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An application of Bacteriocin-Producing Vaginal *Lactobacillus Crispatus* IS30 in A Gel Formula Against Some Vaginal Pathogens

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Abstract

The aim of this study was to evaluate the possibility of using *Lactobacillus* cells as a probiotic to treat some vaginal infections. For this purpose, thirty *Lactobacillus* isolates were collected from vaginal samples subjected to a screening program to investigate their antagonism abilities against four vaginosis species: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia* and *Proteus* spp. Eighteen isolates were selected from the primary screening (agar plug diffusion method) based on their ability to inhibit the growth of 4 indicators which then subjected to a secondary screening program with two methods: detection of bacteriocin activity and biofilm forming capacity. The isolate *Lactobacillus* IS30 was selected based on its high bacteriocin production as well as being biofilm producer. Further identification tests revealed that isolate IS30 was *Lactobacillus crispatus.* Optimization experiments revealed that MRS broth was the best medium for bacteriocin and biomass production with glucose and yeast extract as best carbon and nitrogen sources. The maximum production observed in optimized culture was 15.9 mg/mL and 40 AU/mL for biomass and bacteriocin production respectively, hence this medium was used in the preparation of the formula containing *Lactobacillus* IS30 cells. Study the probiotic properties demonstrated the capability of *Lactobacillus crispatus* IS30 cells to tolerate the high acidity with an ability to adhere to vaginal epithelial cells. Moreover, *Lactobacillus crispatus* IS30 showed ability to auto-aggregation (72.66%) and 58% of *Pseudomonas* co-aggregation percentage after 5 hours. *Lactobacillus crispatus* IS30 cells was successfully incorporated into a gel bases formula and results revealed that cells have kept their viability over 14 days with an ability to produce and releasing the bacteriocin. The formula was tested against *P. aeruginosa* and *E. coli* and results confirmed its activity on both species.

Keywords: *Lactobacillus*; vagina; gel formula; Probiotic; Bacteriocin

استخدام بكتيريا *Lactobacillus crispatus* IS30 المهبليه المنتجة للبكتريوسين في تركيبة هلامية ضد بعض الممرضات المهبلية

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الخلاصة

في هذه الدراسة ، تم جمع 30 عزله من *Lactobacillus* من عينات مهبلية خضعت جميعها للغربله لاختبار قدراتها التثبيطية ضد أربعة أنواع البكتريا المسببة لالتهاب المهبل: *Pseudomonas aeruginosa* و *E. coli* و *Klebsiella pneumonia* و *Proteus*. تم اختيار 18 عزله من الفحص الأولي اعتمادا على قدرتها على تثبيط نمو اربعه انواع من البكتريا المسببة لالتهاب المهبل والتي خضعت بعد ذلك لغربله ثانوية بطريقتين: الكشف عن نشاط البكتريوسين وقابلية تشكيل الأغشية الحيوية. تم اختيار العزلة *Lactobacillus* IS30 بناء على قدرتها على إنتاج بكتريوسين عالي الفعالية بالإضافة إلى كونها منتج للأغشية الحيوية. كشفت اختبارات تحديد النوع أن العزلة IS30 هي *Lactobacillus crispatus*. كشفت تجارب تحديد الظروف المثلى ان MRS broth كانت أفضل وسط لإنتاج البكتريوسين والكتلة الحيوية مع الكلوكوز ومستخلص الخميرة كأفضل مصادر للكربون والنيتروجين على التوالي، وبالتالي تم استخدام هذا الوسط في تحضير التركيبة التي تحتوي على خلايا *Lactobacillus crispatus* IS30. دراسة خصائص *Lactobacillus crispatus* IS30 اثبتت قدرتهاعلى تحمل الحموضة العالية مع القدرة على الاتصاق بالخلايا الظهارية المهبلية. علاوة على ذلك ، أظهرت هذه البكتريا 72.66 ٪ نسبة تجميع التلقائي و 58 ٪ من نسبة التجميع المشترك مع *P. aeruginosa* و*E. coli* بعد 5 ساعات. تم اضافة خلايا *Lactobacillus crispatus* IS30 بنجاح الى تركيبة من الهلام وكشفت النتائج أن الخلايا حافظت على حيويتها على مدار 14 يوما مع القدرة على إنتاج وإطلاق البكتريوسين. تم اختبار التركيبة ضد *P. aeruginosa* و*E. coli* حيث اظهرت النتائج نشاطا تثبيطيا عاليا على كلا النوعين.

