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Molecular Study of Antimicrobial Effect of Bacteriocin Isolated from *Lactobacillus* on *Candida albicans*

Noor Ali Faleh*, Abbas Muhei Muzher
Biology Department, College of Science, Diyala University, Iraq
Corresponding Author: Noor Ali Faleh, **Phone:** +96 407732430481, Email**:** noornoor19481948@gmail.com

ABSTRACT

Background: It is generally known that several lactic acid bacteria (LAB) species have antibacterial properties. Different Lactobacillus species have been shown to have anticandidal effects by numerous researchers. This genus's members benefit the vagina and gastrointestinal systems' health. Additionally, they create a variety of bacteriocins with a wide spectrum of actions against bacteria or fungi.

Objective: The aim of the current study was to examine the antifungal effects of bacteriocin produced by Lactobacillus spp. isolates against Candida spp. with the ultimate goal of gene expression on *C. albicans* biofilm genes before and after bacteriocin treatments.

Material and methods: To test the antifungal activity of lactobacilli that make bacteriocin against the yeast Candida, 50 yogurt and vaginal samples were gathered. Testing on the isolated Lactobacilli included microscopic, macroscopic, and biochemical examinations. Additionally, a screening was done to find the best producer isolation. A total of 100 urine samples from females of various ages were obtained for the isolation of Candida spp. All of the isolated *C. albicans* were examined under a microscope, on a larger scale, and using biochemistry. Primary and secondary screening methods were used to look into the detection of bacteriocin formation from Lactobacillus. The expression of the agglutinin-like sequence 1 (ALS1) gene before and after treatment with bacteriocin was compared using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results: The agar wells diffusion technique was the most effective, according to the results. The results show that folding of this gene was reduced after bacteriocin treatment which means that bacteriocin inhibits the gene expression of biofilm formation of *C. albicans*.

Conclusions: Our findings suggest that bacteriocin treatments may offer an effective substitute for antifungal medications. To verify these results, additional in vivo research is required.

Keywords: Antifungal, Bacteriocin, Candida, C. albicans, Lactobacillus, Experimental Study, Diyala University.

INTRODUCTION

Fungal illnesses, which have a death rate of 45%, are among the hardest diseases to treat in humans [(1)]. Despite the availability of numerous potent antifungal medications, mortality rates remain high. Invasive candidiasis can be caused by a variety of Candida species, the most significant of which is *C. albicans* [(2)].

Like many other microorganisms, *C. albicans'* ability to develop into a biofilm, a densely packed community of cells, determines how harmful it is to humans [(3)]. The creation of a biofilm is thought to start with microbial adhesion. This building serves as a barrier between environmental pressures and the host's defenses [(4)]. Adhesins are specialized proteins that *C. albicans* uses to mediate adhesion to host cells, other microorganisms, abiotic surfaces, and other C. albicans cells [(5)]. The agglutinin-like sequence (ALS) proteins, a family of eight, are what make up *C. albicans* adhesions (ALS1–7 and ALS9). Biofilm formation is frequently linked to *C. albicans* infections.

Previous studies showed that biofilm development on mucosal surfaces is correlated with the expression of ALS1, a member of the ALS (agglutinin-like sequence) gene families [(6)]. Lactic acid bacteria's (LAB) bacteriocins are easy to make, stable at low pH levels, non-toxic to people, and protease-resistant. In general, it is generally recognized that LAB species have antibacterial properties. Different Lactobacillus species have been shown to have anticandidal effects by numerous researchers. This genus's members benefit the vagina and gastrointestinal systems' health. Additionally, they create a variety of bacteriocins with a wide spectrum of actions against bacteria or fungi [(7)].

The purpose of this study is to identify bacteriocin production by Lactobacillus spp. isolates and assess its antifungal activity against Candida spp. The gene expression of the *C. albicans* biofilm before and after bacteriocin treatments will then be examined.

MATERIALS AND METHODS

Samples collection and isolation: A total of 50 samples were collected from Yogurt and vaginal swabs (25 from each) for isolation of *Lactobacillus* sp. Also, 100 samples were collected from urine for isolation of *C. albicans* during the period from November 2021 to February 2022. All samples were collected from females of different ages who attended Al-Batool Teaching Hospital. The clinical diagnosis was done by Consultant Obstetrician. The samples were cultured immediately after sampling for diagnostic purposes.

