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Nabaraj Banjara
University of Nebraska-Lincoln, s-nbanjar1@unl.edu

Kenneth Nickerson
University of Nebraska-Lincoln, knickerson1@unl.edu

Mallory J. Suhr
University of Nebraska-Lincoln, msuhr@huskers.unl.edu

Heather E. Hallen-Adams
University of Nebraska-Lincoln, hhallen-adams2@unl.edu

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Killer toxin from several food-derived *Debaryomyces hansenii* strains effective against pathogenic *Candida* yeasts

Nabaraj Banjara,[1] Kenneth W. Nickerson,[2] Mallory J. Suhr,[1] Heather E. Hallen-Adams[1]

1 Department of Food Science and Technology, University of Nebraska-Lincoln, 1901 North 21st St, Lincoln, NE 68588-6205, USA

2 Department of Biological Sciences, University of Nebraska-Lincoln, 1901 Vine St, Lincoln, NE 68588-0665, USA

Corresponding author — H. E. Hallen-Adams, hhallen-adams2@unl.edu

Abstract

Candida yeasts are the dominant fungi in the healthy human microbiome, but are well-known for causing disease following a variety of perturbations. Evaluation of fungal populations from the healthy human gut revealed a significant negative correlation between the foodborne yeast, *Debaryomyces hansenii*, and *Candida* species. *D. hansenii* is reported to produce killer toxins (mycocins) effective against other yeast species. In order to better understand this phenomenon, a collection of 42 *D. hansenii* isolates was obtained from 22 cheeses and evaluated for killer activity against *Candida albicans* and *Candida tropicalis* over a range of temperatures and pH values. Twenty three strains demonstrated killer activity against both *C. albicans* and *C. tropicalis*, which was pH- and temperature-dependent, with no killer activity observed for any strain at pH 6.5 or higher, or at ≥35 °C (physiological conditions in the human gastrointestinal tract). A cell-free mycocin preparation showed transient killer activity against *C. albicans* at 35 °C and a cheese sample containing a killer *D. hansenii* strain demonstrated sustained killer activity against both *C. albicans* and *C. tropicalis*. Together, these observations raise the possibility that *D. hansenii* could influence *Candida* populations in the gut.

1. Introduction

Debaryomyces hansenii is among the most common yeast species isolated in viable form from foods such as cheeses (Banjara et al., 2015; Breuer and Harms, 2006), due to its ability to grow in the presence of high salt (Breuer and Harms, 2006; Prista et al., 2005), low pH, and low water activity (Capece and Romano, 2009), and its ability to metabolize lactic and citric acids (Ferreira and Viljoen, 2003). In recognition of its food processing potential, *D. hansenii* has QPS status in the European Union, permitting its use in the production of cheeses and fermented sausages, and is considered a useful organism in food fermentations (Bourdichon et al., 2012). *D. hansenii* forms a monophyletic group with human pathogenic *Candida* yeast species (with the exception of *Candida glabrata*, which is more closely related to *Saccharomyces cerevisiae*) and, indeed, the asexual state of *D. hansenii* has been described as *Candida famata* (Nguyen et al., 2009; Suh et al., 2006). Previous studies have reported the presence of *D. hansenii* in the human gastrointestinal tract, likely due to foodborne strains (Borelli et al., 2006; Cosentino et al., 2001; Desnos-Ollivier et al., 2008; Hallen-Adams et al., 2015; Vasdinyei and Deák, 2003).

Candida species are part of the normal microbiota of the human mucosal oral cavity, vagina, and GI tract (Moran et al., 2012; Sardi et al., 2013; Williams et al., 2013). These yeasts are commensal in healthy humans but following disturbances in the host's defense systems, including the physiological intestinal microflora, the gut-associated immune system, and the mucosal barrier, *Candida* species can adopt a pathogenic mode and cause disease (candidemia), ranging from superficial mucosal to life-threatening systemic infections (Liu, 2002; Netea et al., 2008; Walker et al., 2009; Moran et al., 2012).

Some yeasts produce toxic proteins or glycoproteins called killer toxins, or mycocins, which can kill sensitive yeast isolates (Schmitt and Breinig, 2002). The production of mycocins has long been known in the wine industry, where wild killer yeasts can have deleterious effects on fermentation; conversely, killer strains of known fermentation efficacy may be deliberately selected for their ability to prevent the overgrowth of wild yeasts (Bevan and Mitchell, 1979; Santos et al., 2011). Since the first description of the phenomenon in *Saccharomyces cerevisiae* in 1963, mycocin activity has been reported from more than 90 yeast species belonging to more than 20 genera (Buzzini and Martini, 2001). The killer character does not appear uniformly among these species, nor can it be linked to the sources of their isolation (Buzzini and Martini, 2001; Young and Yagiu, 1978). The first years of the 21st century have seen a renewed interest in mycocins, with several papers on mycocin production in fermented foods, and the possibility of using killer strains as biocontrol against spoilage organisms (Hernández et al., 2008; Liu and Tsao, 2009; Santos et al., 2011). Another area of perennial research interest involves the potential of mycocins to kill yeasts pathogenic to humans or animals. Killer activity by some yeasts against *Candida albicans* was first reported by Middelbeek et al. (1980), and in 1984 Morace and colleagues were able to recommend a screen for sensitivity to mycocins as a valuable means of differentiating strains of

pathogenic yeasts (Morace et al., 1984). Since then, several studies have reported in vitro killing of *C. albicans* and other pathogenic yeasts (e.g. (Buzzini and Martini, 2001; Hodgson et al., 1995; Vadkertiova and Slavikova, 2007); however, with few exceptions (Travassos et al., 2004; Walker et al., 2009), in vivo therapeutic applications remain elusive. Because of regulatory considerations, it would be simpler if the relevant mycocin activity resided in a yeast which already had Qualified Presumption of Safety (QPS) status or was otherwise already approved for use in humans.

