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Note

Characterization, enzymatic activity and biofilm formation of *Candida* species isolated from goat milk

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ABSTRACT

Background: Data regarding yeast microbiota in goat milk is scarce.
Aims: To isolate and identify species of the genus *Candida* in milk samples from clinically healthy goats, and evaluate their enzymatic activity and biofilm formation.
Methods: 1092 milk samples from clinically healthy goats were collected and processed. The yeast isolates were identified by phenotypic, methods and their enzymatic activity (phospholipase, hemolysin and protease) and biofilm formation evaluated.
Results: We obtained 221 *Candida* isolates belonging to six species: *Candida kefyr* (35.7%), *Candida guilliermondii* (33%), *Candida famata* (23.5%), *Candida glabrata* (5.9%), *Candida albicans* (1.35%) and *Candida parapsilosis sensu lato* (0.45%). Protease activity was detected in all *Candida* species while hemolysin activity was only present in *C. kefyr, C. guilliermondii*, *C. famata* and *C. albicans*. Only *C. albicans* showed phospholipase activity. With the exception of *C. parapsilosis sensu lato*, all *Candida* species formed biofilm, with 60.19% of the isolates being poor producers, 9.93% moderate producers, and 1.35% strong producers.
Conclusions: The milk of clinically healthy goats contains several species of the genus *Candida* that could play a role as opportunistic pathogens in mastitis.

Caracterización, actividad enzimática y formación de biopelículas de especies de *Candida* aisladas de leche de cabra

Palabras clave:
Levadura
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RESUMEN

Antecedentes: El conocimiento de la microbiota levaduriforme presente en la leche de cabra es escaso.
Objetivos: Aislar e identificar especies del género *Candida* en muestras de leche de cabras clínicamente sanas, evaluar su actividad enzimática y su capacidad de formar biopelículas.
Métodos: Se recogieron y procesaron 1092 muestras de leche de cabras clínicamente sanas. Las levaduras aisladas fueron identificadas mediante métodos fenotípicos, evaluándose posteriormente su actividad enzimática (producción de fosfolipasas, hemolisinas y proteasas) y la formación de biopelículas.
Resultados: Se obtuvieron 221 aislamientos de *Candida* de seis especies: *Candida kefyr* (35,7%), *Candida guilliermondii* (33%), *Candida famata* (23,5%), *Candida glabrata* (5,9%), *Candida albicans* (1,35%) y *Candida parapsilosis sensu lato* (0,45%). En todas las especies de *Candida* se detectó actividad proteolítica, y únicamente *C. kefyr*, *C. guilliermondii*, *C. famata* y *C. albicans* presentaron actividad hemolítica. Por su parte, *C. albicans* fue la única especie con actividad fosfolipasa. Con excepción de *C. parapsilosis sensu lato*, todas las especies de *Candida* formaron biopelícula, con el 60,19% de los aislamientos poco formadores de biopelícula, el 9,93% moderadamente formadores y el 1,35% altamente formadores.

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Conclusiones: La leche de cabras clínicamente sanas presenta diversas especies del género *Candida* que podrían actuar como patógenos oportunistas de mastitis.

The mammary glands in ruminants are known to harbor diverse bacteria; little is known, however, about their fungal microbiota. In dairy cattle, studies report the presence of different species of the genus *Candida*.[7,18,21,25,34,37] A variety of enzymatic activities, mainly proteases, hemolysins and phospholipases, allow different species of *Candida* to adhere, colonize and invade host tissues.[8,10] In addition, biofilm production enables *Candida* infection and resistance to antimicrobials.[13,33] The objective of this study was to isolate and identify *Candida* species in milk samples from clinically healthy goats, and to evaluate their enzymatic activity and biofilm formation.

