ANTI-PATHOGENIC *Candida* Spp. Activity DETERMINATION VIA *Lactobacillus* Spp. ISOLATION AND IDENTIFICATIONS USING CONVENTIONAL AND MOLECULAR METHODS

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ABSTRACT

Two Hundred and fifty samples of cow's milk from different parts of the province of Basrah were collected from clinical and subclinical mastitis reported using the California mastitis test between March 2018 and September 2019 and examined using conventional PCR assay, Candida species was identified in 116/250 (46.4%). Based on conventional method and ID - Yst card system Vitek 2*, Candida albicans* was the predominant 60/116 (51.7%), followed by *Candida parapsilosis* 15/116 (12.9%). Concerning the results of PCR amplification of 18S rRNA gene for identification of *C. albicans* and *C. parapsilosis*, this gene was present in 60 samples in *C. albicans*, and in 15 of *C. parapsilosis*. *Lactobacillus* are an industrially important group of probiotic organisms that play an important function in human health through inhibiting dangerous and pathogenic bacteria growth, boosting immune function, and increasing resistance to infection. Ten out of 250(4%) Lactobacillus isolates were obtained from apparently healthy cow milk samples. *Lactobacillus* isolates were identified according to phenotypic characterization and molecular technique using PCR (16S rRNA) and sequencing, it was seen that *L. acidophilus* formed 5 isolates (50%), *L.amylovorus* was three (30%), while

L.crisaptus formed only two (20%) only. The results of this study revealed that the BLAST analysis at the NCBI gene bank gave 99.39% homology with *L. acidophilus*, 99.19% homology with *L.crispatus* and 97.59% with *L. amylovorus*. *In vitro* antimycotic activity of probiotic bacteria (Lactobacillus) against *C. albicans* and *C. parapsilosis* using agar well diffusion methods was adapted. The cell-free neutralized supernatant (CFS) of Lactobacilli (10^5,10^6,10^7) were inhibited the growth of pathogenic *C.albicans and C. parapsilosis*. It was also noticed that, *L. acidophilus* showed the strongest antifungal activities against pathogenic *C. albicans* and *C.parapsilosis* with different degrees of inhibition zones in comparsion with each of *L.crispatus* and *L. amylovorus*, meanwhile, *L. amylovorus* revealed strongest antifungal activity against pathogenic *C.parapsilosis.*

INTRODUCTION

Bovine mastitis is a disease prompted with the aid of a wide range of microorganisms that causes large economical loses and damages to the dairy industry via lowering milk production and via increasing costs of antibiotic treatment and culling (1). The incidence of mycotic mastitis is on the rise and the most often isolated fungi from milk are *Cryptococcus neoformans* and *Candida albicans* due to extended and indiscriminate use of antibiotics and steroids in intramammary therapy for mastitis as nicely as the occurrence of fungal organisms on dairy farms (2). The early, fast and correct identification of the pathogenic fungus is integral for timely, excellent management. The traditional identification of pathogenic fungi in the clinical microbiology laboratory is primarily based on morphological and physiological tests frequently require three or extra days and can also be inaccurate in latest years a multiplex PCR technique was once developed to identify concurrently multiple fungal pathogens in a single reaction (3).

Lactic acid bacteria (LAB) are an industrially important group of bacteria and used as starter cultures for the production of fermented milk products (yoghurt and some cheeses) in the dairy industry. Natural habitats, which consist of the indigenous flora of raw milk, can be an exact source of novel LAB strains with the manageable proper properties for use raw milk are from inside the udder, the exterior of the teats and the udder, the milking machine, the storage equipment, the housing, bedding, feed, air and water (4). Lactic acid bacteria (Lactobacillus) are among the most

powerful prokaryotes when it comes to antimicrobial potential. A large extent of LAB traces have effective antimycotic the pathogens with the aid of depleting vitamins consumed by the pathogens and modulate the host immune response. In addition, they release endogenous microbicides compounds including; lactic acid, bacitracin and hydrogen peroxide which have microbicidal effect (5). Recently the utility of live microorganism as potential therapeutic towards mastitis has gained interest (6).

The study aims to isolate and identify *Candida spp* that cause mycotic mastitis in cows by using conventional and molecular techniques, identification of *Lactobacillus spp* by molecular methods isolated from apparently healthy cow's milk and finally evaluate the anti-Candida activity by the above isolated and diagnosed Lactobacilli.