Introduction

Urogenital tract infections (UGTI) such as yeast and bacterial vaginosis and sexually transmitted infections are the main medical problem, affecting many women over the world [1-3]. Vaginal microbiota is a complex community of microorganisms in which lactic acid bacteria (LAB) play an important and vital role [4]. The function of lactobacilli is to maintain an environment that restricts or block colonization of pathogenic microorganisms [5]. In the vagina, the predominant role of LAB, in particular lactobacilli, in this specific ecological place is now well recognized and hence, it's the presence or absence is usually considered as a health or disease indicator [6].

Several mechanisms were suggested to explain the role of lactobacilli in the vagina, however the overall suggested that the mechanism is based mainly on the antagonistic ability of this bacteria. This mechanism is mainly based on its ability to adhere to the vaginal epithelium and inhibit or prevent the adhesion of pathogens to the vaginal epithelial surface. The mechanism is also involved competition for nutrients, congregation with some uropathogenic bacteria [7].

Vaginal lactobacilli produce several active compounds such as organic acid, particularly lactic acid, which contributes in the maintenance of low vaginal pH to be 4 to 4.5, hydrogen peroxide (H_2O_2) which is naturally produced by lactobacilli existing in the healthy vagina and production of bacteriocins [8-11], or biosurfactants [12].

Probiotics are non-pathogenic live microorganisms having the potential to exert health benefits to the host [13], such as reducing inflammation, speeding the wound healing process, and strengthening the immune system [14]. Several strains of *Lactobacillus* species have proven to exert a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens, reduction of blood cholesterol levels and others [15-18]. Therefore, lactobacilli were among the first bacteria that described and used as probiotics.

Although, drug and antibiotic therapy are useful for the treatment of common urinary tract infections, numerous problems have occurred, for instance multi resistant bacteria [8,19,20]. For the treatment and prevention of upper genital tract infections, probiotic lactobacilli were

suggested as an alternative to the antibiotics [21, 22]. Furthermore, vaginal lactobacilli have a number of properties that make them very appropriate to be used as probiotic [8,23]. The aim of the current study was to utilize a safe and bacteriocin-producing isolate of *Lactobacillus* as a probiotic against some common vaginal pathogenic bacteria via introducing it in a suitable pharmaceutical formula.

Materials and Methods

Collection of vaginal samples and isolation of *Lactobacillus*

Vaginal samples were collected from 120 women (17-48 years) with healthy vaginal ecosystem as follow: from the vaginal fluid, samples were collected with sterile cotton swabs introduced into the vagina, rotated a few times along the vaginal sidewall, and allowed to absorb for few seconds [24]. The swabs were placed in tubes containing de Man Rogasa Sharpe (MRS) broth which then incubated at 37°C under anaerobic condition in anaerobic jar. MRS broth was used for the selection and isolation of *Lactobacillus*. After incubation, a loopful was spread on MRS agar plate (pH 6.5) and incubated for 24 to 48 h at 37°C under anaerobic condition [2]). All isolates were identified through culture characteristics and biochemical tests [25,2]).

Screening of *Lactobacillus* isolates for bacteriocin production

Primary screening

Agar plug diffusion method [2]) was used in primary screening as follows: After overnight growth in MRS broth, the *Lactobacillus* isolates were cultured into the MRS agar medium by streaking on the plate surface and then incubated at 37ºC for approximately 24 h in a candle jar. After incubation, plugs of about 0.5 cm in diameter were made with a sterile cork borer from each isolate which then placed on plates surface of Mullar-Hintonagar streaked with 200 µl of pure culture of an overnight growth culture of the indicator bacterium containing approximately (1×10^8) cells/ml. Four indicator isolates were used: *Escherichia coli, Pseudomonas aeruginosa, Proteus* sp and *Klebsiella pneumonia* all were obtained from the department of biotechnology / university of Baghdad. Plates were then incubated overnight under the same conditions. The zones of inhibition plugs were used as a measure of antibacterial activity of each isolate.

Secondary screening

Well diffusion assay was used to estimate the production of bacteriocin by isolates as follows [26-**28**] universal tubes contained 10 ml of MRS broth were inoculated with 2% of an overnight culture of the *Lactobacillus* isolate contained approximately 10^8 cells/ml and then, tubes were incubated for 24 h at 37°C. After incubation, the cell-free supernatant (CFS) was collected and NaOH and catalase solution were added to neutralize the effect of organic acid and H_2O_2 activity respectively. The CFS of each isolate was assayed for the presence of bacteriocin using agar well diffusion assay as follows: 100 µl of an overnight growth culture of the indicator bacteria (*E. coli, K. pneumonia, Proteus spp., and P. aeruginosa*) containing approximately 1×10^8cells/ml was mixed with 25 ml of a sterile Mueller Hinton agar and then poured into sterile Petri dishes. Wells of 5 mm diameter were cut and 100 µl aliquots of the filtered CFS were dispensed in each well. Then, plates were incubated for 24 h at 37°C [29,30].