Mycocins from *D. hansenii* have shown activity in the lab against multiple yeast species, including opportunistic pathogenic *Candida* (Buzzini and Martini, 2001; Santos et al., 2002). Hernández et al. (2008), studying six genera of yeasts isolated from olive fermentations, reported the highest percentage of killer strains in *Debaryomyces*, with 66.7% of strains showing killer activity against various species. In this study, we evaluated killer activity of *D. hansenii* strains isolated from different cheeses against *C. albicans* and *Candida tropicalis* to determine whether *D. hansenii* has potential to reduce *Candida* levels in the human GI tract.

2. Materials and methods

2.1. D. hansenii and Candida species in the healthy human gut microbiome

A previous study in our lab generated fungal profiles for a total of 69 fecal samples, taken from 45 healthy adult volunteers (Hallen-Adams et al., 2015). Data from this study were used to generate the Spearman's rank correlation coefficient for *Candida* species vs. *D. hansenii*.

2.2. Collection of D. hansenii strains from cheese

As described in Banjara et al. (2015), 44 cheeses (differing in variety and/or manufacturer) were collected from local retailers and sampled for yeasts and molds. DNA was extracted from pure cultures following the method described by Harju et al. (2004), and polymerase chain reaction (PCR) was performed using the fungal-specific forward primer ITS1F (Gardes and Bruns, 1993) and the universal eukaryotic reverse primer TW13 (White et al., 1990). PCR products were submitted for Sanger sequencing using ITS1F to the Michigan State University Research Technology Support Facility. Samples were identified by sequence homology using nucleotide BLAST against the UNITE curated fungal ITS sequence database (Koljalg et al., 2013) and the curated FUNCBS database, as accessed through the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all). Selected killer and non-killer isolates have been submitted to Centraalbureau voor Schimmelcultures as accession numbers [pending].

2.3. Mycocin assays

Mycocin activity of strains identified as *D. hansenii* was evaluated against the human clinical isolates *Candida albicans* SC5314 (ATCC MYA-2876) and *C. tropicalis* NRRL 10985.Mycocin activity was evaluated by streak-plate agar diffusion assays (Hodgson et al., 1995; Rosini, 1983). YEPD-methylene blue agar (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar, 0.003% methylene blue and 10 mM sodium citrate) was prepared and adjusted to pH 4.5, 5.5, 6.5 and 7.0 with HCl. At pH values ≤4.0 the media did not solidify following autoclaving whereas at pH 4.5 the agar plates were soft but sufficiently solid for all assays. All media were autoclaved, cooled to 45 °C and seeded with *C. albicans* or *C. tropicalis* at a final cell density of 10^5 cells ml^{-1} whereupon 15 ml was poured into each plate. After 3 h, a colony of *D. hansenii* was streaked on the agar surface and the plates were incubated in the dark at 20 °C, 25 °C, 30 °C or 35 °C for 3–7 days. Forty two strains of *D. hansenii* were tested (see Table 1) at one strain per plate and the experiments were performed in triplicate. Development of a clear inhibition zone around the *D. hansenii* colony was considered as a positive indication of mycocin activity whereas the absence of a clear or blue zone indicated a lack of mycocin activity (Hernández et al., 2008).

D. hansenii strains 274, 237 and 65 with killer activity and strain 3 without activity were selected for further investigation. The selected *D. hansenii* strains were inoculated at 10^6 cells ml^{-1} into 200 ml of liquid YEPD media at pH 4.5 in a 250-ml Erlenmeyer flask and shaken at 150 rpm for 72 h at 25 °C. The cells were harvested by centrifugation (Beckman, USA) at 2750×*g* for 10 min at 4 °C and discarded. The culture supernatants were filtered through 0.45 μm filters whereupon the clarified extracts were lyophilized (Labconco, USA) for 3 days and 5 g of each dried sample was reconstituted in 15 ml of sterile YEPD to yield a crude preparation of *D. hansenii* secreted metabolites (henceforth "crude mycocin"). Two hundred milliliters of uninoculated YEPD was also freeze-dried and 5 g of this dry sample was reconstituted as above and used as a control. Thus, the crude mycocin preparations contained salts and other media components as well as variable levels of protein.

The agar diffusion bioassay was performed according to Hodgson et al. (1995). YEPD-methylene blue agar plates were prepared at varying pH values (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) and seeded with either *C. albicans* or *C. tropicalis* as above. Wells were cut in the solidified agar using a sterile 7 mm borer, 6 wells per plate, crude mycocin (100 μl) was added to the wells, and the plates were incubated at 20, 25, 30, and 35 °C for 2 days. A clear zone of inhibition around a well was indicative of killer activity, such that larger zones of inhibition denoted higher mycocin activity. The killer activity of each sample was defined as the mean of three replicate wells (Hodgson et al., 1995).