MATERIALS AND METHODS

1- Samples collection: Two hundred and fifty samples of bovine milk with mastitis according to California mastitis test from different areas of Basrah province, had collected in the course of the period from March 2018 up to September 2019. The udder, teats orifices and milkers hands had been perfectly cleaned with tap water and soap and disinfected with 70% ethyl alcohol before collecting milk samples. Two to five ml of milk have been collected in clean sterile tubes after discarding the first streams of milk to keep away from contamination, samples labeled with serial numbers and saved at 4^{o}C in a cold box for the duration of transportation to the laboratory to run the experiment directly (7).

2- Isolation and identification of *Candida Spp*

Milk samples have inoculated in Sabouraud's Dextrose agar supplemented with 0.05 mg/ml chloramphenicol and then incubated at 37^{o}C for 24h up to 1 week. Macroscopic and microscopic morphology tests have been performed after incubation in order to classify the genus level (8). Commercially available ID –YST card device (Vitek 2 system, Bio Mèrieux, France for pathogenic yeast) was carried out in a range of setting phenotyping identification tests for Candida species (9). Molecular identification of *Candida spp*. in this study, with the aid of the use of conventional PCR for the amplification of a partial gene of 18S rRNA by way of specific primer sequences was used.

3- Molecular characterization: Yeast genomic DNA from milk samples were extracted by using G-spin DNA extraction kit according to the producer protocol. Candida culture as 200 µl was pipetted into 1.5 ml sterile microcentrifuge tubes then 50 mg glass beads with 20 µl of proteinase K have been added and homogenized by cell disruptor vortex mixer for 5 min. Thereafter 200 µl of BL cell lysis buffer was added to every tube and mixed by way of vortex mixer, all tubes have been incubated for 56°C for 10 min with mixing every 3 min, 200 µl of absolute ethanol was added to lysate and right now blended by using shaking vigorously. Spin column placed in a 2 ml collection tube and the mixture was transferred (including any precipitate) to the column, then centrifuged at 13,000 rpm for 1 min. The filtrate used to be discarded and the spin column used to be placed in a new 2 ml collection tube, 400 µl W1 buffer was added to the spin column and centrifuged for 1 min at 13,000 rpm, the flow-through was discarded and re-used the collection tube. Six hundred µl wash buffer was added to every column then centrifuged for 1 min at 13,000 rpm, the flow-through was once discarded, the column was placed into a new 2 ml collection tube, then once more all the tubes were centrifuged for 1 min at 13,000 rpm to dry the column membrane. The spin column was placed into a new 1.5 ml tube and 50 µl of the buffer CE was added directly onto the membrane and incubated for 1 min at room temperature then centrifuged for 1 min at 13,000 rpm. Then all tubes left to stand for 1 min to ensure the elution buffer was absorbed by the matrix, and then centrifuged at 10000 rpm for 30 sec to elute the purified DNA. Nano Drop spectrophotometer was used to check the concentration of the extracted DNA in accordance to the formula: 1OD260=50ng, purity= 260/280. Oligonucleotide primers (forward and reverse) were designed for *C. albicans* and *Candida parapsilosis* genes such as 18S rRNA, table (1).

Table (1): Oligonucleotide primers used for 18S rRNA gene in *Candida albicans* and *Candida parapsilosis*.

Species	Gene	Oligonucleotide Primer sequence (3´-5´) Forward & reverse	Amplicon (bp)	Genbank Accession number
C.albicans	18S rRNA	F=GCCGCCAGAGGTCTAAACTT R=AGTTCAGCGGGTAGTCCTAC	415	AB365317
C.parapsilosis	18S rRNA	F=CTGCGGAAGGATCATTACAGA R=TCCTCCGCTTATTGATATGCTT	507	FM172980

4- PCR amplification: PCR master mix reaction was prepared by using (Maxime PCR premix kit i-Taq protocol). The master mix used to be organized in accordance to the company instruction by adding three µl of template DNA, 1 µl of forward and reverse primers (10 pmol), the volume was completed to 20 µl of nuclease-free water. PCR machine was set up as mentioned in (table 2) for 30 cycles. PCR products have been run on agarose gel electrophoresis for 1 hour (100V), DNA bands had been visualized by using a gel documentation system and photographed.

