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- **A concise and informative title:** Effects of changes in culture conditions on the virulence and antifungal susceptibility of dairy cow mastitis fungal isolates
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Effects of changes in culture conditions on the virulence and antifungal susceptibility of dairy cow mastitis fungal isolates

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Abstract

Background: In recent years, the number of incidences of dairy cow mastitis caused by non-albicans *Candida* (NAC) have increased owing to the virulence factors, including cell surface hydrophobicity (CSH) and phospholipase activity, of the causative agents, namely, *Candida krusei* and *Candida parapsilosis*. Temperature and pH changes in the cow's udder after fungal infection and unreasonable medication can affect the antifungal susceptibility of *Candida* spp. and their expression of virulence factors.

Methods: In this study, the effects of different temperatures and pH on the virulence of NAC strains were tested, and the in vitro susceptibility of the fungal strains to Cu²⁺ and antibacterial agents were examined. Besides, the changes in the virulence factors of *Candida* spp., including biphasicity, hemolytic activity, CSH, and phospholipase activity under these test conditions were investigated, and the internal relationship between these factors was analyzed.

Results: The results showed that the virulence factors and antifungal susceptibility of *Candida* spp. could be altered through changes in various physiological conditions. Both temperature and pH were noted to be important factors affecting *Candida* growth, antifungal susceptibility, and expression of virulence factors. Cu²⁺ inhibited the growth and virulence factors expression of *Candida* spp., whereas antibacterial agents directly promoted the growth of *Candida* spp., making them resistant, which is one of the reasons for breast inflammation symptoms in cows.

Conclusions: These results on virulence factors, antifungal susceptibility, and physiological characteristics of NAC provide a theoretical basis for understanding and treating dairy cow mastitis caused by NAC.

Keywords Cow mastitis, *Candida parapsilosis*, *Candida krusei*, virulence factors, antifungal susceptibility

Background

Cow mastitis causes major economic losses in modern animal husbandry, resulting in decreased milk production, reduced milk quality, reproductive disorders, and higher elimination rates [1]. Among the many factors that cause cow mastitis, pathogenic microbial infection has received widespread attention [2]. Pathogenic microbial infections can be caused by bacteria and fungi. It has been shown that *Candida albicans*, a conditional pathogen, is the main causative agent of mastitis in cows. However, recent studies have suggested that the number of non-albicans *Candida* (NAC) infected cows has increased owing to the biphasic nature, hemolytic activity, cell surface hydrophobicity (CSH), and phospholipase activity of NAC. NAC, such as *Candida parapsilosis* and *Candida krusei*, are weakly pathogenic, but widely distributed, and infect the host due to reduced body resistance and antibiotics abuse [3].

The main pathogens that cause bacterial mastitis are *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli*, and *Trueperella pyogenes* [4]. During bacterial mastitis, the levels of

inflammatory factors in the host increase, and these factors subsequently participate in the intracellular environmental balance and produce systemic effects [5]. The symptoms of bacterial mastitis generally include inflammatory reactions such as redness, swelling, heat, and pain. Besides, the quality of milk significantly changes, with precipitation, flocs, or blood in the milk [6]. Fungal mastitis is often secondary to acute bacterial mastitis that is inappropriately treated with antibiotics [7], and is mainly caused by *Candida albicans*, *Cryptococcus neoformans*, *C. krusei*, and *C. tropicalis* [8]. In addition to the symptoms of bacterial mastitis, fungal mastitis may also present diffuse swelling and dough-like hardness of the affected area. The temperature of the infected cattle could reach 39.5–41.5°C, along with elevated milk pH and color of milk becoming sanguineous and irreversible. These symptoms get worse or become ineffective with antibiotic treatment, because when bacterial pathogens are inhibited or killed, the fungal pathogens can grow and reproduce. After biofilm formation, the bacterial flora in the breast becomes imbalanced, exacerbating the fungal infection and resulting in a long course of repeated disease [9]. Thus, fungal mastitis can lead to irreversible damage of the mammary gland function, producing huge losses to dairy farming.

