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In Vitro Study of Probiotic Inhibitory Effect against *C. Albicans*.

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ABSTRACT
Candida albicans is dangerous yeast pathogens that can cause severe health problem diseases.Resistance to antifungal one of the biggest problems faced in clinical practice Therefore, the most serious challenge in discovery and development of new antifungal or antimicrobial from a live microorganism and plants.
study was performed to evaluate the antifungal activity of Probiotic manufactures powder (ProD, ProL, ProI, ProV, ProM and ProP) and three isolates of *Lactobacillus (L. rhamnosus* (ProR), *L. fermenter* (ProF) and *L. acidophilus* (ProA), against 50 isolates of *C. albicans* by 2– stage agar overlay method in solid media and MMT assay method for two-fold supernatant. Results showed that the ProL showed highest activity against *C. albicans* isolates by 2–stage agar overlay method. While the Concentrated supernatant (two-fold) of probiotic isolates reduced significantly ($P < 0.05$) the viability (CFU/ml) of *C. albicans,*comparing to the control, and the highest antifungal activity by probiotic type ProM and ProL. while the lowest inhibitory effect of probiotics supernatant was present by ProA.The probiotic cell and concentrated supernatant (two-fold) of probiotic isolates reduced the viability (CFU/ml) of *C. albicans*, comparing to the control, in statistical analysis showed significant different ($P \leq 0.05$) between all probiotic cell and probiotic supernatant groups.
Key word:: *Candida albicans*, probiotic cell, probiotic supernatant.

1-INTRODUCTION

The yeast *Candida albicans* is the most frequent human pathogenic *Candida* species(1).*C. albicans* is still the major species related with oral lesions and vulvovaginitis(2) .

C. albicans is the fourth leading cause of nosocomial bloodstream infections(3), with an attributable mortality of 37–44% in severely immunocompromised patients(4,5).

Thus *C. albicans* is an opportunistic fungal pathogen commonly found in the human gastrointestinal and female lower genital tracts. It is a unique parasite capable of colonizing, infecting, and persisting on mucosal surfaces, and stimulating mucosal immune responses(6).

When the immune system is suppressed, this yeast can multiply rapidly, penetrate the intestinal lining and move into the blood stream, Yeast population is controlled by probiotic or "friendly" bacteria(7) .

The term Probiotic literally means "for life"(8) , is used to describe as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host"(9) .

Probiotics have been extensively studies due to their remarkable ability to inhibit the growth of other organisms through bactericidal activity and by producing lactic acid as product of metabolism. Lactic acid production, production of bacteriocins, and the production of hydrogen peroxide have led to an abundance of search involving the ability of probiotic to inhibit pathogens(10) .

Usually, for treatment of systemic fungal infections, azoles such as fluconazole are used, but one of the biggest problems faced in clinical practice is the emergence of resistance due to mutation for most of these azoles drugs currently used. Therefore, the most serious challenge in discovery and development of new antifungal or antimicrobial from a live microorganism and plants(11) . Ajah (12) showed that The *in vitro* study using the seed extracts of *Carica papaya* at concentrations 10%, 25% and 50% inhibited the growth of *C. albicans* with rate 10mm, 9mm, 8mm, respectively compatible with control(0%) 15mm.

Probiotics have several beneficial effects related to increasing digestion, strengthening the immune system and stimulating the production of vitamin. The use of probiotics is aimed to reduce the use of antibiotics(13) .

Due to the high recurrence of *Candida* lesions, and the increased resistance of conventional antifungal drugs in clinical practice, the continuous use of probiotics to prevent fungal infections may be an interesting strategy(14) . when bacterial flora is crucial for body function and overgrowth with pathogenic microorganisms

lead to illness. Thus the concept of supporting the human body's normal flora with live microorganisms conferring a beneficial health effect is important medical strategy(15) .