The stability of crude mycocin from Dh-237, Dh-274, and Dh-65 was assessed following incubation at 25 °C and 37 °C for 48 h. Aliquots (100 μl) were removed at 24 and 48 h and the remaining killer activity was evaluated by the agar diffusion bioassay at 25 °C. The stability of the crude mycocins incubated at 37 °C was also assayed after 20, 30, 40, 60, 80, 100, and 120 min incubation. Liquid YEPD medium at pH 4.5 was seeded with 10^5 cells ml^{-1} of *C. albicans* or *C. tropicalis* and 200 μl aliquots were dispensed in the wells of a microtitre plate. Uninoculated medium was used as a control. Fifty microliters of Dh-237 crude mycocin, Dh-3 culture filtrate or uninoculated control was mixed in each well and incubated at 25 °C, 30 °C and 35 °C. The cell growth of *C. albicans* or *C. tropicalis* was monitored using a microplate reader (Bio Rad, Japan) (595 nm) over a 24 h period. The experiments were performed in triplicate and significant differences between the treatments were determined using two-way ANOVA. Comparisons were done using Tukey's multiple comparison tests. Differences between treatments were considered significant when P-values were less than 0.05. GraphPad Prism6 (GraphPad Software, Inc., 2013)was used to perform statistical tests and to generate graphical images.

2.4. Detecting killer activity in commercial cheese

YEPD-methylene blue agar plates were prepared at pH 4.5 and seeded with *C. albicans* or *C. tropicalis* as above. After 3 h at 25 °C, ca. 1 g samples from the outer surface of Romano and blue cheese as well as from shredded Gruyere cheese were placed on the plates and they were incubated for 3 days at either 25 °C or 37 °C. From sample where killer activity had been observed, three samples (10 g each) from the outer surface of that cheese were homogenized in 50ml of sterile 1% peptone using a stomacher Blender 400 and the samples were serially diluted and plated on yeast extract glucose chloramphenicol agar (YEGC) to prevent bacterial growth and incubated for 5 days at 25 °C (Banjara et al., 2015). Colonies were selected based on morphology and color and then restreaked on YEGC agar plates. Killer activity against both *C. albicans* and *C. tropicalis* was evaluated by the streak-plate agar diffusion bioassay as described in Section 2.3.

Table 1. Killer toxin activity at varying temperatures and pH values of *D. hansenii* strains isolated from cheese.

Origin	Strain	20 °C, pH 4.5		20 °C, pH 5.5		25 °C, pH 4.5		25 °C, pH 5.5		30 °C, pH 4.5	
		Calb	*Ctrop*	*Calb*	*Ctrop*	*Calb*	*Ctrop*	*Calb*	*Ctrop*	*Calb*	*Ctrop*
Parmesan (Parma, Italy)	Dh-242	+	+	+	+	+	+	+	+	+	–
Cheddar (Wisconsin, US)	Dh-76	–	–	–	–	–	–	–	–	–	–
Jarlsberg (Norway)	Dh-63	–	–	–	–	–	–	–	–	–	–
Jarlsberg (Norway)	Dh-107	+/–	+	+/–	+	–	+/–	–	+/–	–	–
Romano (Wisconsin, US)	Dh-3	–	–	–	–	–	–	–	–	–	–
Romano (Wisconsin, US)	Dh-21	+	+	+/–	–	–	–	–	–	–	–
Romano (Wisconsin, US)	Dh-45	+	+	+	+	+	+	+	+	–	+
Colby (Wisconsin, US)	Dh-56	+	+	+/–	+	+	+	+	+	–	–
Colby (Wisconsin, US)	Dh-60	–	–	–	–	–	–	–	–	–	–
Colby (Wisconsin, US)	Dh-72	–	–	–	–	–	–	–	–	–	–
Colby (Wisconsin, US)	Dh-79	–	–	–	–	+	–	+	–	–	–
Gouda (Wisconsin, US)	Dh-33	–	–	–	–	–	–	–	–	–	–
Gouda (Wisconsin, US)	Dh-51	+	+	+/–	+	+	+	+/–	+	–	–
Gouda (Netherlands)	Dh-65	+	+	+	+	+	+	+	+	–	+
Gouda (Netherlands)	Dh-111	–	+/–	–	–	–	–	–	–	–	–
Gouda (Netherlands)	Dh-121	+	+	+	+	+	+	+	+	–	–
Gouda, Smoked (Netherlands)	Dh-61	–	–	–	–	–	–	–	–	–	–
Gouda, Smoked (Netherlands)	Dh-66	–	–	–	–	–	–	–	–	–	–
Provolone (Wisconsin, US)	Dh-255	+	+	+	+	+	+	+	+	+	+
Raclette (Wisconsin, US)	Dh-246	+	+	+/–	+	+/–	+	+/–	+	+	+
Bel Paese (Italy)	Dh-34	+	+	+	+	+	+	+	+	+	+
Bel Paese (Italy)	Dh-43	+	+	+	+	+	+	+	+	–	–
Bel Paese (Italy)	Dh-75	–	–	–	–	–	–	–	–	–	–
Bel Paese (Italy)	Dh-220	+	+	+	+	+	+	+	+	+	+
Bel Paese (Italy)	Dh-237	+	+	+	+	+	+	+	+	+	+
Wensleydale (UK)	Dh-265	+	+	+	+	+	+	+	+	+	+
Blue 1 (Wisconsin, US)	Dh-46	+	+	+	+	+	+	+	+	–	–
Blue 2 (Wisconsin, US)	Dh-274	+	+	+	+	+	+	+	+	+	+
Blue 2 (Wisconsin, US)	Dh-276	+	+	+/–	+	+	+	+	+	–	+/–
Mascarpone (Wisconsin, US)	Dh-254	+	+	+	+	+	+	+	+	–	–
Ricotta (Illinois, US)	Dh-262	+	+	+	+	+	+	+	+	–	–
Mold-ripened (Bavaria, Germany)	Dh-53	–	–	–	–	–	–	–	–	–	–
Mold-ripened (Bavaria, Germany)	Dh-201	+	+	+	+	+	+	+	+	+	+
Spanish-type fresh 1 (Wisconsin, US)	Dh-7	–	–	–	–	–	–	–	–	–	–
Spanish-type fresh 2 (Wisconsin, US)	Dh-10	+	+	+	+	+	+	+	+	–	+
Spanish-type fresh 2 (Wisconsin, US)	Dh-68	–	–	–	–	–	–	–	–	–	–
Novelty (Wisconsin, US)	Dh-44	–	–	–	–	–	–	–	–	–	–
Novelty (Wisconsin, US)	Dh-48	–	–	–	–	–	–	–	–	–	–
Novelty (Wisconsin, US)	Dh-80	–	–	–	–	–	–	–	–	–	–
Novelty (Nebraska, US)	Dh-55	–	–	–	–	–	–	–	–	–	–
Novelty (Nebraska, US)	Dh-103	+	+	+	+	+	+	+	+	–	–
Cream Gouda (Netherlands)	Dh-23	–	–	–	–	–	–	–	–	–	–

