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New approach in diagnosis and treatment of Bovine Mycotic Mastitis in Egypt

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In the present study, 123 collective milk samples from 71 cattle and 52 buffaloes suffering from clinical mastitis with no or poor response to treatment with conventional antibiotics were tested for mycotic mastitis using culture and multiplex PCR (m-PCR) methods. The overall positive percentage of Mycotic mastitis was 25.2 and 30.08% using culture and m-PCR respectively. Milk culture revealed 24 isolates of *Candida albicans* and 7 isolates of *Aspergillus fumigatus*. PCR succeeded to detect *A. fumigatus* from two samples and *C. albicans* out of four samples that were culturally negative. These yeast and fungi isolates were tested for their sensitivity toward different probiotic strains from different sources. Results revealed that *Lactobacillus acidophilus* isolated from goat colostrums followed by *L. acidophilus* isolated from mare colostrums showed the best antifungal activities against *C. albicans* followed by *A. fumigatus*, while, *Lactobacillus plantarum* and *Bacillus subtilis* had no effect. These results showed that Mycotic mastitis is an increasing problem due to the wide misuse of antibiotics as mastitis therapy. Antimicrobials should be used in mastitis in a careful way and effort most be encouraged to apply safe substitutes such as probiotics and bioactive natural compounds for prophylactic and therapeutic use.

Key words: Bovines, milk, mycotic mastitis, m-PCR, culture and probiotic.

INTRODUCTION

Bovine mastitis has been defined as an inflammation of the mammary gland resulting in reductions of milk yield and quality. A wide variety of microorganisms have been implicated as causative agents of bovine mastitis including bacteria and fungi (Krukowski et al., 2006).

Bovine mycotic mastitis is usually caused by yeasts, but mastitis due to filamentous fungi mostly *Aspergillus fumigatus* has been reported. It occurs as sporadic cases

affecting a small percentage of cows, or as outbreaks affecting the majority of animals. In both situations, however, the seriousness of infection depends on the number of organisms present in the glands and the species of yeast involved (Pengov, 2002). Generally, in 2001, studies have suggested that mycotic mastitis is on the increase, and *Candida krusei* is frequently isolated from affected quarters (LasHeras et al., 2000; Malinowski et al., 2001).

The most frequent isolated organisms among the Mycotic mastitis are the *Candida* species (Tarfarosh and Purohit, 2008; Spanamberg et al., 2008) which are a group of unicellular opportunistic organisms, ever present

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in the natural surroundings of dairy cattle (milker's hands, milking machines, treatment instruments, floor, straw, feed, dust, soil, drug mixtures, and sanitizing solutions) and are normal inhabitants of the skin of the udder and teats, in which they exist in low numbers (Santos and Marin, 2005). They can invade mammary glands and cause clinical mastitis characterized by pain, prolonged fever, tenderness, inflammatory reaction in the mammary gland and associated lymph nodes and reductions of milk yield and quality in animals (Şeker, 2010). Some intramammary fungal infections such as *A. fumigatus* and *Candida* Spp may result in death of affected animals (Krukowski et al., 2000; Perez et al., 1999; Heras et al., 2000).

Outbreaks of Mycotic mastitis are generally believed to result from an ascending infection subsequent to incorrect administration of antibiotic preparations during drying-off period (Spanamberg et al., 2008). Contamination of the teat end or cannulas by environmental yeasts and fungi associated with lack of hygiene during the milking and poor equipment cleaning leads could favor further penetration into the mammary gland (Gaudie et al., 2009). The large doses of antibiotics may cause a reduction in the vitamin A, leading to injury to the udder's epithelium and affecting the microflora of the mammary glands, which acts as an animal natural defense, thus facilitating the invasion of fungi and yeasts (Perez et al., 1999; Heras et al., 2000; Şeker, 2010).

The clinical signs are non-specific and, in some cases, their development may be masked by symptoms of an underlying disease. Therefore, these diseases are generally diagnosed by demonstrating and identifying the aetiological agent histopathologically and in culture, respectively (Ferreiro et al., 1989; Perez et al., 1999).