The physiological environment (including temperature, pH, ion concentration, etc.) of dairy mammary glands is dynamic. *Candida* spp. adapt to the host's internal environment by sensing the phenotypic changes in the host's physiological environment. The normal temperature of a cow's breast is generally between 33 °C and 36 °C [10]. When mastitis occurs, the body temperature of the cattle reaches 42 °C. And the optimal growth temperature of fungi is 28 °C -35 °C, temperature determines the ability of the *Candida* spp. to infect the host [11]. Besides, the normal pH of milk is around 6.5. When cows are acidic or alkaloid, the pH can reach <6[12] and > 7[13], respectively. *Candida* spp. can transform to adapt to the changing pH of the environment [14], indicating that pH is also one of the important factors affecting the physiological characteristics of *Candida* spp. Trace elements such as Cu²⁺ can be used as biocides in addition to essential elements in the host. Antibacterial agents as a nitrogen source directly promotes *Candida* spp. growth and easily make them resistant[15]. Factors such as the environment of the cow's udder, unreasonable medication, and trace elements can affect mastitis development and recovery from the disease. In the present study, the virulence factors of *Candida* spp. and the changes in their antifungal susceptibility were examined by altering the in vitro culture conditions of the fungal isolates, and the internal relationship between these factors was analyzed. The results obtained could help in understanding and treating fungal cow mastitis.

Materials and methods

Isolation and identification of pathogenic fungi

The study was submitted to, and approved by, the Animal Research Ethics Committee of Ningxia University, China. A total of 576 milk samples from cows with mastitis were collected from three different dairy farms in Ningxia, China. Clinical mastitis was defined by swelling, reduced milk flow, and abnormal milk appearance (watery to viscous with clots varying from gray-white to yellowish). In addition, other signs of infection such as fever, inappetence, ataxia, and depression were also considered. The milk samples (2 mL) from infected cows were aseptically collected into a 10-mL centrifuge tube, mixed with 7 mL of PBS, and centrifuged at 5000 rpm for 5 min (Sigma, USA) to collect the precipitate. An appropriate amount of physiological saline was added to resuspend the pellet, and 100 µL of the pellet were spread on TTC-Sarbol agar medium (OXIOD, UK) and cultured at 35°C for 48 h. A single colony of suspected fungus was picked and cultured on

Sabouraud agar medium (OXIOD, UK). The isolates were further confirmed by CHROMagar (CHROMagar, France) color identification, Gram staining, internal transcribed spacer (ITS) sequence amplification, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (VITEK® MS, BioMerieux, France).

Growth status

Temperature and pH

Four different temperatures (35°C, 37°C, 40°C, and 42°C) and four pH values (6.0, 6.5, 7.0, and 7.5) were employed for full factor tests. As reference strains for *C. parasilosis* and *C. krusei*, ATCC22019 and ATCC6258 were used, respectively. The fungal cells were incubated at different temperatures and pH for 24 h, and their morphology and number of colonies were determined. All the experiments were performed in triplicate.

Addition of Cu²⁺

Sterile CuSO₄ was added to each experimental group at final CuSO₄ concentrations of 0 (blank control), 1.6, 3.2, 6.4, 16, and 80 µg/mL, respectively. The optimum growth temperature and pH of the test strains were selected, and the number of colonies formed was counted after 24 h of incubation with CuSO₄. All the experiments were performed in triplicate.

Addition of antibacterial agents

Penicillin, lincomycin, and penicillin combined with lincomycin were added to the sterilized milk for 24 h. The final concentrations of penicillin and lincomycin were 7.5 and 35 µg/mL, respectively. The non-antibiotic group was used as blank control. The suspension of each fungal strain was diluted to 10³ CFU/mL, and 60 µL of the suspension were spread on Sabouraud agar medium and cultured for 24 h to observe colony growth. All the experiments were repeated thrice.