2-MATERIAL AND METHODS:

1. Preparation of probiotic

A. Probiotic manufactures powder

The manufactures probiotics used in the study listed in table (1):

Table (1): The manufactures probiotics used in the study

Probiotic	Total Microorganisms	Components of probiotic	Company (organ)
Gastro cell (ProG)	6×10^{12} cfu/capsule	***L. acidophilus, L. salivarius, L .bulgaricus*** *and* ***Bifidobacterium bifidum***.	NutraMed Inc. (USA)
Lactéol® fort (ProL)	1×10^{13} cfu/sachet	***L.delbruekii*** and ***L. fermentum.***	Rameda (Egypt)
Lactodrop (junior) (ProD)	0.55×10^{12} cfu/ drop	***L.rhamnosus, L. reuteri*** *and* ***Bifidobacterium lactis.***	Xenofarma (Iraq)
PRO-IBS (ProI)	4.5×10^{12}, 4.5×10^{12}, 1×10^{12} cfu/sachet	***L. reuteri*** ***L. rhamnosus*** ***Saccharomyces boulardii.***	Xenofarma (Iraq)
Protexin balance (ProM)	2×10^{8} cfu/capule	***L casei, L. rhamnosus, L. bulgaricus, L. acidophilus, Bifidobacterium longum*** *and* ***streptococcus thermophiles.***	Protexin (UK)
Vitalactic B™ (ProV)	2×10^{12} cfu/capsule	***L. plantarum*** *and* ***L. acidophilus***.	Vitane pharma (Germany)

The Probiotic manufactures powder (ProD, ProL, ProI, ProV, ProM and ProP) inoculated in MRS broth at 37°C for 24 hr**s.** in anaerobic condition.

B. *Lactobacillus* isolates

Three isolates of *Lactobacillus* (*L. rhamnosus, L. fermenter* and *L. acidophilus*) were obtained from Dr. Jehan abd alsatar, Department of biology of the Sciences College /Al-mustansyriah university. This isolates inoculated in MRS broth at 37°C for 24 hrs. in anaerobic condition.

2. Preparation of yeast inoculum

Candida albicans isolates inoculated in SDA at 37°C for 24 hr., then *C. albicans* colonies were suspended in 5ml of 0.85% normal saline, suspension was mixed for 15 second with a vortex, and then its concentration was adjusted

to1.5x10 ^{8} CFU/ml based on a standard 0.5 McFarland(16) .

3. Inhibitory effect of probiotic against *C. albicans* by 2- stage agar overlay.

Method described byAjah, (17) was followed to detect of Probiotics inhibition activity by spreading 0.1 ml of probiotics (that prepared in 1) on the MRS agar and incubation at 37°C for 24 hr. in anaerobic condition. After incubation time, sterile filter paper (Whottman no. 2) put on probiotic growth and flow second layer, about 15 ml, from SDA. After agar solidify, 0.1 ml of *candida* suspension (2) spreading over SDA. Plates were incubated aerobically at 37°C for 24 hrs., after incubation period, the colony counting manually or by using colony counter.

4. Cell Proliferation Assay Kit

4-1 Principle of MMT assay:

The viability of *Candida albicans* was measured by using MTT [3-(4, 5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay, is a colorimetric assay system, which measures the mitochondrial enzyme reduction of MTT into an insoluble purple formazan product by the viable cells .

4-2 Kit Contents:

• 10 ml from MTT solution (1ml x 10 vials).

• 100 ml from solubilization solution (50 ml x 2 bottle).

4-3 *In vitro* assay

A. *Candida albicans* isolates

Ten most virulent isolates of *Candida albicans* (Can7, Can69, Can76, Can20, Can77, Can22, Can67, Can37, Can88, and Can84) choose for this study accordant to virulence factor testes. All isolates inoculated in 10 ml of SDB at 37°C for 24 hrs., then adjusted number of yeast to 1.5×10^{8} cell /ml by using haemocytometer slide.

B. Preparation of probiotic supernatant

The four types of different number of strains probiotics overnight cultures contained 1.5×10^{8} CFU/ml were grown in MRS broth at 37°C for 24 hrs. under anaerobic conditions. These cultures were centrifuged at 6000 rpm/min for 10 min at 4°C. The resulting supernatants were filtered through a 0.22 µm membrane filter to remove the remaining bacteria and debris, and then supernatants concentrated to two fold(18) .

The four types of probiotics (ProM, ProD, ProL and ProA) that concentrated 2 fold choose for this study accordant to antagonistic activity of probiotic against *C. albicans* testes.