Table (2): PCR program setting for *Candida albicans* and *Candida parapsilosis*

No	Step	*C. albicans*	*C. parapsilosis*	Time	
1	Initial denaturation	95°C	95°C	2 min	
2	Denaturation	95°C	95°C	30 sec	30 cycles
3	Annealing	59.3°C	57.0°C	30 sec	
4	Extension	72°C	72°C	50-60 sec	
5	Final Extension	72°C		5 min	
6	Hold	4°C		10 min	

5- Isolation of *Lactobacillus:* Two hundred and fifty milk samples were collected from apparently healthy cow's from different areas of Basrah province. For isolation of Lactic acid bacteria, 1 ml of milk sample was homogenized with 9 ml sterile distilled water for about 1-3 min aseptically. Appropriate serial dilution (10^{-1} to 10^{-6}) was prepared for every sample. A volume of 1ml of appropriate dilution used to be spread on De Man, Rogosa and Sharpe agar (MRS) agar and incubated at 37°C under

anaerobic conditions using a candle extinction jar with a moistened filter paper to provide CO^2, the enriched water-vapour saturated atmosphere at 37°C for 48 h. Well, isolated colonies with typical characteristics particularly pure white small (2-3 mm diameter) with entire margin were picked up and purified by streaking two or three times on a fresh MRS agar plate accompanied through macroscopic and microscopic examination (10, 11).

6- Identification of Lactobacillus

Identification of Lactobacilli was performed according to their morphological, cultural and biochemical characteristics, it is Gram-positive ranging from rods to long slender bacilli, Catalase-negative (12).

7- Confirmation of Lactobacillus bacteria by PCR assay

A- DNA extraction: Genomic DNA of Lactobacillus (probiotic) bacteria isolates was extracted by using FavoPrep Genomic DNA Mini bacteria kit, and done according to the company instructions. One ml of the bacterial broth was taken to a sterile 1.5 ml microcentrifuge tube, 200 µl lysis buffer (20 mg lysozyme) and 100 mg cell disruptor glass beads were added and mixed by disruptor homogenizer vortex for 2 min. then, 200 µl TG1 buffer was added to each tube. The cell pellets was re-suspended by shaking vigorously with vortex mixer, and incubated at room temperature for 10 min, all tubes were inverted every 3 min through incubation periods, 200 µl of TG2 buffer was added to each tube and mixed by shaking vigorously for 5 sec, all tubes were incubated at 60ºC for 10 min and inverted every 3 min through incubation period. Two hundred µl absolute ethanol was added to the lysate and immediately mixed by shaking vigorously. A TG Mini column was placed in a collection tube. The mixture (including any precipitate) was transferred to the TG column, then centrifuged at 18,000 for 1 min, the TG column was placed to a new collection tube. Four hundred µl W1 buffer was added to the TG column, then centrifuged at 10000 rpm for 30 sec. The flow- through was discarded and the column back in the 2 ml collection tube. Seven hundred µl wash buffer was added to the TG column, then centrifuged at 10000 rpm for 30 sec. The flow- through was discarded and then column back in the 2 ml collection tube. Dried TG column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of pre-heated elution buffer was added to the center of the column matrix. The tubes were let stand for at least 3 min.

To ensure the elution buffer was absorbed by the matrix, then centrifuged at 15,000 rpm for 30 sec, to elute the purified DNA. NanoDrop spectrophotometer was used to check the concentration of the extracted DNA according to the formula: 1OD260=50ng, purity=260/280.

B- Oligonucleotide primer sequences: Oligonucleotide primers were designed by NCBI- GenBank database and primer design online, and supported by (Macrogen, Korea) company, table (3).

Table (3): Conventional PCR primers used for the detection of *Lactobacillus Spp.*

Genes	Oligonucleotide primer sequences (5'-3'-)	Product Size (bp)	Genbank Accession number
16S rRNA gene *Lactobacillus spp.*	F= GAAGTATCCAGAGCAAGCGGA R= CTCGCAATTTCGCTTACGGG	550	AJ438156

C- Preparation of PCR Master Mix: PCR master mix reaction was prepared by using (Maxime PCR premix kit i-Taq protocol). The master mix was prepared according to the company instructions. PCR procedure was performed using the recommended thermal cycling conditions that outlined in table (4).