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***Lactobacillus* sp. Isolation and identification**:

The vaginal swabs were streaked on MRS agar whereas the yogurt samples were inoculated in MRS broth 1 ml of yogurt in 9 ml broth then incubated at 37ºC for 48hrs using microaerophilic conditions. Thereafter, pure isolates were subjected to identification as macroscopic and microscopic appearance and those isolates readily Gram-positive rods that are catalase-negative are identified negative for oxidase [8].

Candida Isolation and identification:

One isolated pure colony was produced after the isolates were purified by streaking on sabouraud dextrose agar and incubating at 37°C for 2 days. Based on the morphological characteristics of the culture media, particularly chromogenic agar and germ tube development, Candida was recognized [9].

Germ tube test: A tiny portion of the candida colony was transferred to a sterile tube containing 0.5 ml of human serum according to the procedure reported by **Forbes *et al.*** [10], and the mixture was then cultured at 37°C for 1.5 to 3 hours. The germ tube was then observed by dropping a small amount of the suspension onto a clean, glass slide, covering it with a slide cover, and viewing it under a microscope at a power of (40 X).

Susceptibility to antifungal agents' test: The disk diffusion susceptibility method for antibiogram testing (Kirby-Bauer method); Fluconazole (10mg) and Muconazole (10mg). The Clinical Laboratory Standard Institute (CLSI) publication advised applying antifungal discs to MHA, and after incubation, the inhibition zones were determined [11].

Detection of Biofilm: *C. albicans* isolates were tested for their capability to form biofilm after and before bacteriocin treatment by using microtiter plate method, according to a previous study [12].

***Lactobacillus* Screening for bacteriocin production:**

Primary Screening by Agar-Plug Diffusion Method: Primary Screening was done by using Agar-Plug Diffusion Method, according to **Biyari and Fozouni** [13].

Secondary Screening by Agar-Wells Diffusion Method: Agar-Wells Diffusion Method was done for Secondary Screening according to **Ołdak *et al.*** [14].

Bacteriocin Activity Assay: Crude bacteriocin was serially diluted twice with saline solution to measure the bacteriocin activity. By using an agar well diffusion experiment, these dilutions were used to test the antibacterial activity of bacteriocin against the indicator bacteria (as previously described). The reciprocal of the highest dilution showing a clearly defined inhibition zone of the indicator yeast was considered to be the bacteriocin activity, which was given as AU/ml. (1000 / 100) D, where 1000 is a constant, 100 is the volume of supernatant in a well (l), and D is the dilution factor, was used to calculate the amount of AU [14].

Partial Purification of Bacteriocin:

The bacterial isolate was added to MRS broth, and it was then incubated at 37°C for 48 hours. Centrifugation was used to separate the cells for 15 minutes at 6000 rpm. For 15 minutes, CFS was centrifuged at 6000 rpm after being heated for 10 minutes at 80°C. The supernatant and n-butanol were thoroughly combined in a 1:1 ratio. Phase separation was achieved by centrifuging the mixture for 10 minutes at 4000 rpm. PPB, or partially purified bacteriocin, is the result of re-suspending the sediment in 1.0 mM sodium phosphate buffer (pH 6), which was used to evaporate the organic phase at 65°C [15]. The agar well diffusion method was used to ascertain the antibacterial activity of bacteriocin.

Extraction and purification of bacteriocin: Ammonium sulfate precipitation, cation exchange chromatography, and gel filtration were the 3 procedures employed to extract and purify bacteriocin (**Table 3**).

Determination of (MIC) of bacteriocin: Serial dilutions were performed for bacteriocin and well diffusion method was used to determine the minimum concentration of bacteriocin that inhibits the *C. albicans* growth. The results have been determined by the inhibition zone formation and measured the diameter of the zone.

Molecular study (Quantitative RT-qPCR, Discovering ALS1 and ITS)

A. Extraction of DNA and Polymerase Chain Reaction: Amplification Three *C. albicans* isolates were chosen, and DNA was extracted from them using the ABIOpure Extraction protocol. Nanodrop evaluated the DNA's concentration and purity.