Calb=*Candida albicans* SC5314; Ctrop=*Candida tropicalis* NRRL 10985.+Killer activity;–No killer activity; +/–Killer activity variable. No killer activity was detected at pH 5.5 at 30 °C, at pH 6.5 or pH 7.0 at any temperature, or at 35 °C at any pH.

2.5. Proteolysis, susceptibility and production

Crude mycocin (500 µL) from strains Dh-237, Dh-274, and Dh- 65 was mixed with 125 µl of proteinase K (10 mg ml^{-1}, Invitrogen, Germany) and incubated at 25 °C for 24 or 48 h. After incubation, the remaining killer activity was evaluated by the agar diffusion bioassay. Additionally, every strain of *D. hansenii* was screened for production of proteolytic enzymes by the gelatin-agar plate method of Smith and Goodner (1958). Colonies of *D. hansenii* 237 were streaked on the agar surface (0.4% peptone, 0.1% yeast extract, 1.2% gelatin, and 1.5% agar) and incubated at 25 °C for 7 days.

3. Results

3.1. D. hansenii and Candida in the healthy human gut microbiome

Our pyrosequencing data of gut-associated fungi from 45 healthy humans detected *D. hansenii* in 16% of all fecal samples, while *Candida* species other than *C. famata* (a synonym of *D. hansenii*) were detected in 75% (Hallen-Adams et al., 2015). The presence of *Candida* and *D. hansenii* were negatively correlated (Spearman's R = −0.30553; two-tailed P-value = 0.01011).

3.2. Mycocin assays

A total of 42 *D. hansenii* strains isolated from multiple samples of 22 distinct cheeses were grown on agar seeded with *C. albicans* and *C. tropicalis* to evaluate killer activity. At low temperature (20 °C) with low pH (4.5), 22 strains (52%) showed mycocin activity against *C. albicans* and 23 strains (54%) had killer activity against *C. tropicalis* (Table 1). At higher temperature and/or pH, fewer strains demonstrated killer activity. Above 30 °C, we did not observe any killer activity against either *Candida* species at any tested pH. The killer activity of crude mycocin preparations from selected *D. hansenii* strains against *C. albicans* and *C. tropicalis* was evaluated at different pH values (4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) and temperatures (20 °C, 25 °C, 30 °C and 35 °C). The results in Table 2 show that numerous variables influence the performance of mycocin against susceptible yeast cells. The impact of crude mycocin on *Candida* was dependent on several factors: the pH and temperature at which the assay was conducted; the *D. hansenii* strain that produced the mycocin; and the sensitive

Table 2. Effect of temperature on activity of crude toxin from selected *D. hansenii* killer strains at different pH values.