Current diagnostic methods have not proven to be sufficiently sensitive and specific to enable an early and effective diagnosis of the disease, with the result that the search for an optimal diagnostic method continues (Garcia and Blanco, 2000). Among nonculture methods under investigation, Polymerase Chain Reaction (PCR) offers advantages over classical approaches, because theoretically, low level fungal infections (e.g. with *Candida albicans*) can be detected from minimal volumes of clinical samples such as blood, and DNA from both dead and viable organisms could serve as a target template for the amplification reaction (Polanco et al., 1999).

Random use of growth promoters, antibiotics and antimycotic in animals lead to immune-suppression and development of multiple drug resistance strains to compounds used in human medicine. These drugs should be substituted by safer ones (Magnusson et al., 2003).

Lactic acid bacteria (LAB) are among the most powerful prokaryotes when it comes to antimicrobial potential. A large number of LAB strains have effective antimycotic the pathogens by depleting nutrients consumed by the pathogens and modulate the host immune response. Also, they release endogenous microbicides compounds

including; lactic acid, bacitracin and hydrogen peroxide which have microbicidal effect (Magnusson et al., 2003; Ström et al., 2002). Probiotic bacteria present in the alimentary tract and vagina of man and animals prevent the overgrowth of *Candida* spp. and thereby decrease the occurrence of mucosal or systemic candidiasis (Balish, 1986; Zwolinska-Wcislo et al., 2006).

The increasing incidence of mycotic infection, the increasing resistance of *mycotic* spp. to antifungal agents and the rise in mortality associated with infections by *Candida* spp. demand a safe way to prevent and treat infections such as mycotic mastitis caused by opportunistic yeasts and fungi.

Therefore, the present study reports mycotic mastitis in two Egyptian governorates, which is the first time to be diagnosed directly using multiplex PCR on milk samples. This draws attention to the disease in Egypt. Besides, anti-mycotic activity of seven probiotic bacterial isolates was investigated *in vitro* against the isolated fungi and yeast.

MATERIALS AND METHODS

Milk samples

During summer 2010, one hundred and twenty three collective milk samples were collected from 71 dairy cattle and 52 buffaloes in different stages of lactation (from small farms in Kalubia and Menofia Governorates in Egypt) showing clinical signs of mastitis such as abnormal milk (e.g. signs of chunks or clots in milk), abnormal udder (swollen, red, or hard), or fever; whether the cow was off food and teat injury. These animals were treated with conventional antibiotics and the response was poor or absent. The samples were taken under possible aseptic condition in a sterile screw capped bottles and transferred in an ice box to the laboratory.

Sample culture and identification

10 ml of each milk sample was centrifuged for 20 min at 3000 rpm and the sediment was plated onto Sabouraud Dextrose Agar media (Difco) and solid yeast peptone dextrose media (YPD) (Difco) at 25 and 37°C for 4 to 6 days. Identification was performed according to Refai et al. (1969) and Morcos et al. (1990).

Multiplex polymerase chain reaction (mPCR)

Reference strains preparation

A. fumigatus and *C. albicans* reference strains used as positive controls were kindly offered by the Dept of Chemistry of Natural and Microbial Products-National Research Center, Egypt. *A. fumigates* was grown in Sabouraud Dextrose Agar media (Difco) at 25 and 35°C for 2 to 5 days, while *C. albicans* was cultured on solid yeast peptone dextrose media (YPD) (Difco) at 30°C for 48 h prior to DNA isolation.