We collected 1092 milk samples during a six month-period from a farm with Alpina goats clinically healthy ($n = 100$) in Querétaro, Mexico. The samples were centrifuged (4000 rpm, 10 min), the pellets resuspended into 2 ml of yeast extract peptone dextrose (YEPD) broth (casein peptone 2%, dextrose 2%, yeast extract 1%), and incubated 48 h at 37 °C. Yeasts were isolated by plating 50 μl from each suspension on Sabouraud dextrose agar (SDA) supplemented with 50 mg/l chloramphenicol. Conventional phenotypic methods were used for yeast identification: Gram staining, germ tube production, pseudohyphae formation, sensitivity to 0.1% cycloheximide, film formation in liquid culture, urease production, carbohydrates assimilation and fermentation, and development in Biggy medium and CHROMagar *Candida*. [2,5,6,14,24,27,28,30,32,40] Each yeast isolate was evaluated for enzymatic activity and biofilm production. Phospholipase and protease activity were performed according to Kantarcioglu and Yücel,[20] and hemolysin activity according to Luo et al.[26] Briefly, 6 mm diameter sterile filter discs were embedded with 10 μl yeast suspension (1×10^6–5×10^6 CFU/ml). These were placed onto SDA agar supplemented with 4% egg yolk, 0.2% bovine serum albumine or 7% sheep blood for phospholipase, protease and hemolysin activity evaluation, respectively. After incubation (37 °C, 7 days), the yeast colony and the corresponding clear halo diameters were measured. The enzymatic activity was determined through the enzyme activity index according to Williamson's formula.[41] *Candida* biofilm formation was evaluated using the Gokce et al. safranine method.[15] For each isolate a 1×10^8 CFU/ml yeast suspension was prepared in yeast nitrogen base medium (YNBG) with 8% glucose. From this stock suspension a 1:100 dilution was made, and 96-well polystyrene flat-bottom microplates were filled (200 μl/well) and incubated 48 h at 37 °C. Afterwards, the plates were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4). The biofilms formed were fixed with methanol for 15 min (200 μl/well); methanol was discarded and the microplates were allowed to dry for 10 min at 37 °C. Thereafter, 1% safranin solution was added (200 μl/well), and after 20 min at room temperature a washing with PBS was done. Immediately after, 95% ethanol was added (200 μl/well, 20 min at room temperature). Finally, the optical density (OD) was determined at 490 nm using a Biotek ELISA reader. Categorization of biofilm production was established through OD intervals as described by Stepanovic et al.[16,39] Descriptive statistics were used to analyze the results.

Candida was the solely yeast genus found in the goat milk samples analyzed. We obtained 221 *Candida* isolates, further identified as *Candida kefyr* 35.74%, *Candida guilliermondii* 33%, *Candida famata* 23.53%, *Candida glabrata* 5.9%, *Candida albicans* 1.35%, and *Candida parapsilosis (sensu lato)*[44] 0.45% (Table 1). Concerning enzymatic activity, 98.65% of the isolates showed protease activity, 55.2% hemolytic activity, and 1.36% phospholipase activity. Protease activity varied among the species and within the isolates of a given species. *C. kefyr*, *C. guilliermondii*, and *C. famata* showed the whole range of protease activity, having more than 87% of them medium and high activity. All *C. glabrata* presented protease activity, *circa* 70% of them showing high activity. *C. albicans* and *C. parapsilosis* isolates exhibited only high protease activity. Hemolysin activity was observed in *C. albicans* (100%), *C. guilliermondii* (71.23%), *C. famata* (67.30%) and *C. kefyr* (40.50%). Phospholipase activity was just detected in *C. albicans* (Table 2). Regarding biofilm formation, four categories were determined: no biofilm formation, low biofilm formation, moderate biofilm formation, and high biofilm formation. Biofilm was produced in various degrees by 71.49% of all *Candida* isolates but *C. parapsilosis (sensu lato)* (Table 3).

Yeast identification was performed by conventional methods, which are the routine diagnostic methods used in our laboratory. Although known to be less sensitive and more time consuming than molecular methodologies, they are considered the gold *Candida* identification standard.[1,43] Predominance of non-*C. albicans Candida* species in milk from clinically healthy goats, and from other ruminants with and without mastitis have been reported.[9,17,19,21,22,25] Our findings support this since, out of the six *Candida* species found, *C. albicans* accounted only for 1.35% of the isolates. Little is known about the association of *Candida* spp. enzymatic production and biofilm formation with the yeasts virulence. Both proteases and hemolysins were detected in *C. albicans*, *C. famata*, *C. kefyr*, *C. guilliermondii* and *C. parapsilosis*, whereas phospholipases were only detected in *C. albicans*. These features are in agreement with previous studies.[3,4,12,15,26,29,36] *In vitro* production of these virulence factors are strain-dependent and may differ according to the anatomical site infected or the yeasts involvement in a pathological process.[4,33] Biofilm formation has been described in *C. albicans, C. tropicalis, C. parapsilosis* and *C. glabrata*, and correlation between yeast virulence and high biofilm production has been demonstrated in human beings.[11,13,15,23,31,33,42] In animals, a research studying milk from buffaloes with mastitis have shown high biofilm production in *Candida zeylanoides*, *Candida rugosa* and *Candida kefyr*.[35] In this study, *C. albicans*, *C. kefyr*, *C. guilliermondii*, and *C. famata* isolates mostly showed a low production of biofilms. This might be related to the samples origin, clinically healthy animals, where formation of high density biofilms might not be crucial to yeasts persistence. Nevertheless, all these microorganisms could be opportunistic pathogens in mastitis.[35,38]

More research on *Candida* presence, both in milk of clinically healthy and diseased goats, and their virulence factors is needed to determine their relationship with the development of fungal mastitis in this animal species. Our study is the first of its kind in Mexico, demonstrating the presence of different species of the genus *Candida* in goat milk from clinically healthy animals; the isolates exhibited various virulence factors under laboratory conditions.