		Candida albicans Zone of inhibition (mm) at:					*Candida tropicalis* Zone of inhibition (mm) at:				
pH	Strain	20 °C	25 °C	30 °C	35 °C	SE	20 °C	25 °C	30 °C	35 °C	SE
	Dh-274	$8.00^{a,1}$	$7.72^{a,1}$	$4.83^{b,1}$	3.93b	0.37	$15.09^{a,1}$	$13.80^{b,1}$	$3.27^{c,1}$	-	
	Dh-237	$8.13^{a,1}$	$8.70^{a,1}$	$5.79^{b,2}$	$4.30^{c,1}$		$15.67^{a,1}$	$13.86^{b,1}$	$3.20^{c,1}$	-	
4.5	Dh-65	$7.15^{a,2}$	$7.44^{a,2}$	$4.59^{b,1}$	$2.43^{c,2}$		$14.59^{a,2}$	$13.45^{b,2}$	$2.22^{c,1}$	-	0.31
	Dh-274	$4.74^{a,1}$	$3.74^{b,1}$	$3.30^{c,1}$	$2.94^{c,1}$	0.23	$14.34^{a,1}$	$7.76^{b,1}$	-	-	
	Dh-237	$4.84^{a,1}$	$4.35^{a,2}$	$3.81^{c,2}$	$3.03^{c,1}$		$15.62^{a,2}$	$8.59^{b,1}$	-	-	
5.0	Dh-65	$4.50^{a,1}$	$3.54^{b,1}$	$2.60^{c,1}$	$2.54^{c,1}$		$14.52^{a,1}$	$7.79^{b,1}$	-	-	0.35
	Dh-274	$3.59^{a,1}$	$3.15^{a,1}$	$2.27^{b,1}$	-	0.14	$10.34^{a,1}$	$6.55^{b,1}$	-	-	
	Dh-237	$3.89^{a,2}$	$3.53^{a,2}$	$3.38^{a,2}$	-		$9.38^{a,2}$	$6.84^{b,1}$	-	-	
5.5	Dh-65	$2.76^{a,1}$	$2.43^{a,3}$	$2.27^{a,1}$	-		8.62a,3	$5.09^{b,2}$	-	-	0.19
	Dh-274	-	-	-	-		$4.92^{a,1}$	$2.29^{b,1}$	-	-	
	Dh-237	-	-	-	-		$4.79^{a,1}$	$3.09^{b,2}$	-	-	
6.0	Dh-65	-	-	-	-		$3.25^{a,2}$	$2.10^{b,1}$	-	-	0.23

Zone of inhibition= diameter of cleared zone surrounding the well containing the toxin; data are given as means with standard error (SE); n = 3. Different superscript letters signify statistically significant differences in zone of inhibition between different temperatures, and different superscript numbers signify statistically significant differences between toxins isolated from different strains. - No zone of inhibition observed. No zone of inhibition was observed for material isolated from non-killer strain Dh-3, or uninoculated YEPD medium (negative control).

Candida isolate. For instance, activity of Dh-237 crude mycocin differed significantly from that of Dh-65 under most conditions (Table 2). Dh-237 mycocin consistently displayed the greatest activity of the three tested killer strains, and Dh-65 displayed the least. Activity of all killer strains was lowest at the higher tested temperatures and pH values. Culture filtrate from killer negative *D. hansenii* strain Dh-3 and the negative control had no significant effect on *Candida* growth.

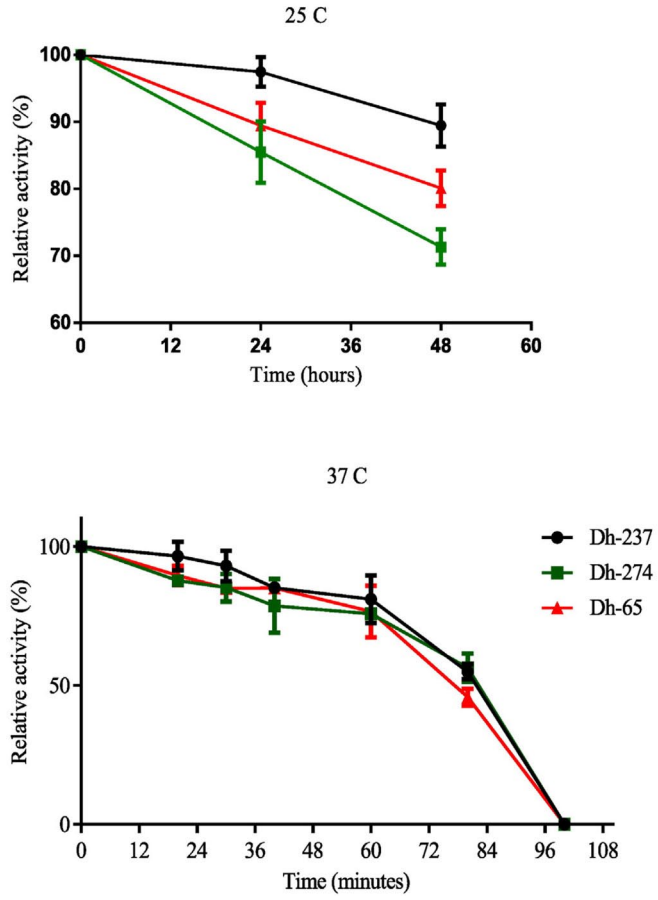

Figure 1. Effect of temperature on the stability of mycocins from Dh-237, Dh-274 and Dh-65 from *D. hansenii*. Error bars represent the mean ± standard deviation for three replications.

Incubation of mycocins from Dh-237, Dh-274 and Dh-65 at 25 °C decreased killer activity by 3%, 15% and 11% after 24 h, and 11%, 29% and 20% after 48 h, respectively (Figure 1). In contrast, we observed killer activity for only a limited time period when mycocins were incubated at 37 °C: killer activity of Dh-237, Dh-274 and Dh-65 mycocins were decreased by 20%, 25% and 24% within 60 min of incubation but activity decreased sharply, by 46%, 44%, and 55% (compared with initial activity) after incubation for 80 min, and all activity was destroyed within 100 min.