DNA extraction

DNA was extracted directly from a loopful of fungal cells using DNeasy Blood and Tissue Kit (Qiagen Co. Cat no. 69504) following the manufacturer instruction. While DNA extraction from milk was

Table 1. *A. fumigatus* and *C. albicans* -specific primer pairs characteristics.

organisms	Primer pairs	Sequence (5'→3')	Amplicon size (bp)
<i>A. fumigatus</i>	ASP1	CGGCCCTTAAATAGCCCGGTC	357bp
	ASP7	CCTGAGCCAGTCCGAAGGCC	
<i>C. albicans</i>	SC1F	CGGAGATTTTCTCAATAAGGACCAC	670bp
	SC1R	AGTCAATCTCTGTCTCCCTTGC	

performed with modified method from Nebbia et al. (2006) and Dalmasso et al. (2011). Milk samples (1 ml) were centrifuged at 1500 g for 15 min to collect somatic cells. The pellets were rinsed three times in 1 ml of PBS, centrifuged at 12,000 g for 5 min and resuspended in 200 µl of PBS, followed by an enzymatic digestion with lysozyme buffer (lysozyme 18 mg/ml, 15 mM Tris-HCl pH 8.0, 1 mM EDTA and 1% Triton X-100) and proteinase K. Finally DNA was extracted following the protocol of the Dneasy Blood and Tissue kit (Qiagen).

Optimization of individual PCR assays

A. fumigatus primers and PCR amplification

The PCR primers used in this study were designed according to Yamakami et al. (1996) and Garcia et al. (2008). These primers amplify the region V7 to V9 of subunit 18S of *A. fumigatus* rRNA as shown in Table 1.

C. albicans primers and PCR amplification

The oligonucleotide primer pair (Table 1) was designed by Galan et al. (2006) in corresponding to sequences of *C. albicans* KER1 gene.

Each PCR reaction mixture (50 µl) contained 25 µl PyroStart™ Fast PCR Master Mix (Fermentas Co. Cat. no. Ko211), 1 µl for each primer (100 pmol) with 6 µl of DNA extracted from pure fungal culture or 17 µl of DNA extracted from milk samples.

PCR was performed in PTC-100 Peltier Thermal Cycler (MJ Research, USA). The first cycle included 2 min of denaturation at 95°C. This first step was followed by 35 cycles of 2s of denaturation at 94°C, 10s of annealing at 63°C for *A. fumigatus* and 15s at 60°C for *C. albicans* and 10s of primer extension at 72°C and a final extension step of 72°C for 10 min. The presence of specific DNA amplicon was verified in 1% agarose gels.

Optimization of the multiplex PCR assay

The multiplex PCR mixture was optimized for primers, temperature and fast master mix volume. Each PCR reaction mixture (50 µl) contained 30 µl PyroStart™ Fast PCR Master Mix, 0.5 µl for each primer (100 pmol) with 6 µl of DNA extracted from pure fungal culture or 17 µl of DNA extracted from milk samples. The reaction profile was as previous in the individual PCR with 61°C annealing temperature that was a compromise between the annealing temperatures of the original individual PCR assays.

Milk samples collection for isolation of LAB

Colostrums were collected from goat and mare and milk samples

were collected from cow and buffalo-cow, in a separate sterile screw-cap bottles and kept under low temperature using an ice-cooled box to be brought to the laboratory where they kept in a refrigerator (around 4°C) till the time of use.

Isolation of lactic acid bacteria (Tserovska et al., 2002)

For better growth of bacteria, 1 ml samples were homogenized with 9 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1000 ml distilled water, pH 7.0). The homogenate was serially diluted and the appropriate dilutions were surface plated on MRS agar (De Man Rogosa and Sharp, 1960). Plates were then incubated at 30°C for 3 days under anaerobic conditions using anaerobic jars.

Individual isolates from De Man Rogosa Sharpe (MRS) agar plates were randomly-picked, representatives from all morphologically distinct colonies and were sub-cultured and purified 5 times on the appropriate agar medium. Pure strains, as judged by microscopic observations for homogeneity of cellular morphology and were maintained in MRS slants at -20°C. Bacterial isolates were further tested for gram reaction, catalase production, oxidase activity and cell morphology. Isolates of the gram-positive, catalase-negative, rods, occurring singly, in pairs or in chains and grown under anaerobic incubation, were randomly selected as presumptive LAB. All isolates were overnight cultured in MRS broth and tested individually for the identification of the bacterial strains. The determination of the strains was performed according to their morphological, cultural, and biochemical characteristics by the procedures described in the Bergey's Manual (Boone et al., 1984) for characterization of carbohydrate fermentation pattern of the LAB isolates.