Table (4): PCR program setting for *Lactobacillus spp.*

Step	Temperature	Time	
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	Repeat steps 2-4 for 29 more times
Annealing	59.5°C	30 sec	
Extraction	72°C	50-60 sec	
Final extension	72°C	5 min	
Hold	7:4°C	10 min	

D- DNA sequencing and phylogenetic analysis of isolated Lactobacillus: The PCR product of 16S rRNA gene was sent to Macrogen Company in Korea for sequencing. Phylogenetic analysis was performed according to NCBI-Blast alignment

identification and unweighted pair group method with arithmetic mean (UPGMA Tree) in (MEGA 6.0 version).

8- *In vitro* antimycotic activity of probiotic bacteria (*Lactobacillus spp.)* against *C. albicans* and *C.parapsilosis* using agar well diffusion methods: The sixteen *C.albicans* and fifty *C.parapsilosis* isolates had examined for their sensitivity towards three species of already identified probiotic Lactobacillus.

Based on a unique CFU number used to be produced for probiotic (Lactobacillus) sterile brain heart infusion broth was placed as 9 ml in each tube. One ml of exponentially growing bacteria broth subculture (stock) was added to the first tube and blended well. From the first tube, one ml was once then added to a 2nd tube and so on to obtain a tenfold serial dilution series (10^{-1} to 10^{-7}) of exponentially growing bacteria probiotic (Lactobacillus). From every dilution, 1µl was inoculated onto MRSA and then incubated at 37ºC for 24h in the triplicate plate. The mean number of CFU used to be counted, until the obtaining of a suspension containing 10^5,10^6,10^7 cells expressed as CFU ml^{-1}. Each species of Lactobacillus was cultured in MRS agar of the following (10^5,10^6,10^7 cells expressed as CFU ml^{-1}) where incubated at 37 ^oC with 5% CO^2 for 18-24h, where recultured on MRS broth and was used as the broth culture bacteria (BCB). Cell-free supernatant (CFS) was obtained by centrifuging the culture after 24h of incubation at 10000 for 10 min. CFS for each Lactobacillus species was filtered by using Millipore filter (0,22 µ) which is essential to prevent further growth of bacterial cells, then neutralized to PH 7.0±0.2 by way of adding 1M NaOH. Concerning *C.albicans* and *C.parapsilosis* they had been inoculated on nutrient broth (NB) and incubated at 37^{o}C for 18-24h. Petri dishes containing 20 ml of nutrient agar were prepared, then inoculated with 100 µl of 24h broth culture of *C. albicans* and *C.parapsilosis* the usage of the spread plating method and left for 1h at room temperature, five wells have been cut via cork pore onto the agar of every plate. Each well was filled with 50µl of MRSB (control), culture broth of Lactobacillus, and neutralized CFS (10^5,10^6,10^7) in accordance to (13). Aliquots of fresh MRSB were used as controls for Candida and Lactobacillus species respectively, in accordance to (14). Agar plates have been incubated at 37^{o}C for 24 h. After incubation, the

diameter of the inhibition zones was measured (in mm) with vernier callipers. Isolates with clear zones less than 11 mm, 11-16 mm, 17-22 mm and more than 23 mm, were grouped as negative (-), mild (+), strong (++), and very strong (+++) (15).

RESULTS

1- Isolation and identification of *C. albicans* and *C. parapsilosis*

One hunderd and sixteen (46.4%) Candida isolates out of 250 were obtained from milk samples of cows with mastitis based on cultural, morphological and commercially available ID –YST card system (Vitek 2 system) the highest percentage among *Candida spp* was belong *C.albicans* which was 60/116 (51.7%) followed by *C. parapsilosis* 15/116 (12.9%). Molecular identification of *Candida spp* done by conventional PCR by amplification of a part of the mitochondrial gene encoding for large subunit of 18S rRNA gene by specific primer sequences. The yield of the detected *C. albicans* and *C. parapsilosis*, was 60/60 (100%) and 15/15 (100%), respectively, figure (1and 2).