The effect of *D. hansenii* mycocin on *Candida* growth kinetics was examined at pH 4.5 because most killer strains produce mycocins that are active at this pH (Middelbeek et al., 1980; Table 1). Crude mycocin from Dh-237 was selected for this study due to its displaying consistently high mycocin activity against *C. albicans* and *C. tropicalis* (Table 2). *C. albicans* and *C. tropicalis* growth was monitored by microplate reader (OD^{595}) over 24 h (Figure 2). *C. albicans* reached log phase after 15 h at 25 °C and 30 °C, and 11 h at 35 °C. Log phase occurred earlier for *C. tropicalis*: in 10 h at 25 °C, 8 h at 30 °C and 5 h at 35 °C. When *Candida* cells were incubated with mycocin there was a reduction in the turbidity of the culture compared with control treatments. Following lag phase, growth inhibition was observed in the mycocin treatments, while *C. albicans* and *C. tropicalis* showed the same, uninhibited growth pattern at all three temperatures (25, 30 and 35 °C) in theDh-3 and control treatments. Dh-237 crude mycocin had a greater inhibitory effect on growth at lower temperatures and a lower effect at higher temperatures. At the highest tested temperature (35 °C) *C. albicans* growth in the presence of Dh-237 mycocin remained low compared to growth with Dh-3 culture filtrate and control treatment, while mycocin treatment showed no effect on *C. tropicalis* growth at 35 °C.

3.3. Killer activity in cheese

The Romano cheese exhibited killer activity against both *C. albicans* and *C. tropicalis* at 25 °C and 37 °C; no killer activity was exhibited by the blue cheese or Gruyere (Figure 3). *D. hansenii* isolated from Romano cheese (1.075×10^4 CFU/g, not figured in Table 1) also demonstrated killer activity against these two *Candida* species; no other mold or yeast species isolated from this cheese sample (five species) demonstrated killer activity.

3.4. Proteolysis

To confirm the proteinaceous nature of *D. hansenii* mycocins, Dh-237, Dh-274 and Dh-65 mycocins were subjected to protease K treat-

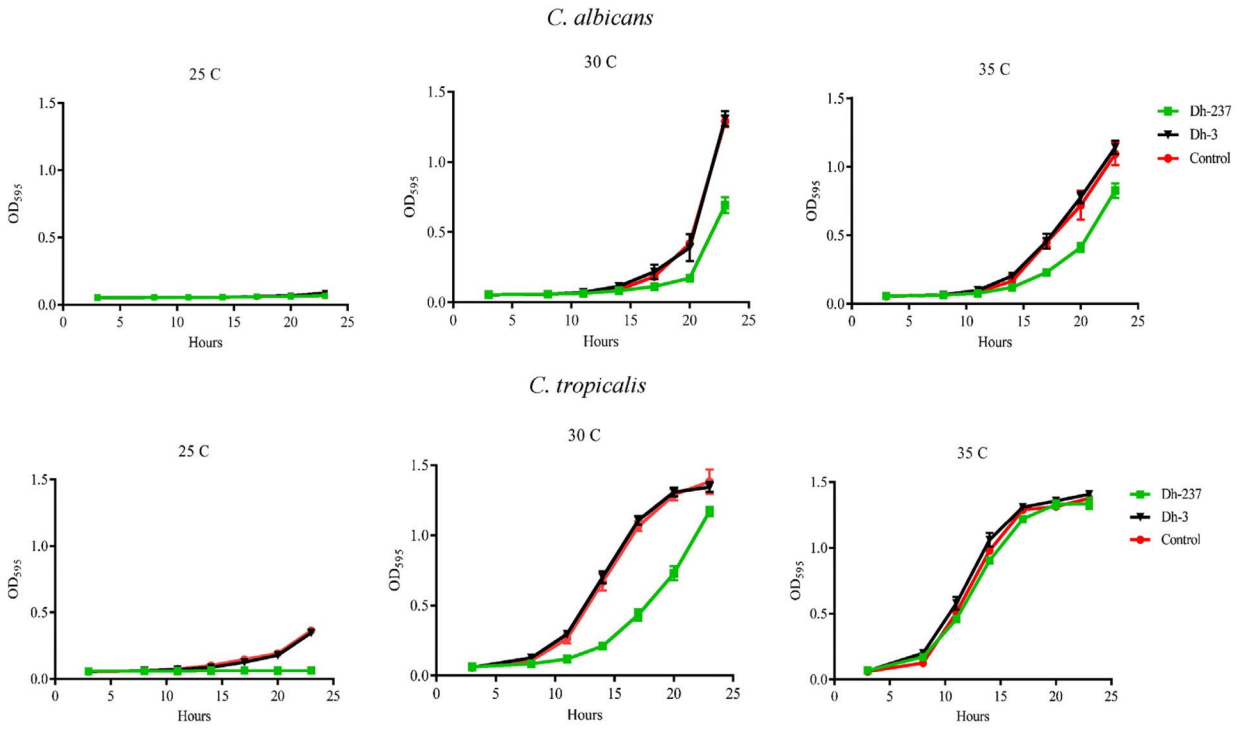

Figure 2. Influence of Dh-237 mycocin on the growth kinetics of *C. albicans* and *C. tropicalis* over 24 h.