In vitro antimycotic activity of probiotic bacteria against *C. albicans* and *Aspergillus* species using well diffusion assay (Sgouras et al., 2004)

The twenty four *C. albicans* and seven *A. fumigatus* isolates were tested for their sensitivity toward different probiotic strains from different sources (Table 3) as follows; *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium* and *Bacillus subtilis*. The *In vitro* antimycotic activity of the tested probiotic strains was carried out using agar well diffusion test as follows; Mueller-Hinton agar plates supplemented with 2% glucose and 0.5 µg/ml methylene blue dye medium (Bansod and Rai, 2008) were prepared and wells were drilled out using Rosseta. The plates were inoculated with fungal cultured isolates prepared in concentration equivalent with 0.5 McFarland tube 1.5×10^5 CFU/ml. and streaked onto the agar plates using sterile swabs, then 50 µl aliquots of cell free cultures supernatant in fresh De Man Rogosa Sharpe (MRS) broth of the probiotic strains were suspended in the agar wells after centrifugation 3000 rpm for cells precipitation. The plate was sealed with a parafilm immediately after adding. Plates

Table 2. Percentage of Mycotic infection detected by culture and PCR methods in milk of cattle and buffaloes with clinical mastitis.

	<i>A. fumigatus</i>		<i>C. albicans</i>		Total	
	Milk culture	Milk PCR	Milk culture	Milk PCR	Milk culture	Milk PCR
Cattle (71)	5 (7.04)	5 (7.04)	12 (16.90)	12 (16.90)	17 (23.94)	17 (23.94)
Buffaloes (52)	2 (3.84)	4 (7.69)	12 (23.07)	16 (30.76)	14 (26.92)	20 (38.46)
Total (123)	7(5.69)	9(7.31)	24 (19.51)	28(22.76)	31 (25.20)	37 (30.08)

Two buffalo's milk samples (3.84%) were positive for both *A. fumigatus* and *C. albicans* using both culture and PCR methods.

Table 3. The antimycotic activity of probiotic isolates against fungal isolates.

Probiotics	Source	<i>C. albicans</i> (24 isolates) (mm)	<i>A. fumigates</i> (7 isolates)
<i>L. acidophilus</i>	mare colostrum	13	12 mm
<i>L. acidophilus</i>	goat colostrum	15	13 mm
<i>L. acidophilus</i>	buffaloe –cow milk	12	11 mm
<i>L. plantarum</i>	cow milk	11	-ve
<i>L. rhamnosus</i>	cow milk	12	10 mm
Bifidobacterium	cow milk	10	9 mm
<i>B. subtilis</i>	Mare feaces	10	-ve

-ve: no zone of inhibition was shown around the well containing the probiotic strain.

were incubated at 28°C for 24 to 48 h and the diameters of inhibition zones around wells were measured in mm. The experiment was carried out in triplicate and the mean of the zone of inhibition was recorded as shown in Table 3.

RESULTS

Microbiological findings

For diagnosis of Mycotic mastitis, 123 collective milk samples from 71 cattle and 52 buffaloes suffering from clinical mastitis were subjected for mycological cultivation. The total positive percentage of Mycotic mastitis was 25.2% of all the examined samples.

From Table 2, *A. fumigatus* showed higher percentage in cattle (7.04%) than in buffaloes (3.84%) while *C. albicans* showed higher percentage in buffaloes (23.07%) than in cattle (16.90%). The total Mycotic mastitis percentage was higher in buffaloes (26.92%) than cattle (23.94%).

For Confirmation of the culture method, a multiplex PCR targeting both *A. fumigatus* and *C. albicans* was performed directly on the same milk samples as shown in Table 2. Using mPCR technique, it showed higher total infection percentage (30.08%) than culture method (25.2%). PCR and cultivation method results were identical in cattle, while in buffaloes, PCR succeeded to detect *A. fumigatus* (2 samples) and *C. albicans* (4 samples) in culturally negative milk samples.