Antifungal susceptibility

According to the guidelines of the Institute of Antifungal Agents Sensitivity Standards in Clinical Laboratories [16, 17], the paper diffusion method was used to determine the sensitivity of the fungal isolates to antifungal agents. The effects of different pH, temperatures, Cu²⁺ addition, and antibacterial agents addition on the sensitivity of the fungal isolates to five antifungal agents (fluconazole, ketoconazole, itraconazole, amphotericin B, and 5-fluorocytosine) were determined.

Expression of virulence factors

The effects of different temperatures, pH, Cu²⁺ addition, and antibacterial agents addition on the expression of virulence factors by the fungal isolates were examined.

Phenotypic transformation

After culturing for 24 h, the pathogenic fungal cells were subjected to Gram staining and observed under a microscope.

Hemolytic activity

After adjusting the concentration of the fungal isolates to 1×10⁷ cells/mL, 5 µL of the fungal suspension were added to the surface of Columbia blood plate and cultured at 5% CO₂ for 48 h. Three replicates were prepared for each group.

CSH

The fungal isolates were inoculated in YPD liquid medium and cultured for 24 h. Then, the concentration of the fungal suspension was adjusted to OD_{600nm} of 1.0 using a spectrophotometer.

Subsequently, 1.2 mL of the fungal suspension was mixed with 0.3 mL of n-octane, vortexed for 3 min, and allowed to stand for 10–15 min until the two phases separated. The ambient temperature was controlled between 16–25°C. The lower aqueous phase was transferred into a 96-well plate, with fungal suspension used as a negative control, and the OD value at 600 nm was measured using a fluorescence microplate reader. Relative hydrophobicity was calculated as follows: Relative hydrophobicity (CSH) = (OD600 control – OD600 test)/OD600 control. The experiment was performed in triplicate.

Phospholipase activity

After adjusting the concentration of the fungal suspension to 2×10^7 CFU/mL, 10 μ L of the suspension were added dropwise to the surface of yolk agar medium, and incubated under same pH and temperature combination. The phospholipase activity (PZ) was calculated as follows: PZ = colony diameter/total diameter. The smaller the PZ value, the higher is the phospholipase activity.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software. One-way ANOVA was used for analysis of variance, and $P < 0.05$ indicated statistical significance.

Results

Isolation and identification of pathogenic fungi

A total of 284 pathogenic yeasts were isolated from 576 milk samples collected from cows with clinical mastitis (284/576, 49.3%) in Yinchuan, Ningxia Province, China. Among them, 82 (82/284, 28.87%) were identified to correspond to 10 *Candida* spp., including *C. krusei* (32.93%, 27/82), *C. parapsilosis* (18.29%, 15/82), *C. lipolytica* (7/82, 8.54%), *C. lusitaniae* (7/82, 8.54%), *C. pararugosa* (6/82, 7.32%), *Trichosporon mucoides* (6/82, 7.32%), *C. sphaerica* (5/82, 6.1%), *C. tropicalis* (5/82, 6.1%), and *C. utilis* (4/82, 4.88%). Therefore, a *C. krusei* isolate (CK1) and a *C. parapsilosis* isolate (CP1), each with the highest infection rate, were selected. In addition, *C. pararugosa* (CA1), which is less common in clinical infections, was also selected.

Growth status

Temperature and pH

Figure 1-1 shows the effects of temperature and pH on the growth of strains ATCC6258, CK1, ATCC22019, CP1, and CA1. The optimum growth conditions for ATCC6258 were noted to be 37°C and pH 6.0, while those for CK1 were 35°C and pH 6.5. With the increase in pH and temperature, the growth of these two strains was inhibited. ANOVA results showed that the effect of temperature and pH on the growth of ATCC6258 and CK1 was significantly different. With the increase in pH, the morphology of these two strains changed from shrinking to smooth, and the colonies transformed from large to small.

The optimal growth conditions for ATCC22019, CP1, and CK1 were 37°C and pH 6.5. With the increase in pH and temperature, the growth of the three fungal strains was inhibited. When the temperature reached 42°C, the fungal suspension at a concentration of 10^3 cells/mL did not grow on the culture medium.