In addition, the biofilm detection for *Lactobacillus* isolates was integrated in the secondary screening by using the congo red agar [31] and microtiter plate assay (TCP) [32].

Biofilm formation

The ability of the selected *Lactobacillus* to form biofilm was detected via two methods as follow: Congo red agar method**,** this method was achieved according to Freeman, *et al***.,** [31].

Lactobacillus sp. isolate was streaked on congo red agar and then incubated for 24-48 h at 37°C. After incubation, the appearance of sparkle dark colonies indicated biofilm formation.
Microtiter plate method*:* briefly *Lactobacillus* isolate was grown in MRS broth with 1% glucose for 48 h at 37 ◦C in microaerophilic conditions. An amount of 180 µl of sterile TSB was added to each well in a microtiter plate, and then 20 µl of 48hrs grown *Lactobacillus* was added. The broth in the well was mixed 10 times by pipette and then incubated for 72 h at 37°C without shaking. After incubation, the supernatant was removed, and each well was washed 3 times by phosphate buffer. Aliquots of 200 µl of 1% crystal violet were added for 15 min. The wells were washed 3 times with phosphate buffer and then dried by air for 30 min. An amount of 200 µl ethanol (96%) was added for 15 min. Sterile medium was used as a negative control. The result was read using ELISA reader **(**Huma reader HS, Germany) at 630nm [32].

Identification using API 50-Strep (BioMérieux -France)

API 50 CHl was used for the identification of *Lactobacillus* and related genera. It is a ready to use set of media which allow the fermentation of 49 carbohydrates. The results were recorded on recording sheet and the biochemical profile obtained for the strain after the final reading was identified online using the identification software with bioMérieux, Inc. online database.

Optimization of optimal culture medium

Different nutritional media (nutrient broth, brain heart infusion broth, tryptic soy broth, MRS broth, mueller hinton broth) were tested in order to choose the medium that support maximum production of bacteriocin and biomass by the selected isolate. In addition, seven different carbon sources (glucose, fructose, arabinose, mannose, maltose, dextrose, and starch) were tested**.** Each carbon source was replaced with the original carbon source in the best production medium (MRS) to elevate biomass and bacteriocin production. Moreover, MRS medium is already contained three organic components that may serve as nitrogen sources (peptone, yeast extract, meat extract). All these compounds were subjected in this study to an optimization process in order to choose one source that support both growth and bacteriocin production. The method was based principally on the removal experiment optimization approach as follow: first, each nitrogen source was studied separately, then half amount for each two-nitrogen sources and finally equally amount for the three different nitrogen sources. In all experiments, production of bacteriocin was determined based on the well diffusion assay method as described earlier and biomass estimation.

Study the probiotic properties of *Lactobacillus*

Antibiotic Susceptibility

Antibiotic susceptibility was conducted using the disc diffusion method [33] on a Mueller-Hinton agar which was previously seeded with the selected *Lactobacillus*. The antibiotics used were Erythromycin, Cefotaxime, Cefixime, Ceftazidim, Amikacin, Clindamycin, Penicillin, Meropenem, Tetracyclin, Gentamicin, Azithromycin, Chloramphenicol, Ciprofloxacin, Norfloxacin, Ceftriaxone, amoxicillin clavunic acid, Rifampicin and Piperacillin

Acid tolerance

The selected *Lactobacillus* was incubated overnight in MRS broth at 37°C and then cells were harvested by centrifugation (7000 rpm, 4◦C,10 min). A fresh MRS medium was prepared and pH was adjusted to 2.0 with 1N HCl. Harvested cells were resuspended in acidic MRS and incubated at 37°C. Samples were withdrawn at zero time and after 1 and 2 h which serially diluted in phosphate buffer saline (PBS). Samples were then plated on MRS agar and incubated at 37°C for 48 h. MRS broth with pH 6.5 was used as a control. Cells viability was assessed by the plate count method (34).