Added 1 ml, from each isolates of *Candida albicans*, in 150 test tubs which divided into 50 groups each group had 3repeater for treatment and for control, added 1ml from each 2fold supernatant probiotic (ProA, ProD, ProL and ProM) on treatment group tube and incubation at 37 °C in 24 hrs. and zero time, the control group, for each *Candida albicans* isolates, without addition supernatant probiotic .The number of viability of *Candida albicans* was calculated by MTT cell proliferation assay.

4-4 Procedure of MTT Kit

1. After treatment, put 200 µl per well of microplates 96 wells flat bottom from each treatment group and control group.

2. Added 10 µl of the MTT solution to each well. Incubate the microplate for 4 hrs. in a CO2 incubator (e.g. 26 °C, 5% CO2).

3. Carefully remove media. Do not disturb cells and do not rinse with PBS.
4. Added 100 µl of the Solubilization solution into each well.
5. Checked for complete solubilization of the purple formazan crystals then measured the absorbance of the samples by using a microplate (ELISA) reader, at wavelength was 600-570 nm.
To measure the number yeast for drawing stander curve to use in measured the viability of yeast by insertion in a standard curve, Different number of yeast were prepared (1.27×10^{7}, 3.82×10^{7}, 6.36×10^{7}, 9.54×10^{7}, $12.08 \times 10^{7}$and 14.63×10^{7}) cell /ml by using haemocytometer slide. Put 200 µl per well of microplates 96 wells, and the number of viability of *Candida albicans* was calculated by MTT cell proliferation assay.

5. Statistical analysis

Minitab software version 6 was used data analyzing. The ANOVA - test has been done to calculate the P value between the control and test groups in the previous studies. Least significant difference- LSD test was done also to compare means between groups in this study. The results were presented as mean ± SD. A P value equal or less than 0.05 was considered as the level of statistical significance.

3-RESULTS AND DISCUSSION

1. Inhibitory effect of probiotics against *C. albicans* by 2-stage agar overlay

The ability of probiotics (ProP, ProL, ProG, ProV, ProM, ProD, *lactobacillus rhamnosus* (ProR), *lactobacillus fermenter* (ProF) and *lactobacillus acidophilus* (ProA) to inhibit 50 isolates of *C. albicans* were separately test on MRS agar and SDA agar.

The result in table (2) showed a variation among these probiotics in their effect. There was a high (good) inhibitory effect which presented in no growth *C. albicans* by probiotic type ProL and followed by probiotics type ProG, ProV, ProA, ProR, ProF and ProD respectively, while low (simple) effect with probiotic type ProM, and some isolated had no inhibitory effect or less than 5% by type ProI. The results of statistical analysis showed significant ($P \leq 0.05$) for all probiotics type excepted ProI was not significant.

Table (2): Effectiveness of Probiotic against *C. albicans* by 2stage agar overlay.

Culture filtrate	**ProM**	**ProF**	**ProR**	**ProG**	**ProI**	**ProD**	**ProV**	**ProL**	**ProA**
Control Mean×10^4±SD	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34	14.04 ± 5.34
Test Mean×10^4±SD	6.94 ± 4.04 e	2.25 ± 1.96 c	2.35 ± 1.77 C	0.84 ± 0.71 B	17.73 ± 13.49 f	4.35 ± 2.21 D	0.95 ± 0.12 b	0.21 ± 0.05 A	2.59 ± 1.3 c
p-value	0.04	0.02	0.02	0.001	1.47	0.03	0.001	0.000	0.02

These results close near with result of Tang *et al*., (19) that found in clinical studies of candidiasis showed oral probiotics could enhance the clearance of *C. albicans* from intestine and vagina patients. That also agree with Saha *et al*., (20) that investigates the probiotic inhibition growth of *C. albicans.*

Ogunshe *et al*., (21) indicated that vaginal *L. acidophilus* and *L. plantarum* have inhibitory effect between 57.1% and 68% of *candida* species in vitro. When they study the in vitro antagonistic activities of vaginal *L. acidophilus* and *L. plantarum* against *Candida spp.*

Also several clinical trials have been performed to investigate the effects of specific strains, mainly with probiotics such as *L. acidophilus*, *L. rhamnosus* and *L. fermentum* species as a protective agent against both bacterial vaginosis and candida vaginitis owing to its ability to adhere to vaginal and cervical cells and to antagonist the growth of vaginosis-associated pathogens(15) .