Addition of Cu^{2+}

For statistical analysis, data obtained for 1000-fold Cu^{2+} dilution were selected. When compared with the control group, Cu^{2+} at lower concentrations significantly inhibited the growth of *Candida*

spp. Figure 1-2 shows that Cu^{2+} inhibited *Candida* growth in a dose-dependent manner.

Addition of antibacterial agents

Addition of antibacterial agents significantly promoted the growth of *Candida* spp. (e.g. ATCC6258) (Fig. 1-3).

Antifungal susceptibility

Temperature and pH

The change in pH significantly altered the sensitivity of *C. krusei* to triazole. The antifungal susceptibility of the fungal strains increased with the increase in pH (Fig. 2-1a, b). Based on the effect of different pH on the antifungal susceptibility of *C. parapsilosis*, it was found that the susceptibility of ATCC22019 to fluconazole, ketoconazole, and amphotericin B significantly changed with the variation in pH. Furthermore, the susceptibility of CP1 to fluconazole, 5-fluorocytosine, ketoconazole, and amphotericin B significantly increased with the increasing pH (Figure 2-1c, d). Similarly, with the increase in pH, the susceptibility of CA1 to fluconazole, ketoconazole, itraconazole, and amphotericin B significantly increased (Fig. 2-1e).

With regard to temperature, the fungal strains could not grow at 42°C. At 40°C, the susceptibility of ATCC6258 to ketoconazole, itraconazole, and amphotericin B was significantly increased, while the susceptibility of CK1 to ketoconazole and itraconazole was significantly increased. In contrast, both *C. parapsilosis* and *C. parapsilosis* could not grow at 40 °C, and there was no significant difference in the MIC values at 35°C and 37°C (Fig. 2-2).

Addition of Cu^{2+}

There was no significant difference in the effects of different concentrations of Cu^{2+} addition on the antifungal susceptibility of ATCC6258 and CK1.

Addition of antibacterial agents

Addition of antibacterial agents significantly increased the resistance of *Candida* spp. (e.g. ATCC6258 and ATCC22019) to some antifungal agents such as fluconazole, 5-fluorocytosine, and ketoconazole. However, the antifungal susceptibility was restored when the temperature was increased to 40°C (Fig. 2-3).

Expression of virulence factors

Phenotypic transformation

Variation in the temperature or pH altered the Gram staining morphology of the fungal strains. The *Candida* cells remained as hyphae at near-neutral pH (Fig. 3-1). Addition of Cu^{2+} did not significantly inhibit mycelial growth without causing cytotoxicity; however, formation of *Candida* mycelium was significantly inhibited at 20 mM Cu^{2+} concentration. In contrast, addition of antibacterial agents had no effect on the phenotypic changes of the fungal strains.

Hemolytic activity

Both ATCC6258 and CK1 presented no hemolytic activity. The hemolytic activity of each fungal strain was significantly different under different temperature conditions. When compared with the standard strain, the fungal isolates were more likely to produce high hemolytic activity at high temperatures; however, ATCC22019 and CP1 exhibited no hemolytic activity at 42°C (Fig. 3-2). There was no significant difference in the effect of Cu^{2+} and antibacterial agents addition on the hemolytic activity of the fungal strains.

CSH

The CSH of the five fungal strains was not noted after the temperature reached 40°C. The changes in the temperature and pH led to significant differences in the hydrophobicity of each strain (Fig. 3-3). While CSH was not detected when Cu²⁺ was added, it was significantly reduced when antibacterial agents were added (Fig. 3-4).

Phospholipase activity

The phospholipase activity of the fungal strains was inhibited at high temperature and pH. Both ATCC22019 and CP1 produced maximum phospholipase activity at pH 6.0 and 35°C. The interaction of temperature and pH on the phospholipase activity of *C. parapsilosis* was extremely significant. In contrast, ATCC6258, CK1, and CA1 did not exhibit phospholipase activity with the change in temperature and pH (Fig. 3-5). Besides, there were no significant differences in the effects of Cu²⁺ and antibacterial agents addition on the phospholipase activity of the fungal strains.