Results revealed that *L. acidophilus* isolated from goat colostrums showed the best antifungal activities against

C. albicans followed by *A. fumigatus* with zone of inhibition equal 15 and 13 mm respectively. This was followed by *L. acidophilus* isolated from mare colostrums with antifungal activities against *C. albicans* followed by *A. fumigatus* with zone of inhibition equal 13 and 12 mm; while *L. plantarum* and *B. subtilis* have no effect against *A. fumigatus* as shown in Table 3 and Figures 2 and 3.

DISCUSSION

Mycotic mastitis existed in cattle and buffaloes before the discovery of antibiotics. However, since then there has been a sharp increasing number of cases reported (Lagneau et al., 1996). The reasons of this increase may be intensive and prolonged use of non specific antibiotics for the treatment of mastitis (Pérez et al., 1998; Crawshaw et al., 2005) or as a prophylaxis before parturition) inadequate milking hygiene or the contamination of syringes used for the intramammary treatments could be the source of infection (Jensen et al., 1996).

Mycotic mastitis in cattle is relatively common and is rarely diagnosed before laboratory diagnosis. Its low frequency of diagnosis is attributable to its bizarre clinical presentation and uncharacteristic findings. Hence it is often forgotten in the differential diagnosis of bovine mastitis (Morcos et al., 1990).

The percentage of bovine mycotic mastitis in surveys carried out in many countries varies considerably between 1 to 25%, with 1.3% rates reported in South Korea (Yeo and Choi, 1982), 1.3% in Denmark (Aalbaek

et al., 1994), 9.6% in Poland (Krukowski et al., 2000), 2 to 7% in central, northern Europe and in the USA (Kirk and Bartlett, 1986; Aalbaek et al., 1994). In tropical countries, the percentage can be higher such as in Brazil (12.07 to 25.4.3%) as reported by Costa et al. (1993) and Dos Santos and Marin (2005).

Here, in the present study (Table 2), the percentage of the bovine mycotic mastitis in two governorates of Egypt was high (25.2%) in comparison with previous reports in Egypt (1.1, 3, 6.13 and 20%) as reported by El-Kholy and Hosein, (1990), Farid et al. (1975), Awad et al. (1980) and Abd El-Halim, (1979) respectively. While it was lower than that of Morcos et al. (1990) who reported an infection incidence of 32%.

Regarding the main causes of mycotic mastitis, yeast infection due to *C. albicans* (19.51%) was higher by about 4 times than that of *A. fumigatus* infection (5.69%). This was in agreement with that of all the Egyptian authors mentioned previously and may be due to Yeast-associated mastitis is previously mentioned following unhygienic intramammary treatments as reported by Gaudie et al. (2009).

This high incidence rate may be due to the extensive and prolonged use of antibiotics nowadays in Egypt not only for treatment of mastitis but also as a prophylaxis during dry period.

As fungi and yeasts are ubiquitous in the environment, their presence here could be as a result of contamination but the growth of the isolated yeast and fungi at 37°C and above presents some evidence for pathogenicity. Further evidence was the presence of clinical mastitis in some of the herds with history of poor or no response to treatment with conventional antibiotics. This came in agreement with that of Farnsworth and Sorensen (1972) and Lagneau et al. (1996).

The conventional microbiological methods such as culture are time-consuming, lack sensitivity and specificity and prone to the appearance of false negatives (Kawazu et al., 2004). Besides, possibly hazardous cultures for the health of laboratory personal (Mirhendi et al., 2001). In all cases the growth is very difficult to interpret as truly etiological in the process (Jensen et al., 1996). Because of its ability to directly detect extremely small quantities of DNA with high level of sensitivity and specificity, PCR technology offers potentially earlier detection of infection and permits earlier treatment with antifungal therapy which finally improves survival and reduces morbidity (Holmes et al., 1994). It allows researchers to detect and identify fungal species in swabs, fluids and tissues (Pham et al., 2003; Shin et al., 2003; Scheuller et al., 2004; El-Razik et al., 2011), in food as contaminants (Zur et al., 2002; Millar et al., 2003), also in indoor air, water, soil and building surfaces (Haugland et al., 2004; Portnoy et al., 2004).