B. Primer Selection: Table 1 lists the specific primers that were required for amplifying an ALS1 and ITS segment. To achieve a final concentration of 100 pmol/l, the primers were given in lyophilized form and dissolved in sterile water devoid of nuclease. They were then kept until use in a deep freezer.

Table (1): Primers used in candida gene detection.

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)
ALS1-F	GACTAGTGAACCAACAAATACCAGA	55	318
ALS1-R	CCAGAAGAAACAGCAGGTGA		
ITS-F	TCCGTAGGTGAACCTGCGG		541
ITS-R	TCCTCCGCTTATTGATATGC		

Gene Expression

RNA Purification: RNA was extracted from the sample using the TRIzolTM Reagent's instructions. After being converted to cDNA, RNA/miRNA concentration is a factor in the analysis and calculation of gene expression levels. All procedures include data analysis, qPCR amplification, and whole RNA purification. Comparative Ct technique was used to quantify the degree of gene transcription and determine the level of gene expression for the ALS1 and ITS genes (mRNA level). As an endogenous control, the Ct of the ITS gene was utilized to calibrate the Ct values of other genes.

The calculations for folding gene expression are as follows:

* Ct= cycle of threshold (number of cycles required for florescent signal to cross threshold).
* HK= housekeeping gene.

Ethical Approval:

This study was ethically approved by the Institutional Review Board of the Diyala University. Written informed consent was obtained from all participants. This study was executed according to the code of ethics of the World Medical Association (Declaration of Helsinki) for studies on humans.

RESULTS

Identification of *Lactobacillus*: About 15 (30%) isolates of *Lactobacillus* were obtained from vaginal swab cultured on MRS agar after incubation period and 5 (20%) isolates from yogurt samples (**Table 2**).

Identification of Yeast Isolates: About 25% *C. albicans* were isolated from urine samples (**Table 2**).

Table 2: The frequency of bacterial isolates under study.

Samples	No. of samples	No. of positive isolates	Frequency %
Vaginal swab	30	15	30%
Yogurt	20	5	10%
Total	**50**	**20**	**40%**
Urine	100	25	25%

Antifungal susceptibility test for *C. albicans*: The results showed that all isolates 100% resistance for both antibiotics (MCL, FLU). After primary and secondary screening for bacteriocin production, the results showed that yogurt samples were the best and higher activity bacteriocin producer and one isolate was picked up for further purification and extraction. Purification steps of bacteriocin are depicted in **Table 3**.

Table (3): Purification and activity of bacteriocin produced by Lactobacillus.

Steps of Purification	Volume of bacteriocin (ml)	Activity (Au/ ml)	Protein Con. (mg/ml)
Crude extract	200	640	22.82
Ammonium sulfate precipitate 80%	50	1280	34.96
Ion exchange	15	2560	32.28
Sephadex -S-1000 for gel filtration	15	2560	31.45

AU: (Arbitrary Unit) was defined as the reciprocal greatest dilution that prevented the indicator strain from growing.

Minimum Inhibitory Concentration of bacteriocin on *C. albicans*: MIC is used to assess antifungal capability of bacteriocins against four isolates from tested isolate with concentration of bacteriocin about 100mg/ml to 1.5 mg/ml. The lowest concentration that inhibits fungal growth, it was discovered, is 6.25 mg/ml.

Biofilm Formation of *C. albicans* before and after bacteriocin treatment: In the current investigation, *C. albicans* produced biofilms of varying strength depending on the OD value and crystal violation. Treating the biofilm formed by *C. albicans* with crude and purified bacteriocin caused a reduction in absorbency (i.e., biofilm thickness), so 3 of isolates that showed the higher reduction in biofilm were tested for gene expression.

Molecular study:

Concentration and purity of DNA for fungi: The concentration for all DNA samples was between (10, 12, 15) ng/µl, respectively. These concentrations were sufficient amounts for PCR amplification.