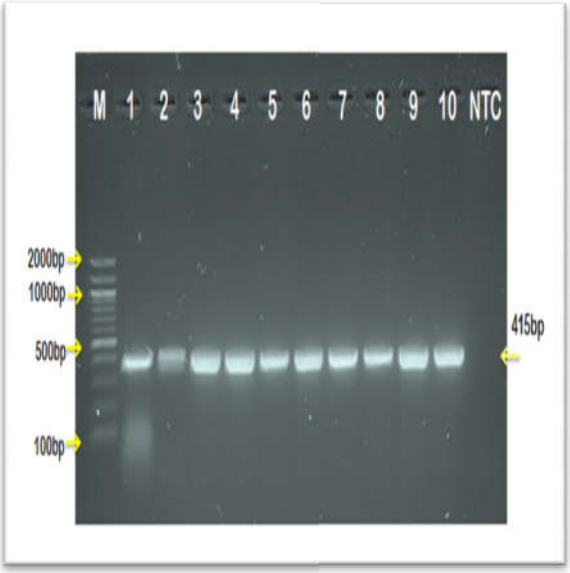


Figure (1): Agarose gel electrophoresis image shows the PCR product analysis of pathogenic *C. albicans*. Lanes M Marker ladder (2000 bp), lane (1-10): 18 SrRNA gene of *C. albicans* isolate with 415 bp. Lane (NTC): None template (negative control).

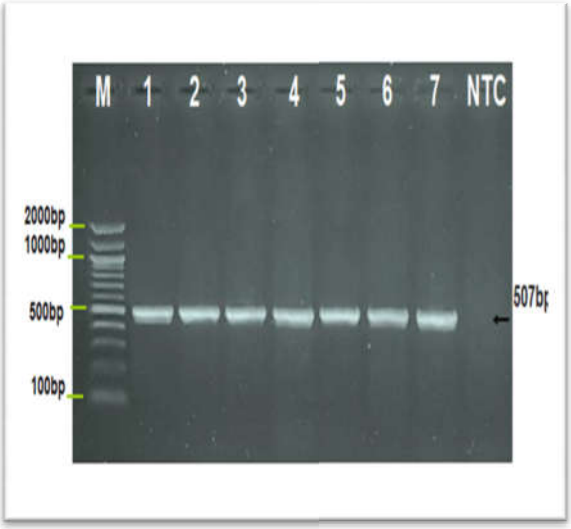


Figure (2): Agarose gel electrophoresis shows the PCR product analysis of pathogenic *C. parapsilosis*. Lanes M Marker ladder (2000 bp), lane (1-7): 18S rRNA gene of *C. parapsilosis* isolate with 507 bp. Lane (NTC): None template (negative control).

2. Isolation and identification of *Lactobacillus Spp.*

Ten Lactobacilli out of 250 (4%) milk samples were isolated from appearently healthy cows, were identified on the base of morphology Gram positive, catalase negative, long rods shaped bacilli, occurring singly or in chains, non-spore forming.

By molecular technique using 16S rRNA and sequencing, it was seen that *L. acidophilus* formed 5 isolates (50%), *L.amylovorus* was 3 (30%), while *L.crisaptus* formed only 2 (20%). The results of this study revealed that the BLAST analysis at the NCBI gene bank gave 99.39% homology with *L. acidophilus*, 99.19% homology with *L.crispatus* and 97.59% with *L. amylovorus*. The amplicons size was of 550 bp, figure (3) and table (5).

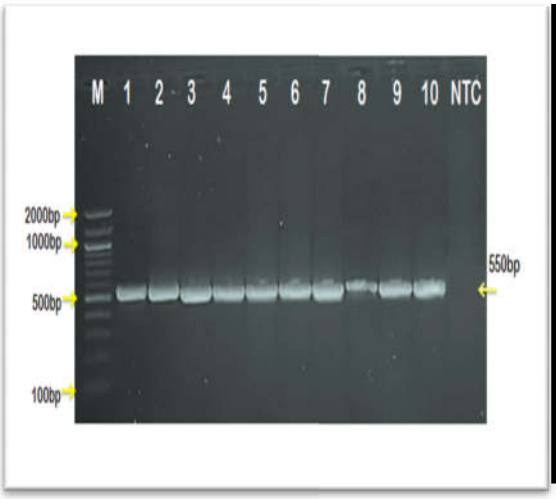


Figure (3): PCR product of 16S rRNA gene Lactobacillus spp.. On agarose gel electrophoresis. Lane 1: Molecular size marker (2000bp); Lanes 1-10: 16S rRNA gene *Lactobacillus spp*. with 550bp; Lane NTC (negative control).