Adhesion to Vaginal Epithelial Cells (VECs)

Vaginal epithelial cells were collected from healthy women by sterile cotton swabs, immersed in 0.04 M citric acid -Na_2HPO_4 buffer pH 4.5 and stored at 4°C for less than 3h. The vaginal epithelial cells were washed using the same buffer, centrifuged at 800rpm for 4 min and resuspended to a concentration of 1×10^6 vaginal epithelial cells.ml^{-1} [33]. An overnight culture of the lactobacilli was suspended to reach 10^8 CFU.ml^{-1} in normal saline. Equal volumes of the bacterial suspension and the vaginal cells were mixed and incubated for 1h at 37°C. Next, the cells with adherent bacteria were collected and washed three times in citric acid $Na_2H_2PO_4$ buffer (800 rpm,7 min). Bacterial adhesion to vaginal epithelial cells was assessed by microscopy (×100) after staining with 1% of crystal violet. The number of bacterial cells attached to 50 consecutive vaginal epithelial cells smears was counted and Vaginal epithelial cells control smear was made to confirm that the presence of native bacteria was negligible [24].

Auto-aggregation and Co-aggregation Assay

Lactobacillus cells of an overnight culture were harvested by centrifugation at 5000 rpm for 15 min, washed twice in PBS pH 6.0 to give viable counts of 10^8 CFU.ml-1. Four ml of cell suspension were mixed by vortex for 10 seconds and auto-aggregation was determined during 5 h of incubation at room temperature. 0.1 ml of the upper suspension was transferred to another tube containing 3.9 ml PBS and the absorbance was measured at 600 nm [35].

The percentage of autoaggregation was calculated by the following expression:

Autoaggregation (%) = [ODi - ODf / ODi] ×100

Where ODi is the OD at initial time (t=0 h) of autoaggregation assay, and ODf is the OD at t=1 h, 2 h,3 h, 4 h and 5 h.

For the co-aggregation method, equal volumes (2 ml) of the lactobacilli and *P. aeruginosa* (indicator strain) cultures were mixed together vigorously for 10 s. Four ml of each bacterial suspension were used as a control. Then, the absorbance was determined at 600nm after 5 h of incubation at room temperature (35). The percentage of coaggregation was calculated using the following equation:

% Coaggregation =([(Ax+Ay)/2] - A (x+y)/ [Ax+Ay] /2)×100

x and y represent strains in the control tube and (x+y) the mixture

Pharmaceutical formula including *Lactobacillus* cells

***Preparation of Lactobacillus* biomass**

Preparation the inoculums of *Lactobacillus* isolate were as follows: from an overnight culture on MRS agar, a few loopfuls of *Lactobacillus* growth were inoculated into 10 ml of the optimized medium (modified MRS broth) and then incubated at 37°C, pH 6.5 for 24 h. After incubation, the number of cells were estimated with 0.5 McFarland Standard tube to be approximately 1×10^8 cell/ ml. Thereafter, biomass was collected using centrifuge at 12000 rpm for 15 min, and then a known amount of the optimized medium was added to the precipitant. This cells suspension was kept as usable biomass to be mixed later with the formula [36].

Preparation of gel formula (100 g)

An aliquot of 4g of carbopol 941 was dissolved in 95 ml D.W and then mixed very well using magnetic stirrer. Next, 0.1 g of methyl paraben was added and the mixture was left soaking for 24 h. Then, 2% of cells suspension containing 1×10^8 cell/ ml prepared in the previous section was added and mixed well. The final volume of the formula was then completed to 100g. All compounds included biomass, optimized medium and other formula chemicals (mentioned above) were mixed together for 1hr. Sufficient amount of triethanolamine was added subsequently to obtain gelling formula texture and pH was adjusted to pH 6.5. The formula was stored in a close container at 4ºC **[36]**.

***In vitro* evaluation experiment**

One hundred µl from a mixture of an overnight growth culture of the indicator bacterium (*P. aeruginosa* or *E. coli*) containing approximately 1×10^8cells/ml and a sterile muller hinton agar (100 ml) was poured into sterile plastic petri dishes and left to solidify. Circular wells of 5mm in diameter were made. Then, aliquots of 100µl of the gel formula containing *Lactobacillus* cells were dispensed in the well. After the incubation, the inhibition zone diameter around the wells was detected indicating of antibacterial activity [37].

