, they are rare human fungal pathogens, causing fungemia not exceeding 5% of nosocomial systemic infections worldwide. However, in certain geographical areas such as Brazil, India and Italy, over 10% of all the candidemia cases were caused by these species [39, 40]. All isolates molecularly identified to species level in this study showed very low intraspecific and high interspecific variability. An increasing number of studies reported that ITS-based identification system was accurate and applicable, even to strains with atypical morphological features [41]. Here, *C. guilliermondii*, *C. fermentati*, as well as *C. parapsilosis* and *C. metapsilosis*, were molecularly differentiated, confirming the utility of ITS sequence polymorphism analysis. Sequence analysis of the data revealed the circulation of four different STs for *C. albicans*, and one ST for others *Candida* spp. (*C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. fermentati*, *C. slooffiae*, *Clusitanae*, *Pfermentans*). These findings confirmed previous results suggesting that the genetic variability observed in ITS region, was more likely to be found in *Candida* species primarily involved as commensal organisms while species predominantly associated with an exogenous origin, such as, *C. guilliermondii*, *C. fermentati*, *C. slooffiae*, *C. lusitanae* and *C. lambica* showed low intraspecific variability [2, 3]. The ML tree clearly indicates that all strains of each *Candida* species from wild boars were phylogenetically close to the same species recovered from immune-compromised patients suffering for a variety of lesions (e.g., oropharyngeal candidiasis, onychomycosis, persistent candidemia, candiduria, atopic dermatitis and vulvovaginal candidiasis). The phylogenetic analysis also showed that strains of *C. albicans*, *C. parapsilosis*, *C. metapsilosis*, and *C. guilliermondii* from wild boar clustered with those from domestic animals (i.e., sheep, cat and dog), from insect (i.e., *Anopheles darling*) and from environmental sources (i.e., tree, herb, egg, orange, water, soil) from Italy, as well as from other geographical locations (i.e., France, Poland, Hungary, Brazil, Argentina, Iran) [42]. However, MLST analysis should be done in the future in order to confirm the molecular epidemiology of the fungal diseases caused by this yeast species. Overall, all these findings might suggest a clonal origin of these strains and their ability to circulate amongst different hosts and environment thus reinforcing the hypothesis that pathogens may be transmitted from wild boar (e.g., emerging zoonoses) to environment and to humans. Nowadays, wild boar populations are widespread, with considerably large home ranges, overlapping their habitat with livestock and humans and serving as an interface between human-influenced settings and natural areas [43].

As far as the antifungal susceptibility profile of these yeasts the finding of high susceptibility for POS, VOR and ISA for all *Candida* spp. confirms the results of previous studies and suggests the usefulness of these drugs for treating tested infections [44, 45]. However, a low susceptibility for azoles and echinocandins were detected among non-*albicans* *Candida* spp. strains. The high MICs for fluconazole in *C. krusei* and *C. guilliermondii* are a constant finding and may reflect an intrinsic resistance or acquisition of resistance followed to drugs exposure [46]. On the contrary, the high FLZ MIC values registered for *C. lambica* and *C. slooffiae* are of interest. In particular, these yeast species are mainly isolated from environment or as commensal organism of porcine gut [47]. Usually, a very low MIC values for FLZ were registered in yeasts collected from their natural habitat whereas high FLZ MIC values were reported in isolates from human infections thus suggesting an acquired resistance phenomena during therapy. Consequently, the isolation of low FLZ susceptible yeast in faeces from wild boar might be due the acquisition of these yeasts from anthropized environments or for the acquisition of azole resistance from environment azole exposition. However due to the small number of studies on antifungal susceptibility of these yeast species no any straightforward conclusions can be herein allowed. Accordingly, the poor susceptibility to echinocandins in *C. guilliermondii* and *C. fermentati* due to naturally occurring polymorphisms in the FKS1 hotspot regions [48], are usually registered in clinical isolates but the therapeutic level of drugs seems to be enough to successfully treat their infections [49].

In addition, the finding of the low level of azole or echinocandin resistance phenomena in the many *Candida* spp. herein registered is in accordance with the fact that azoles and echinocandins resistance is uncommon (< 1%) for *Candida* spp causing invasive fungal infections (IFIs) in patients from Southern Italy hospitals [50, 51]. All these findings might suggest that wild boars have no previous history of exposure to drugs or antifungal residues in the environment [52]. However, since azole fungicides are frequently used to treat fungal infections in agriculture, the acquisition of resistant phenomena is becoming a worldwide treat in the recent [53] and could at some point affect wild animals. Thus, the resistance rate registered from yeasts, isolated from wild boar, associated to the fact that wild boar is unlikely of being treated with antifungal drugs, might also suggest the importance of these animal species as bio-indicator of a good environmental quality.