Table(5): NCBI BLAST Homology sequence identity

Local Bacterial Isolates	**Genbank Accession Number**	**NCBI-BLAST Identical Isolates**	**NCBI BLAST Homology sequence identity**		
			Genbank Accession Number	**Country**	**Identify**
Lactobacillus spp. isolate No.1	MK863556	*Lactobacillus acidophilus*	MG827269.2	India	99.19
Lactobacillus spp. isolate No.2	MK863557	*Lactobacillus amylovorus*	MH819604.1	China	97.59
Lactobacillus spp. isolate No.3	MK863558	*Lactobacillus acidophilus*	MG827269.2	India	99.19
Lactobacillus spp. isolate No.4	MK863559	*Lactobacillus crispatus*	MH392998.1	China	99.39
Lactobacillus spp. isolate No.5	MK863560	*Lactobacillus acidophilus*	MG827269.2	India	99.39
Lactobacillus spp. isolate No.6	MK863561	*Lactobacillus acidophilus*	MG827269.2	India	99.17
Lactobacillus spp. isolate No.7	MK863562	*Lactobacillus crispatus*	MH392998.1	China	99.39
Lactobacillus spp. isolate No.8	MK863563	*Lactobacillus acidophilus*	MG827269.2	India	99.39
Lactobacillus sp isolate No.9	MK863564	*Lactobacillus amylovorus*	MH819604.1	China	97.81
Lactobacillus spp. isolate No.10	MK863565	*Lactobacillus amylovorus*	MH819604.1	China	97.59

3- Evaluation of antifungal activity by probiotic (Lactobacillus).

The cell-free neutralized supernatant (CFS) of Lactobacilli (10^5,10^6,10^7) were inhibited the growth of pathogenic *C.albicans* and *C.parapsilosis* by well diffusion method. It was also noticed that, *L. acidophilus* showed the strongest antifungal activities against pathogenic *C. albicans* and *C.parapsilosis* with different degrees of

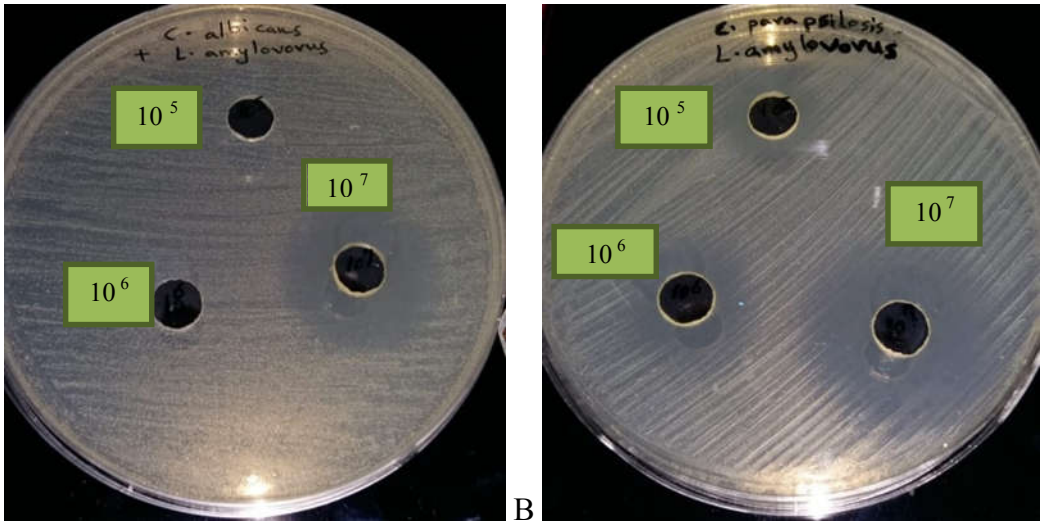
inhibition zones in comparsion with each of *L.crispatus* and *L. amylovorus*, meanwhile, *L. amylovorus* revealed strongest antifungal activity against pathogenic *C.parapsilosis*. Zone of inhibition of probiotic (Lactobacillus) against *C. albicans* was compared among 10^5, 10^6 and 10^7 concentrations. Regarding *L. acidophilus,* the best zone of inhibition was obtained at 10^6 concentration. *L.crispatus,* the best zone of inhibition was obtained at 10^7 concentration. While the best inhibition zone of *L. amylovorus* was obtained at 10^6 concentration, table (6). In case of *C.parapsilosis*, zone of inhibition of probiotic was compared among 10^5,10^6 and 10^7 concentrations. Regarding *L. acidophilus,* the best zone of inhibition was obtained at 10^7 concentration. In case of *L. crispatus,* the best zone of inhibition was obtained at 10^6 concentration. The best zone of inhibition was obtained at the concentration of 10^6 of *L. amylovorus*, table (7) and figure (4).