The result of our study partially agreement with the result of local study done byHabib and Farhan, (22) that found *L.* plantarum given higher inhibition effects against *C. albicans*

followed by *L. acidophillus* and *L. fermentum* than *L. casei.*

Multicellular lactobacilli strain aggregates are believed to be crucial for the colonization of mucosal surfaces such as those of the oral and urogenital cavities. The aggregation properties of lactobacilli are divided into auto-aggregation, which is demonstrated by the formation of a clump (aggregate) of the lactobacilli strain only, and co-aggregation, which is characterized by formation of aggregates between lactobacilli and other genetically distinct cells such as bacterial or fungal pathogens. The ability to adhere on the mucosal surface of epithelial cells is regarded as one of the most important criteria for probiotic selection(23), that result had similarity with our study result.

2. Inhibitory effect of probiotics supernatant against *C. albicans* by MTT:

Four probiotics supernatant chosen in this experiment ProL, ProD, ProM and ProA for study inhibitory effects against (10 most virulence isolated) of *C. albicans* in MTT assay.

The mechanisms of action behind the observed inhibition need to be investigated further. *In vivo*, several mechanisms may be involved in a successful probiotic inhibition of pathogens, namely: 1) inhibition of pathogenic molecules by production of substances (such as organic acids, hydrogen peroxide, and bacteriocins) 2) inhibition of exo-polysaccharide (EPS) secretion, responsible for accelerated growth of opportunistic organisms 3) blocking of pathogen adhesion sites 4) pH modulation 5) nutrient competition and 6) Probiotic strains activate mucosal immunity and stimulate cytokine production, IgA secretion, phagocytosis, and production of substances (21,24).

The results of the current study showed a clear effect of ProM and ProL probiotics that had biggest inhibitory effects compared with other probiotics type used in reducing the growth or concentration of cells for all *C. albicans*, followed by ProD, and ProA respectively as in table (3). The results of statistical analysis showed significant ($P \leq 0.05$) between all probiotics supernatant type and control.

Table (3): Antagonist effective of probiotics supernatant against *C. albicans* by MTT assay.

Type	**Control** Mean×10^7± SD	**ProM** Mean×10^7± SD	**ProA** Mean×10^7± SD	**ProL** Mean×10^7± SD	**ProD** Mean×10^7± SD	P value
Test zero time	3.72±0.26	3.72±0.26	3.72±0.26	3.72±0.26	3.72±0.26	(NS)
After 24 hr	7.4±1.8	0.86±0.6	2.19±1.6	1.11±0.37	1.82±0.5	0.001
						LSD 0.46
P value	NS))	0.001	0.001	0.001	0.001	

Different letters mean significant difference ($P \leq 0.05$) between all probiotics supernatant types and control.

This results partially agree with the results done by Shino *et al*., (25) that show probiotics have inhibitory effectiveness against *C. albicans*. Our study result has related with local study result done by Abood, (11) that found *Lactobacillus* supernatant probiotics can reduce number of pathogenic *C. albicans*. Ajah(26) showed that the probiotics and Seaweed extract(*Ascophyllum nodosum*) possesses significant *in vitro* and *in vivo* antifungal activity again *Candida albicans* and therefor it could be used in the treatment of *C.albicans* infection .

Another study done by Dasari *et al.*, (27) showed ability of probiotics to producer of antimicrobial compounds, that have able to inhibit the growth of cervical pathogens.

Antimicrobial substances of probiotics supernatant produced by beneficial

microorganisms as a potential inhibitory agent include lactic acid, acetic acid, formic acid, phenyllactic acid, benzoic acid as well as other organic acids, short chain fatty acids, hydrogen peroxide, carbon dioxide, acetaldehyde, acetoin, diacetyl, bacteriocins and bacteriocins-like inhibitory substances and others(28) .

4- CONCLUSION

The probiotic cell and concentrated supernatant (two-fold) of probiotic isolates reduced the growth and viability (CFU/ml) of *C. albicans*, comparing to the control by 2– stage agar overlay method in solid media and MMT assay method for two-fold supernatant.

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