In conclusion, this study showed for the first time the important role of wild boars in dissemination of pathogenic fungi in the environment, highlighting their potential role in zoonotic transmission of these microorganisms to immune-compromised human/animal hosts. The absence of resistance phenomena in the *C. albicans* and *Candida* non-*albicans* spp. strains from wild boar might reflect environmental free from residues of azoles antifungals pollution or chemical. Due to the ability of wild boar to passively carry yeasts without affecting their susceptibility, these animals should be proposed as bio indicators of environment quality and/or as sentinel animal species for revealing the emergence of azole resistance phenomena. Further studies are needed in order to better understand the life cycle of pathogens and to disclose the evolution of resistance during transmission of yeast from wildlife animal to livestock/environment or human and vice versa.

Declarations

Authors Contributions

Rhimi Wafa and Claudia Cafarchia conceptualised the study and wrote the manuscript. Sgroi Giovanni and Vincenzo Veneziano collected and verified animal information. Rhimi Wafa and Aneke Chioma Inyang performed the research. Giada Annoscia, Maria Stefania Latrofa and Ana Alastruey-Izquierdo have contributed on the identification of organism. Wafa Rhimi and Cafarchia Claudia and Ana Alastruey-Izquierdo analyzed and interpreted data. Cafarchia Claudia, Otranto Domenico and Ana Alastruey-Izquierdo revised, edited, and made intellectual inputs in the manuscripts. All authors read and approved the final manuscript.

Funding

This study has been partially financed by the Fondo de Investigaciones Sanitarias from the Instituto de Salud Carlos III (grant number PI20CIII/00043).

Availability of Data and Materials

Sequences were deposited in GenBank under accession numbers MW279244– MW279253.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

Ethical Statement

This study is a part of the project 'Piano Emergenza Cinghiali in Campania - PECC 2016-2019' (protocol number: Decreto Dirigenziale no. 210-Piano B7 DPAR 2018) that was approved by Institute for Environmental Protection and Research (ISPRA). The approval letter has been included in Supplementary Material.

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Figures

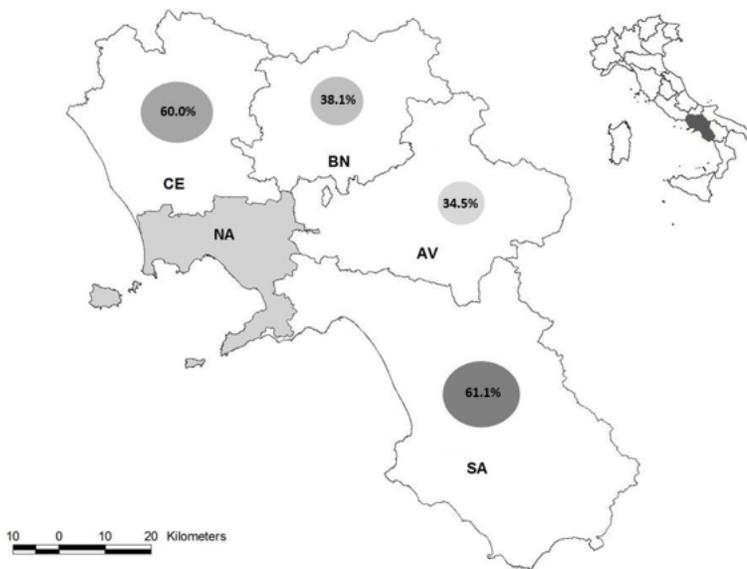


Figure 1
 Map with proportioned circles showing wild boars positive to different kind of yeasts collected from each province examined (Avellino - AV; Benevento - BN; Caserta - CE; Salerno - SA) in Campania region, southern Italy. The province of Napoli (NA) was not investigated



Figure 2

Phylogenetic tree based on internal transcribed spacer (ITS) sequence data with those of other yeasts available in the GenBank database. The tree was constructed using the Maximum Likelihood (ML) method on 8000 replicates and rooted against *Penicillium oxalicum* as outgroup. Bootstrap value >50% are indicated