The most important step in the detection of yeasts and fungi using PCR is the ability to efficiently extract DNA from hyphae and or conidia. This step is even more

critical when attempting to detect small quantities of mycotic material in biological samples such as blood (Garcia et al., 2004), mucus, milk (El-Razik et al., 2007, 2008 and 2010) and tissues (Karakousis et al., 2006; El-Razik et al., 2011).

This is the first report about using PCR, specifically the multiplex one for the direct diagnosis of mycotic mastitis in milk of bovines in Egypt.

From Table 2 and Figure 1, with simple modification for the used DNA extraction kit, simple modifications of the multiplex PCR though increasing the fast PCR master mix volume and decreasing both primers concentrations and the using of an annealing temperature (61°C) that was a compromise between the annealing temperature of original individual PCR assays, the used multiplex PCR showed a high sensitivity and specificity in identifying both *A. fumigatus* and *C. albicans* from milk in one step. Additionally, the designed primer pairs did not cross-react with animal DNA and not affected by potential PCR inhibitory substances in milk.

The used multiplex PCR succeeded in detection of additional two *A. fumigatus* and four *C. albicans* isolates from milk samples that was negative in culture as shown in Table 2. This may explain that mycotic cultivation lack sensitivity (Mennink-Kersten et al., 2004) and prone to the appearance of false negatives, primarily due to growth failure, overgrowth by contaminating microorganisms (Garcia et al., 2008), problems originating in the availability of clinical samples and poor viability of fungal elements in clinical samples (Mirhendi et al., 2001).

The multiplex PCR-based identification method described here, using optimized PCR components in combination with a simple and fast DNA extraction protocol, could be useful for the unequivocal identification of both *A. fumigatus* and *C. albicans* in one step. Also, the identification of both pathogens using these specific primers would avoid cross reactivity with bacteria, other fungi or other yeast species.

Probiotic antifungal activities shown in Table 3 and Figures 2 and 3 revealed that *L. acidophilus* isolated from goat colostrums showed the best antifungal activities against *C. albicans* and *A. fumigatus* followed by *L. acidophilus* isolated from mare colostrums, then *L. acidophilus* isolated from buffalo-cow milk with zones of inhibition equal (15, 13), (13, 12) and (12,11) mm respectively. Results agree with that of Magnusson et al. (2003) who screened 1200 LAB for antifungal activity against *A. fumigatus* and showed that 4% have strong inhibition and added that *L. acidophilus* LMG 9433, inhibition zones were observed against several fungi. These results agreed with that of Plockova´ et al. (1997a, b).

Our results proved that *L. rhamnosus* and *Bifidobacterium* isolated from cow milk showed antifungal activities against *C.albicans* and *A.fumigatus* with zone of inhibition equal (12, 10) and (10, 9) mm respectively.