Discussion

Adaptability of *Candida* spp. is an important factor for their pathogenicity, and strong adaptability is demonstrated when exposed to different temperatures, pH, nutritional sources, and immune system attacks in the host environment. Being a conditional pathogen, it is important to investigate the effects of different conditions on the physiological characteristics of *Candida* spp. By sensing various external stimuli, *Candida* spp. induces the transformation from yeast phase to hypha phase through intracellular signal transmission. The biphasic nature of *Candida* spp. helps in resisting the changes in the external conditions, ultimately resulting in adaptation to the environment [18]. Previous studies have shown that *Candida* hyphae are more virulent and activate the inflammatory responses of the host, while *Candida* yeast cells are also pathogenic, enabling the strain to colonize the host [19]. Thus, *Candida* virulence is a complex phenomenon in which the formation of hyphae plays an important role. In the present study, changes in the temperature and pH conditions had very significant effects on the growth status of the *Candida* isolates examined. When compared with *C. parapsilosis* and *C. pararugosa*, *C. krusei* was noted to be more resistant to high temperatures, which may be one of the reasons for the highest proportion of *C. krusei* isolates among all the *Candida* isolates investigated. The results obtained suggest that *Candida* spp. survive in two forms within the temperature and pH range of the cow's mammary gland, with near-neutral pH being more conducive for hypha formation, although *Candida* spp. could still form hyphae at high temperatures.

In addition to its biphasic characteristic, the virulence of *Candida* spp. includes phospholipase activity, CSH, and hemolytic activity. Among the *Candida* spp., *C. albicans* has strong virulence and pathogenicity, exhibiting adaptability to environmental changes, adhesion to host cells, and secretion of hydrolytic enzymes such as phospholipase, acid protease, etc. [20]. Phospholipase enzymes degrade phospholipid membrane of the host cells, increase the permeability of the host cell membrane, and impair cell integrity, promoting fungal invasion. Hemolytic activity destroys the ability of heme to extract iron, which is very important in the infection process [21]. When compared with *C. albicans*, *C. parapsilosis* and *C. krusei* have been found to exhibit higher CSH, which plays a key role in the process of fungal colonization of the host surface. CSH helps to attach *Candida* spp. to the extracellular matrix components, and an increase in hydrophobic activity reduces the ability of the host cells to phagocytose and increases fungal resistance to blood clearance, thus enhancing the virulence of *Candida* cells.

Metal ions such as Ca²⁺, Cu²⁺, Zn²⁺, and Fe³⁺ are essential trace elements for cell growth. They participate in a variety of metabolic pathways in the cell in the form of prosthetic groups, and play

an important role in cell growth and metabolic regulation [22-23]. In particular, Cu^{2+} has broad-spectrum antibacterial properties and inhibits the growth of viruses and fungi [24]. However, despite being a physiologically necessary ion, when the concentration of Cu^{2+} exceeds the physiological requirements, it causes serious problems [25]. Numerous studies [23,26-27] have shown that concentration of Cu^{2+} higher than 100 $\mu\text{g/mL}$ could cause cytotoxicity and damage the cells. Although the National Food Safety Standards for Milk [28] has not stipulated a limit for Cu^{2+} , we referred to the limit of Cu^{2+} in drinking water [29] and wine [30] as 1.0 mg/L, and employed 80 $\mu\text{g/mL}$ as the maximum concentration of Cu^{2+} in the present study. The results showed that Cu^{2+} could significantly inhibit fungal growth, CSH, and phenotypic switching, but had no effect on hemolytic and phospholipase activities. Thus, an appropriate amount of Cu^{2+} can be added to prevent the growth of fungi without causing damage to cow's breast. Nevertheless, the molecular mechanism of changes in fungal virulence factors caused by physiological conditions must be further studied.