Molecular identification of some virulence genes by PCR for fungi: The detection of 2 virulence genes (*ALSI* and *ITS*) by PCR (Polymerase Chain Reaction) of 3 isolates of *C. albicans* from urine. The amplified virulence genes were identified based on annealing temperature and the molecular weight of the products (amplicon) **Figure 2**. The presence of a single band of extracted DNA, which shows how effectively the DNA extraction process worked.

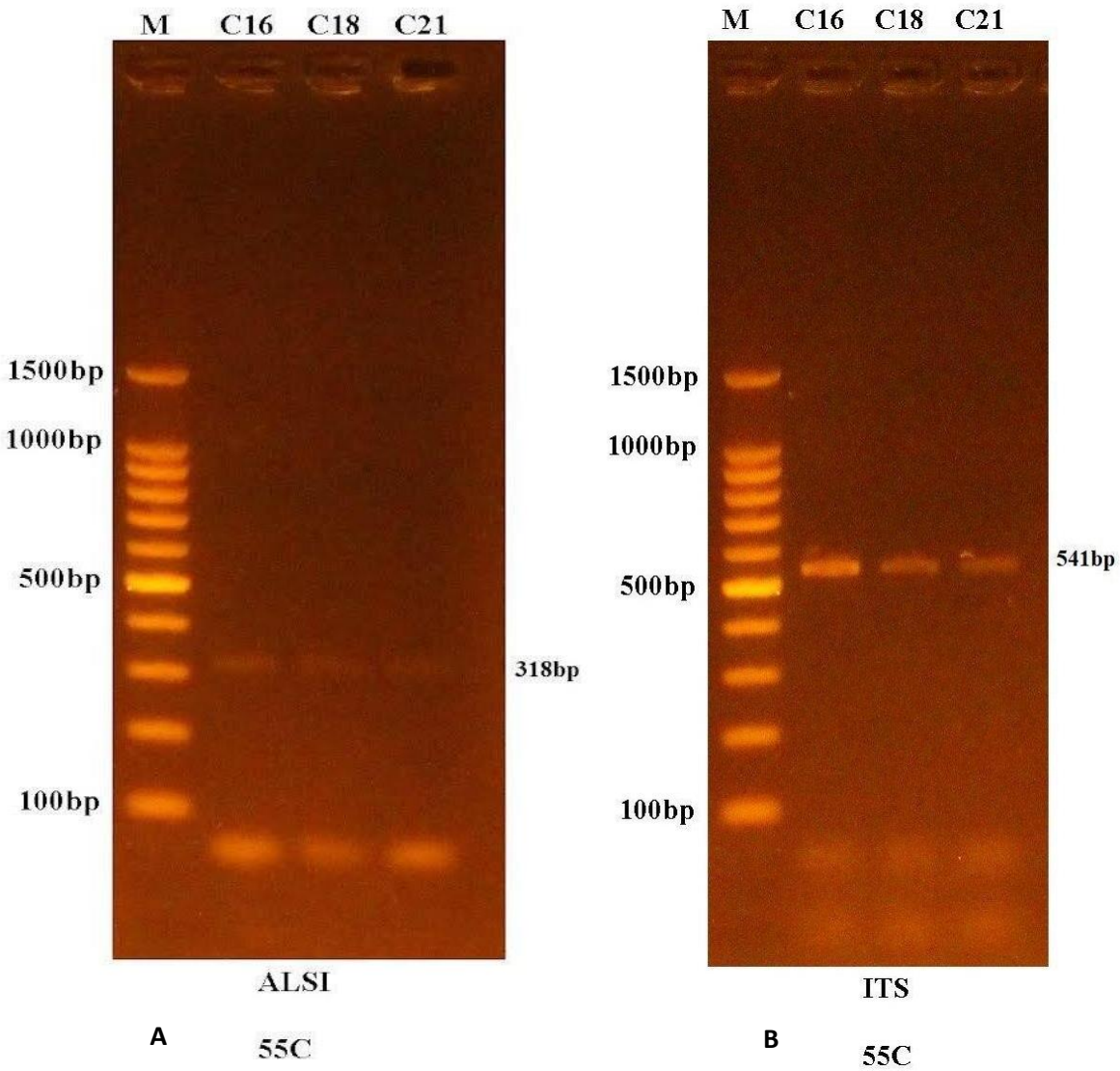

Figure 4-14: (A) Results of the electrophoresis on 1.5% agarose gel stained with Eth.Br. M: 100bp ladder markers were fractionated from the ALSI gene amplification of *C. albicans* samples. 318bp PCR products can be seen in lanes C16–C21. (B) The ITS gene of *C. albicans* samples was amplified, and the results were separated on a 1.5% agarose gel and stained with an Eth.Br. M: 100bp ladder marker. Comparable to 541bp PCR products are lanes C16–C21.