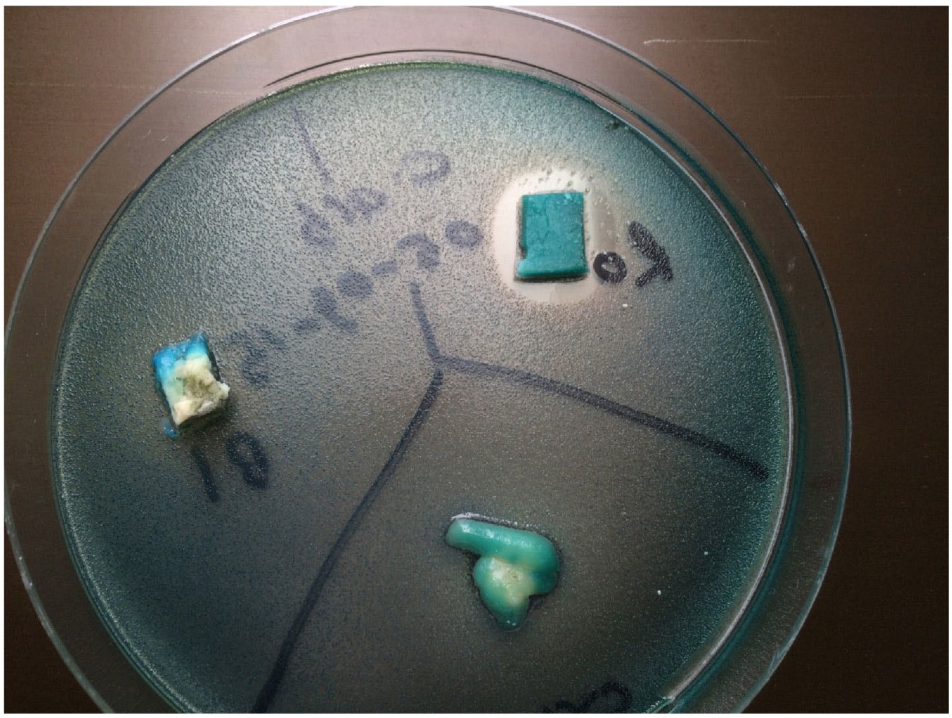

Figure 3. Mycocin produced by *D. hansenii* in cheese, on a YEPD-methylene blue plate seeded with 10^5 CFU ml^{-1} *C. albicans*, after 48 h at 37 °C. A zone of clearing, denoting killer activity, is clearly visible around the Romano (top right). Killer activity continued for at least 10 days (not shown).

ment. The killer activity of three mycocins mixed with protease K against *C. albicans* and *C. tropicalis* was completely eliminated within 48 h, and no activity was detected from mycocins from Dh-274 after 24 h, while killer activity persisted through 48 h in all controls incubated without protease K. This observation suggests that the mycocin was a protein. The instability of mycocin at higher temperatures and/or pH values may be due to proteolytic destruction; when *D. hansenii* 237 was grown on gelatin agar medium for 3 days, a zone of proteolysis was produced (not shown).

4. Discussion

Among 42 strains of *D. hansenii*, our studies found 23 killer strains, while nineteen strains lacked killer activity against either *C. albicans* or *C. tropicalis* under all conditions tested. Activity and response to temperature, pH and protease treatment differed among the killer strains, suggesting that the mycocins may differ between strains (Hernández et al., 2008; Hodgson et al., 1995). In some cases, *D. hansenii* strains differing in killer activity were isolated from same cheese (Table 1). No statistically-significant correlation between killer activity and cheese type was observed.

The activity of all characterized mycocins is strongly dependent on pH and temperature (Hernández et al., 2008; Marquina et al., 2001). We found that mycocin from the strains Dh-274, Dh-237 andDh-65 exhibited temperature-dependent killer activity against *C. albicans* up to pH 5.5 and against *C. tropicalis* up to pH 6.0,with activity decreasing abruptly at nonpermissive temperatures and pH values. Most published studies on mycocin activity report that mycocins are generally stable only over narrow pH ranges and each mycocin has a defined optimal pH for killer activity against the sensitive yeast species (Chen et al., 2000; Soares and Sato, 2000). A previous report by Marquina et al. (2001) stated that mycocin from *D. hansenii* isolated from olive brines had an optimal stability and activity against *Candida boidinii* (IGC3430) between pH 4.5 and pH 5.1, congruent with our observations. We found crude mycocin activity against *C. albicans* up to 35 °C and against *C. tropicalis* up to 30 °C. At the lowest tested temperature (20 °C), killer activity was high but as temperature increased, killer activity against both *Candida* species decreased. That might be due to inactivation of *D. hansenii* mycocin at higher temperatures (Hernández et al., 2008; Marquina et al., 2001) possibly in combination with more robust growth of the *Candida* isolates at higher temperature.

D. hansenii has lower optimal growth temperatures than *C. albicans* or *C. tropicalis* and does not grow at 37 °C; crude mycocin experiments demonstrated that the mycocin itself lost activity at higher temperatures. Sensitivity of *C. albicans* and *C. tropicalis* significantly differed at multiple pH values (4.5, 5.0 and 5.5) and temperatures (20, 25, 30 and 35 °C). *C. tropicalis* was more sensitive to the *D. hansenii* mycocins than *C. albicans* at lower temperatures, while *C. albicans* was more sensitive than *C. tropicalis* to mycocins at high temperature. This result indicates that it is possible that the same mycocin displays a different spectrum of activity against different species or strains (Hernández et al., 2008). Our conclusions assume concentration-dependent diffusion of mycocin through agar during the experiment and an equal number of sensitive cells in all experiments.