With regard to antifungal susceptibility, NAC, such as *C. krusei*, are naturally resistant to fluconazole, which is currently one of the most commonly used antifungal agents [31], and the results of the present study are consistent with this observation. While the increase in temperature and pH and addition of antibacterial agents significantly altered the susceptibility of the fungal strains to antifungal agents, Cu^{2+} had no effect on the antifungal susceptibility of the isolates. Some reports have indicated that lack of nitrogen sources led to low expression of *Candida* growth factors, and that antibiotics added bind to proteins, thereby promoting the expression of *Candida*-related growth genes, ultimately affecting the fungal growth rate [15]. In the present study, addition of antibacterial agents, which are commonly used for treating cow mastitis, was found to directly promote the growth of *Candida* spp., making them resistant to some antifungal agents. These results suggest that unreasonable use of antibacterial agents can produce serious effects on the antifungal susceptibility of fungal isolates that cause dairy cow mastitis, and further research on the physiological characteristics of pathogenic fungi is necessary.

Conclusions

The results showed that the virulence factors and antifungal susceptibility of *Candida* spp. could be altered through changes in various physiological conditions. Both temperature and pH were noted to be important factors affecting *Candida* growth, antifungal susceptibility, and expression of virulence factors. Cu^{2+} inhibited the growth and virulence factors expression of *Candida* spp., whereas antibacterial agents directly promoted the growth of *Candida* spp., making them resistant, which is one of the reasons for breast inflammation symptoms in cows. These results on virulence factors, antifungal susceptibility, and physiological characteristics of NAC provide a theoretical basis for understanding and treating dairy cow mastitis caused by NAC.

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analysis and interpretation, and writing of the manuscript.

Availability of data and materials

All data generated or analyzed in this study are presented in the published article.

Authors' contributions

YY, XW, and XZ conceived and designed the experiments; YY, SM, and XW analyzed the data and drafted the manuscript; YY, SM, and XW performed the experiments and acquired data; and XZ interpreted the data and critically revised the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was conducted in accordance with the Law on Animal Protection and Welfare of Ningxia Hui Autonomous Region of China. Samples were recovered after acquiring permission from the study participants. As only milk samples, and not mammary tissues, were obtained for this study, verbal consent was obtained from all the farm owners who participated in this study. This study was submitted to and approved by the Ethics Committee of Animal Study, Ningxia University, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Fig.1-1 Effect of different temperature and pH on the growth of strains. **a** ATCC6258; **b** CK1; **c** ATCC22019; **d** CP1; **e** CA1. Different lowercase letters indicate significant differences, $P < 0.05$.

Fig.1-2 Effect of Cu²⁺ on the growth of *C. krusei* antifungal susceptibility. *significant 0.01<P<0.05, **extremely significant P<0.01.

Fig.1-3 Effect of antibacterial agents on growth. *significant 0.01<P<0.05, **extremely significant P<0.01

Fig. 2-1 Effect of different pH on antifungal susceptibility of *Candida*. a ATCC6258; b CK1; c ATCC22019; d CP1; e CA1. Different lowercase letters indicate significant differences, $P < 0.05$.

Fig. 2-2 Effect of different temperature on antifungal susceptibility. **a** ATCC6258; **b** CK1. *significant 0.01 < P < 0.05.

Fig.2-3 Effect of antibacterial agents on antifungal susceptibility. **a** ATCC6258; **b** ATCC22019. *significant 0.01 < P < 0.05, **extremely significant P < 0.01.

Fig.3-1 37°C pH6.0 Gram stain (1000×). **a** ATCC6258; **b** CK1

Fig.3-2 Effect of different temperatures on hemolytic activity. Different lowercase letters indicate significant differences, $P < 0.05$.

Fig.3-3 Effect of different temperature and pH on CSH. **a** 35°C; **b** 37°C. Different lowercase letters indicate significant differences, $P < 0.05$.

Fig.3-4 Effects of adding antibacterial agents on the CSH of *Candida*. **a** ATCC6258; **b** CK1. *significant $0.01 < P < 0.05$, **extremely significant $P < 0.01$.

Fig.3-5 Effect of different temperature and pH on phospholipase activity. **a** ATCC22019; **b** CP1. Different lowercase letters indicate significant differences, $P < 0.05$.