Use of the quantitative real-time PCR technique (qRT-PCR) to determine gene expression is summarized in Table 4.

Table 4: gene expression values for biofilm genes (*ALS1*) in *C. albicans.*

Sample	ITS	ALS1	Δct	ΔΔct	Folding
C16	23.65	29.42	5.77	0.00	1.00
C18	24.29	31.12	6.83	0.00	1.00
C21	22.23	31.30	9.08	0.00	1.00
CB16	21.66	31.63	9.97	4.20	0.05
CB18	21.58	33.00	11.42	4.59	0.04
CB21	21.99	32.56	10.57	1.50	0.35

C= *C. albicans* before bacteriocin treatment, CB= *C. albicans* after bacteriocin treatment.

DISCUSSION

The isolates of Lactobacillus were recognized using biochemical and microscopic methods. Gram-positive, non-spore producing, soft, large or tiny, convex, creamy, smooth, and circular colonies of Lactobacillus isolates were observed on MRS agar; they also exhibited oxidase positivity and catalase negativity.

Yeast (Candida) cells were much larger than bacterial cells and had a spherical to oval shape under a microscope. They also had budding. On SDA, yeasts emerged as elevated, glossy, smooth, glabrous yeast-like organisms with colonies that ranged in shape from a circle to an oval and had a unique fragrance [16], Green smooth-type colonies form on HiChrom agar in contrast. Each isolate was created as a germ tube.

The key genes involved in the formation of the *C. albicans* biofilm are typically identified by their level of gene expression. In the current investigation, the gene expression of three randomly selected candidal isolates (isolates 16, 18, and 21) was examined. The expression levels for each gene were calibrated and normalized using the housekeeping gene of *C. albicans* internal transcription spacer sequence, which was immediately isolated purified RNA from each isolate (ITS1). **Table 4** enlists the Delta Ct values, housekeeping gene expression levels, and tested gene expression levels, before and after bacteriocin treatment. The current findings demonstrate that all tested isolates expressed the ALS1 gene. The expression levels demonstrate that these genes were up-regulated in isolates that formed biofilms (BF), with the maximum expression folding 1.00 before bacteriocin treatment and the expression decreasing 0.05, 0.04, and 0.35 correspondingly after bacteriocin treatment for the isolates C16.18.21.

Reverse transcriptase-PCR was used by **Green *et al.*** [17], reverse transcriptase-PCR analysis of the expression patterns of the ALS gene family in clinical oral, vaginal, and urine samples (RT-PCR). They demonstrated that the ALS1 gene was expressed in all inoculation densities and time points. Additionally, they stated that the clinical strains of these samples' samples showed similar gene expression patterns. In another investigation, RT-PCR was used to assess the expression of ALS1 genes in clinical samples and vaginal candidiasis models. The ALS1 gene was found to be the most frequently expressed gene in the ALS gene family. In the same study, clinical samples and model systems also revealed similar expression frequencies and patterns.

CONCLUSION

The antagonistic activity and bacteriocin production of the Lactobacillus isolates against Candida spp. varied in the current study. It is obvious that the isolation and culturing conditions, as well as the used method for detecting antifungal activity and also the processes which involved in the inhibition process, are related to the ability of bacterial isolates to suppress the growth of yeast in vitro [18]. Using a quantitative real-time PCR method, it was demonstrated that the housekeeping gene ITS1 was expressed consistently under all biofilm conditions, while ALS1 was expressed at 100% in all 10 *C. albicans* tested. These symbiotic compositions may offer an effective substitute for antifungal medications. To verify these results, additional in vivo research is required.

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