Binding of mycocin to the cell wall is commonly the first event in the action of mycocin against sensitive cells and numerous mycocin receptors have been reported on the yeast cell wall (Hodgson et al., 1995; Hutchins and Bussey, 1983; Santos and Marquina, 2004; Santos et al., 2000; Schmitt and Radler, 1988). Sensitive cells die due to various causes, including formation of ion channels, inhibition of cell division at the early G2 stage, inhibition of DNA synthesis and β-1, 3-glucan synthesis. β-Glucans are the major components of the yeast cell wall and have been proposed as primary receptors of mycocins. Mycocin produced by *D. hansenii* strain CYC 1021 is primarily adsorbed by (1□6)-β-D-glucan but the detail of the killing mechanism remains unknown (Santos et al., 2000). Growth inhibition is also affected by the number of free receptors present on the cell wall of each sensitive strain. Thus, it is possible that our chosen strains of *C. albicans* and *C. tropicalis* may differ in availability of binding sites, leading to differential killing of the two species.

D. hansenii cultures grew minimally or not at all at 35 and 37 °C, and did not produce detectable levels of mycocin at those temperatures. Crude mycocin was isolated from cultures growing under optimal pH and temperature conditions. A cell-free preparation, it was consequently not subject to the physiological limitations of living *D. hansenii*, and initially showed high activity at all temperatures; however, as the time of incubation increased, activity decreased. That might be due to *C. albicans* and *C. tropicalis* outgrowing available mycocin molecules, or deactivation of mycocin with temperature and time of incubation (Bajaj et al., 2013); more likely, our crude preparation may contain additional components that degrade or inhibit the mycocin.

Denaturation temperatures vary between proteins, but it is rare for denaturation to begin much below 41 °C, and seems improbable that mycocins would be unable to withstand 35 or 37 °C. An alternate possibility is inactivation of mycocin due to secreted proteases (Woods and Bevan, 1968), either from *C. albicans* and *C. tropicalis*, or *D. hansenii* proteases present in the crude preparation. We have demonstrated both the susceptibility of *D. hansenii* mycocin to protease treatment and the production of secreted proteases by *D. hansenii*; therefore, active proteases may serve as a sufficient explanation of the observed temperature sensitivity of these and other mycocins.

Several papers have reported the ability of mycocins from foodborne yeasts to kill pathogenic yeasts in vitro (e.g., (Buzzini and Martini, 2001; Hodgson et al., 1995; Middelbeek et al., 1980; Vadkertiova and Slavikova, 2007). Only one paper (Buzzini and Martini, 2001) detected killer activity at physiologically-relevant temperatures. Buzzini and Martini (2001) report broad-spectrum killer activity at 37 °C, pH 4.5 from two *D. hansenii*, two *Candida maltosa*, and five *Pichia anomala* strains using crude mycocin preparations in an agar diffusion well bioassay. We observed killer activity at 35 °C and pH ≤ 5.0 against *C. albicans* from the three strains chosen for crude mycocin assays. We found no killer activity against *C. tropicalis* at 35 °C at any pH; one of Buzzini and Martini's *D. hansenii* strains killed 13 clinical isolates of *C. tropicalis* at 37 °C, while the other strain was incapable of killing any *C. tropicalis* isolates at this temperature (Buzzini and Martini, 2001). Both studies testify to the benefit of evaluating multiple strains. Marquina et al. (2001) showed that incubating mycocin produced by *D. hansenii* CYC 1021 at temperatures above 15 °C led to an increasingly rapid loss of activity against sensitive cells, in agreement with our observations. We detected killer activity at 37 °C for 80 min against *C. tropicalis*; by 100 min, all killer activity was lost.

Intriguingly, one of three tested cheese varieties exhibited sustained killer activity against *C. albicans* and *C. tropicalis* at 37 °C, and this killer activity could be linked to *D. hansenii*. We anticipate that a broader sampling would yield more cheeses with killer activity, correlated with colonization by killer strains of *D. hansenii*. The high salt concentration, low pH, and moderate to low temperatures during cheese ripening and aging may provide optimal conditions for mycocin production by *D. hansenii* (Santos and Marquina, 2004), while the abundant proteins of the cheese matrix may serve to buffer the mycocin from the effects of secreted proteases. The duration of observed killer activity is more than sufficient for ingested cheese to reach the colon (De Vries et al., 1988) where most gastrointestinal *Candida* reside (Schulze and Sonnenborn, 2009); this, in turn, may account for the observed influence of *D. hansenii* on gut *Candida* populations. Conversely, *D. hansenii* mycocins could be destroyed by gut proteases and any observed reduction in *Candida* populations could be due to transient ecological interactions. *In vivo* feeding studies are warranted to determine whether killer *D. hansenii* reproducibly reduces *Candida* populations in the gut and, if so, the duration of such effect. If killer activity persists in the gut, or can be retained through encapsulation, *D. hansenii*, an approved food additive, deserves further study for its anti-*Candida* properties.

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