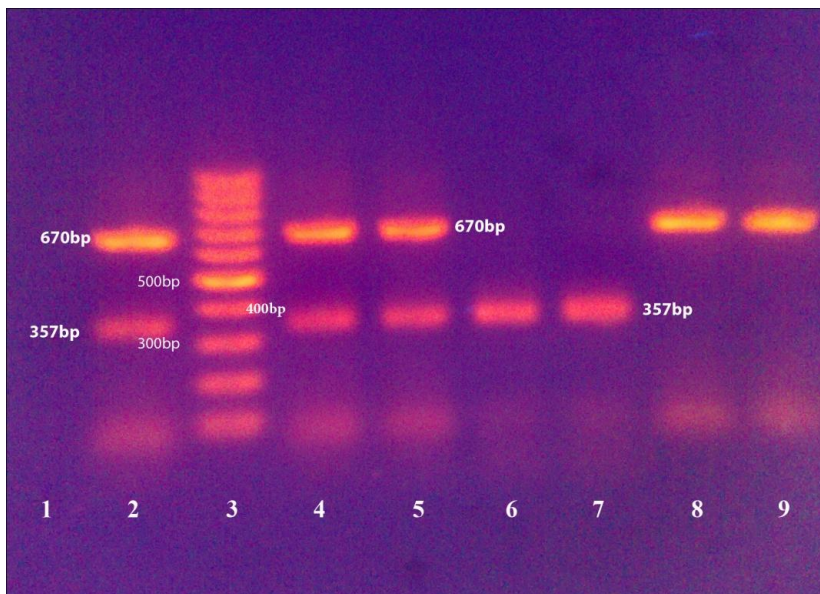


Figure 1. Multiplex PCR of *A. fumigatus* and *C. albicans* DNA in bovine milk. Lane1, negative control; Lane 2, shows PCR products (357 and 670bp) from a mixture of *A. fumigatus* and *C. albicans* DNA respectively; Lane 3, 100 bp ladder (Fermentas); Lanes 4 to 9, amplicon of *A. fumigatus* and or *C. albicans* DNA in bovine milk samples.



Figure 2. Zone of inhibition of different probiotics showing fungicidal activity of *L. acidophilus* (mare colostrums, goat colostrums, buffalo –cow milk), *L. palantarum* (cow milk), *L. rhamnosus* (cow milk), *Bifidobacterium* (cow milk) and *B. suibtlus* (mare fecal sample) in sequence with the arrow show in *C. albicans*.



Figure 3. Zone of inhibition of different probiotics showing fungicidal activity of *L. acidophilus* (mare colostrums, goat colostrums, buffalo –cow milk), *L. palantarum* (cow milk), *L. rhamnosus* (cow milk), *Bifidobacterium* (cow milk) and *B. suibtlus* (mare fecal sample) in sequence with the arrow show in *A. fumigatus*.

Falagas et al. (2006) proved the efficacy of using *L. acidophilus*, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 in reducing the vaginal candidiasis colonization.

Romeo et al. (2011) found that the use of *Lactobacillus reuteri* and *L. rhamnosus* probiotics seems to be effective in the prevention of gastrointestinal colonization by *Candida*. Also, Lakhtin et al. (2010) used preparations of

probiotic bifidobacterial and lactobacillus lectins which possess fungistatic and fungicidal activities against nystatin-resistant *C. albicans* strains.

In this study, *L. plantarum* and *B. subtilis* showed hindrance activity against *C. albicans* with zone of inhibition (11 and 10 mm). Also Feio et al. (2004) proved that *B. subtilis* inhibited the growth of several fungi. Magnusson et al., (2003) found that *Lactobacillus salivarius*, *L. plantarum* and *Pediococcus pentosaceus* strains inhibited some fungi. *L. acidophilus* indicated that the antifungal effect of these lactic acid bacteria could not simply be assigned to the low pH, but most probably to the formation and secretion of antifungal organic metabolites. Furthermore, it has been suggested that some lactic acid bacteria produce a wide spectrum of compounds that might act synergistically towards filamentous fungi and yeasts (Magnusson et al., 2003).

Conclusion

Mycotic mastitis will become an increasing problem due to the wide use of antibiotics in mastitis therapy. Therefore, a correct handling of syringes and strict hygienic measures should be considered in the intramammary injection of antibiotics, otherwise severe cases of mastitis may occur, beside the public health hazard humans. Effort should be encouraged to apply substitutes of antimicrobials such as probiotics and bioactive natural compounds for prophylactic and therapeutic use. Our study emphasizes the use of new antifungal agents including probiotic bacteria that provides effective mechanisms for the prophylaxis and therapy of this serious disease in animals. Multiplex PCR proved to be a simple, sensitive and specific test for the direct diagnosis of bovine mycotic mastitis.

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