Results and Discussion

In this study, 30 *Lactobacillus* isolates from vaginal samples were collected and used. The morphological and biochemical identification of *Lactobacillus* isolates were achieved based on Bergey's manual of systematic bacteriology [38]. For this purpose, all isolates were grown on the selective medium of the genus *Lactobacillus* (MRS), and subjected to catalase and oxidase tests. Then, identified isolates were subjected to a screening program to investigate their antagonism capabilities via inhibiting the growth of four common bacterial vaginosis species (indicators): *P. aeruginosa*, *E.coli*, *K.pneumonia* and *Proteus* spp.. These indicators are frequently causing bacterial vaginosis in women, for example, 90% of all UTIs is caused by *E. coli* [39-41].

The results of primary screening which was achieved by agar plug diffusion method showed that 80% of isolates exhibited inhibitory activities against indicators, the findings of the current study are consistent with those of Noor-alhuda and coworkers [34] who found that most isolates were able to produce bacteriocin with different size of inhibition zones against indicator strains. However, only38 isolates were showed an activity against all the indicator strains used in this experiment. Based on results, most isolates showed an ability to inhibit the growth of 4 indicators with different inhibition zone ranging from 8 to 25mm (Table 1). Results also showed that *P. aeruginosa* was inhibited by most isolates of lactobacilli (Table 2) and therefore, was selected to be used as an indicator for the next experiments in the secondary screening. Moreover, *P. aeruginosa* was chosen due to its multi drug resistance.

Table 1-Primary screening of vaginal *Lactobacillus* isolates under anaerobic conditions against *E. coli, K. pneumonia, Proteus, and P. aeruginosa*.

Isolate No.	Diameter zone of inhibition (mm)			
	P. aeruginosa	***Protous***	***K. pneumonia***	***E. coli***
Is1	**-**	**-**	**-**	**-**
Is 2	**-**	**-**	**-**	**-**
Is 3	**-**	**10**	**10**	**-**
Is 4	**-**	**-**	**-**	**8**
Is 5	**-**	**-**	**-**	**-**
Is 6	**14**	**12**	**11**	**11**
Is 7	**13**	**13**	**12**	**13**
Is 8	**10**	**-**	**-**	**10**
Is 9	**12**	**9**	**10**	**10**
Is 10	**10**	**10**	**8**	**8**
Is 11	**-**	**-**	**-**	**-**
Is 12	**12**	**10**	**10**	**10**
Is 13	**13**	**15**	**14**	**12**
Is 14	**11**	**-**	**-**	**-**
Is 15	**9**	**-**	**-**	**-**
Is 16	**-**	**10**	**8**	**-**
Is 17	**-**	**-**	**-**	**-**
Is 18	**10**	**-**	**-**	**-**

Is 19	**15**	**10**	**10**	**11**
Is 20	**13**	**10**	**8**	**9**
Is 21	**16**	**15**	**14**	**10**
Is 22	**15**	**14**	**14**	**12**
Is 23	**20**	**20**	**8**	**12**
Is 24	**25**	**15**	**8**	**8**
Is 25	**-**	**-**	**-**	**-**
Is 26	**20**	**22**	**10**	**10**
Is 27	**14**	**8**	**8**	**8**
Is 28	**10**	**-**	**-**	**-**
Is 29	**20**	**19**	**8**	**8**
Is 30	**22**	**20**	**8**	**8**