Table (6): Zones of inhibition for *Candida albicans* (N= 60) with a cell free supernatant of probiotic *Lactobacillus spp*.

Lactobacillus spp	**Concentration of probiotic (Lactobacillus)**			**LSD**	**T test/ p value**
	10^5	**10^6**	**10^7**		
L. acidophilus	21.92±0.72	33.57±2.12	25.92±1.97	2.75	-------
L.crispatus	-------	16.9±0.90	35.3±1.88	-------	8.805/0
L. amylovorus	-------	38±1.56	24.15±1.51	-------	6.030/0
$LSD_{0.05}$	-------	2.014	1.075		
T test	-------				

Table (7): Zones of inhibition for *Candida parapsilosis* (N=15) with a cell free supernatant of probiotic *Lactobacillus spp*.

Lactobacillus spp	**Concentration of probiotic (Lactobacillus)**			**LSD**	**T test/ p value**
	10^5	**10^6**	**10^7**		
L.acidophilus	-----	28.28±0.75	45±1	-----	22.2333/0 (S)
L.crispatus	19±1	38.57±0.92	------	-----	21.224/0 (S)
L.amylovorus	15±0.57	23.5±1.3	20±0.31	2.85	-----
LSD $_{0.05}$	------	1.08	------		
T test	3.754/0.032 (S)	------	33.408/0 (S)		



A B

Figure (4): The cell-free neutralized supernatant (CFS) of Lactobacilli (10^5,10^6,10^7) were inhibited the growth of pathogenic *C.albicans* and *C.parapsilosis* by well diffusion method, A- *C.albicans* + *L. amylovorus*, B- *C.parapsilosis* + *L. amylovorus.*

DISCUSSION

Mastitis is the most essential health hassle in bovine dairy herds. Multifarious microorganisms have been implicated as causative agents of bovine mastitis,

including bacteria and fungi (16). Fungal infections of the mammary gland are usually caused by yeast, the important genus of which is Candida (17).

A total of 116 (46.4%) Candida isolates out of 250 milk samples were collected from cows with mastitis based on cultural, morphological and commercially available ID –YST card system (Vitek 2 system Bio Mèrieux, France for pathogenic yeast) were carried out in various setting phenotyping identification tests for *Candida spp*. The highest percentage among isolated *Candida spp.* was belong *C.albicans* 60/116 (51.7%) followed by *C. parapsilosis* was 15/116 (12.9%). In the present study and regarding the percentage of *C. albicans* it is agree with this of (18), who isolated *C.albicans* from cattle suffering from mastitis with no response to therapy with common antibiotic in Egypt, the proportion was 24(60%). However it is not compatible with these results obtained by other studies done in Iraq (25%), Egypt (29.3%) and Nigeria (24.3%) (19, 20, 21). The discrpareies in the result of the current study with the others may come from the differences in the methods used for diagnosis, or due to the number of included samples in the studies are not compatible with each or due to the application hygienic of instructions different from area to another.

In the present study the percentage of *C.parapsilosis* was higher than those obtained in a study done in India were (7.69%) isolated from cows with clinical mastitis (22). The presence of *C. parapsilosis* were also incompatible with those reported in Poland which was (45%) and in Turkey as (12.7%) (17,23). The geographical variation may considered one of the reasons of discrepancy in distribution of species or due to the number of included samples or may due to the differences in the methods used for diagnosis.