Table 1
Identification of *Candida* isolates from the milk of clinically healthy goats.

Candida species	Pseudohyphae formation	Yeast development			Urease	Germ tube formation	Assimilation and fermentation																Yeast development 37 °C	
		SDA 37 °C	YNB + cycloheximide (0.1%)	Sabouraud broth			glu		sac		lac		mal		xyl		raf		tre		mel		Chromagar *Candida*	BIGGY
							A	F	A	F	A	F	A	F	A	F	A	F	A	F	A	F		
C. kefyr ($n = 79$)	+	V	+	–	–	–	+	+	+	+	V	V	–	–	V	–	+	+	–	–	–	–	Matte light pink	Light brown
C. guillier-mondii ($n = 73$)	V	V	+	–	–	–	+	+	+	+	–	–	+	–	+	–	+	+	+	V	+	–	Bright pink	Reddish brown
C. famata ($n = 52$)	V	V	–	–	–	–	+	V	+	V	V	–	+	V	–	–	+	V	+	V	V	V	Matte pale pink	Reddish brown
C. glabrata ($n = 13$)	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	V	–	V	–	–	–	Matte pale pink	Light brown
C. albicans ($n = 3$)	+	+	+	–	–	+	+	+	V	–	–	–	+	+	+	–	–	–	V	V	–	–	Emerald green	Brown and silver appearance on the surface
C. parapsilosis (sensu lato) ($n = 1$)	+	+	–	–	–	–	+	+	+	–	–	–	+	–	+	–	–	–	+	–	–	–	Matte pale pink	Reddish brown

A: assimilation; F: fermentation; SDA: Sabouraud dextrose agar; YNB: yeast nitrogen base; glu: glucose; sac: saccharose; lac: lactose; mal: maltose; xyl: xylose; raf: raffinose; tre: trehalose; mel: melezitose; V: variable strains.

Table 2
Enzymatic activity of *Candida* isolates from the milk of clinically healthy goats.

Candida species	Number of isolates (*n*)	Scores of enzymatic activities (Pz)											
		Protease				Phospholipase				Hemolysin			
		1	2	3	4	1	2	3	4	1	2	3	4
C. kefyr	79	1	9	20	49	79	–	–	–	47	23	9	–
C. guilliermondii	73	1	5	7	60	73	–	–	–	21	46	6	–
C. famata	52	1	–	9	42	52	–	–	–	17	31	4	–
C. glabrata	13	–	–	4	9	13	–	–	–	13	–	–	–
C. albicans	3	–	–	–	3	–	2	1	–	–	1	2	–
C.parapsilosis	1	–	–	–	1	1	–	–	–	1	–	–	–
Total	221	3	14	40	164	218	2	1	–	99	101	21	–

Enzyme activity: 1 = null (Pz = 1); 2 = low (Pz = 0.61–0.99); 3 = medium (Pz = 0.41–0.60); 4 = high (Pz ≤ 0.40).
$Pz = \frac{\text{Diameter of the colony}}{\text{Diameter of the halo}}$

Table 3
Biofilm formation of *Candida* isolates from the milk of clinically healthy goats.

Candida species	Number of isolates (*n*)	No biofilm		Low biofilm formation		Moderate biofilm formation		High biofilm formation	
		OD ≤ 0.120[a]	%	OD (0.121–0.240)	%	OD (0.241–0.480)	%	OD (≥0.481)	%
C. kefyr	79	21	9.5	50	22.62	7	3.16	1	0.45
C. guilliermondii	73	21	9.5	44	19.9	8	3.61	–	–
C. famata	52	18	8.14	26	11.8	6	2.71	2	0.9
C. glabrata	13	2	0.9	10	4.52	1	0.45	–	–
C. albicans	3	–	–	3	1.35	–	–	–	–
C.parapsilosis	1	1	0.45	–	–	–	–	–	–
Total	221	63	28.49	133	60.19	22	9.93	3	1.35

OD: optical density.
[a] Cut-off point (mean OD value of negative controls + 2 standard deviation).

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Conflict of interests

The authors declare that they have no conflict of interest.

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