Molecular identification of *Candida Spp.* in this study, done by conventional PCR by amplification of a part of the mitochondrial gene encoding for large subunit of 18S rRNA gene by specific primer sequences. The yield of the detected *C. albicans* and *C. parapsilosis*, was 60/60 (100%) and 15/15 (100%), respectively. The result concordant with those obtained Vitek 2 system. In the present study the percentage result of *C. albicans* was higher than these obtained in Egypt by (20) 8 (29.3%) who applied PCR using species specific primer of the 26S rRNA gene of *C.albicans* involved in dairy cattle mastitis. (23), applied rapid diagnostic tests and nested PCR method, and he obtained 46 (17.7%) positive samples for *Candida spp*

out of 260 mastitic milk samples by nested PCR technique 6, different species of Candida were identified, *C. tropicalis* was the predominant one (26.1%), followed by *C. parapsilosis* (21.7%), *C. kefyr* and *C. krusei* (17.4%) for each, *C. rugosa* (13%), and *C. glabrata* (4.4%). These differences in the proportion of each study may come from the difference in the primer used for PCR techniques, the discrepancy in the number of isolates enrolled in each study, and in the skills of laboratory investigators. PCR has increasingly used for Candida diagnosis, as it is quick, simple, specific, sensitive and reliable (24).

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (25). Some LAB species (Lactobacillus, Streptococcus, Enterococcus and Pediococcus) had reported as active candidates for probiotic use in humans and animals by several researchers (26). Selection of Lactobacilli as potential health-promoting probiotic in food and pharmaceutical preparations entails in vitro screening for certain criteria, which include antibiotic tolerance, bile tolerance, inhibiting the growth of other microorganisms and gastric juice which allow them to be established in the intestinal tract. Therefore the present study was undertaken with the objective of isolating and identifying Lactobacilli from fermented cow milk (nono) and in vitro determination of tolerance to antibiotic, bile and microbial inhibition (27).

Ten Lactobacilli out of 250 (4%) milk samples were isolated from appearently healthy cows, as Gram positive, catalase negative, long rods shaped bacilli, occurring singly or in chains, non-spore forming. By molecular technique using 16S rRNA and sequencing, it was seen that *L. acidophilus* formed 5 isolates (50%), *L.amylovorus* was three (30%), while *L.crisaptus* formed only two (20%). The results of this study revealed that the BLAST analysis at the NCBI gene bank gave 99.39% homology with *L. acidophilus*, 99.19% homology with *L.crispatus* and 97.59% with *L. amylovorus*.

The results of the proportion of Lactobacillus in the current study is lower than these obtained in France as (22.4%) in a study done by (28) who isolated this bacteria from bovine mammary, each isolate was identified by sequencing the 16S rRNA gene. In addition it is lower than the obtained in Iran (70%) which were isolated from local dairy products (29). These differences in the results among these different studies from the current ones mainly might come from the differences in the number

of samples enrolled, in addition to the hygienic condition, weather effect, intraspecific variation of the isolates.

The cell-free neutralized supernatant (CFS) of Lactobacilli (10^5,10^6,10^7) were inhibited the growth of pathogenic *C.albicans* and *C.parapsilosis* by well diffusion method. It was also noticed that, *L. acidophilus* showed the strongest antifungal activities against pathogenic *C. albicans* and *C.parapsilosis* with different degrees of inhibition zones in comparsion with each of *L.crispatus* and *L. amylovorus*, meanwhile, *L. amylovorus* revealed strongest antifungal activity against pathogenic *C.parapsilosis* .

Zone of inhibition of probiotic (Lactobacillus) against *C. albicans* was compared among 10^5, 10^6 and 10^7 concentrations. Regarding *L. acidophilus,* the best zone of inhibition was obtained at 10^6 concentration. *L.crispatus,* the best zone of inhibition was obtained at 10^7 concentration. While the best inhibition zone of *L. amylovorus* was obtained at 10^6 concentration.

In case of *C.parapsilosis*, zone of inhibition of probiotic was compared among 10^5,10^6 and 10^7 concentrations. Regarding *L. acidophilus,* the best zone of inhibition was obtained at 10^7 concentration. In case of *L. crispatus,* the best zone of inhibition was obtained at 10^6 concentration. The best zone of inhibition was obtained at the concentration of 10^6 of *L. amylovorus.*

Agar well diffusion method on SD agar plates after culture on MRS liquid medium was achieved of selected isolates of Lactobacillus and incubated for 2 days and cell-free culture supernatants had been aseptically separated (30). The test showed that 4 of the 40 isolates had been fantastic in terms of antagonism towards *C.albicans*, the anti- *C. albicans* exercise of cell-free culture supernatant was lost for the duration of prolonged storage. No activity could be recovered after storage for 1 day at -30^{o}C. The bad balance of anti - *C. albicans* activity may be to no longer only due to irreversible precipitation- denaturation process however additionally during accidental thawing of culture supernatant during the- freeze-drying process (31).

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