- **Negative result**

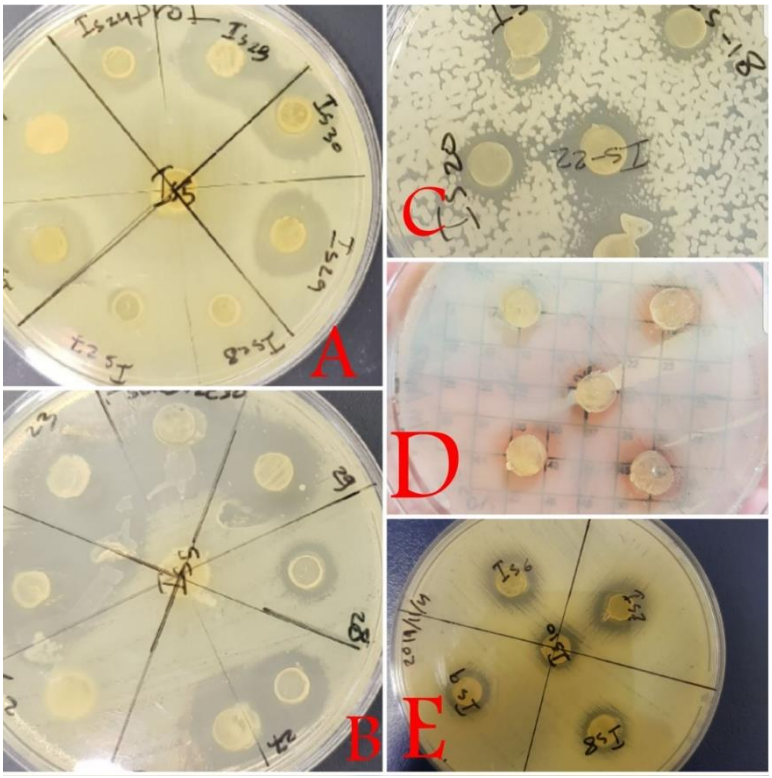

Figure 1-Primary screening by using agar pluq diffusion method to detect bacteriocin production against four pathogenic bacteria (A: *Proteus*, B: *P. aeruginosa*, C: *K. pneumonia*, D: *E. coli* and E: *E. coli*)

Eighteen isolates were selected and subjected to a secondary screening program with two methods: detection of bacteriocin activity by well diffusion method and biofilm forming capacity using, congo red agar and tissue culture plate method (TCP to evaluate the biofilm forming ability. Based on results listed in Table 2, 13 isolates have exhibited an inhibitory activity against indicators. By comparing the results with those obtained from the primary screening, it can be seen that 16 isolates of *Lactobacillus* had the ability to inhibit the 4 pathogenic isolates used as indicators, whereas only two isolates (IS26 and IS30) exhibited the same effect in the secondary screening. This is of course, is due to the antagonistic effect in the agar plug diffusion method that used in the primary screening which certainly induced *Lactobacillus* isolates to exhibit significant antimicrobial activity. These results agree with those reported by Arena *et al*. [42] who showed that *L. plantarum* strains had the ability to

inhibit the growth of food pathogens in agar spot test, while in well diffusion method, no effect was seen.
On the other hand, biofilm formation was also used as a basis to select the most suitable isolate in this study. Biofilm formation by *Lactobacillus* is important for their existence and maintenance in the in vivo ecosystem of vagina. As can be seen in table 2, out of 18 isolates, 14 showed a weak adherent and moderate, whereas 3 isolates had no ability to form biofilm. In addition, only one isolate exhibited a strong adherent ability.

Table 2-Secondary screening of *Lactobacillus* isolates for bacteriocin production and biofilm formation.

Isolate No.	Bacteriocin production (AU/ml)				Biofilm formation
	P. aeruginosa	***Protous spp.***	***K. pneumonia***	***E. coli***	
Is 3	-	-	-	-	Weak
Is 4	10	-	10		Weak
Is 6	10	-	-	-	Weak
Is 7	-	-	-	-	Moderate
Is 9	10	-	10	10	Weak
Is 10	10	-	-	10	Weak
Is 12	10	-	-	10	Weak
Is 13	-	-	-	-	Non-adherent
Is 19	20	-	-	10	Weak
Is 20	20	-	-	10	Non-adherent
Is 21	10	-	-	-	Weak
Is 22	10	-	10	10	Weak
Is 23	-	-	10	10	Moderate
Is 24	-	-	10	-	Strong
Is 26	20	10	10	10	Non-adherent
Is 27	-	-	-	-	Weak
Is 29	-	-	-	-	Weak
Is 30	20	10	10	10	Moderate

By combining the data from both primary and secondary screening, including the wide range of activity against 4 indicators and high bacteriocin activity as well as being biofilm producer, it was logical to select the isolate *Lactobacillus* IS30 as an elected candidate among the other isolates.
Lactobacillus IS30 was subjected to an identification process based on using the API 50 CHL test. Based on results, out of 49 carbohydrates, *Lactobacillus* IS30 isolate utilized 14 types and did not utilize 25. The results of API 50CHl were compared and identified online using the identification software via online API database (Bio Mérieux, Inc) which revealed that *Lactobacillus* IS30 isolate was *Lactobacillus crispatus* IS30.
Next, 5 media (nutrient broth, brain heart infusion broth, tryptic soy broth, MRS broth, mueller hinton broth) were used in order to investigate the best medium that can support maximum bacteriocin production and biomass by the selected isolate. As can be seen in

Figure 2, MRS broth was the best medium with 20 AU/ml and 10.7 mg/ml for bacteriocin activity and biomass respectively. Therefore, this medium was used in the next experiments as well as in the preparation of the formula containing *Lactobacillus* IS30 cells as a probiotic.

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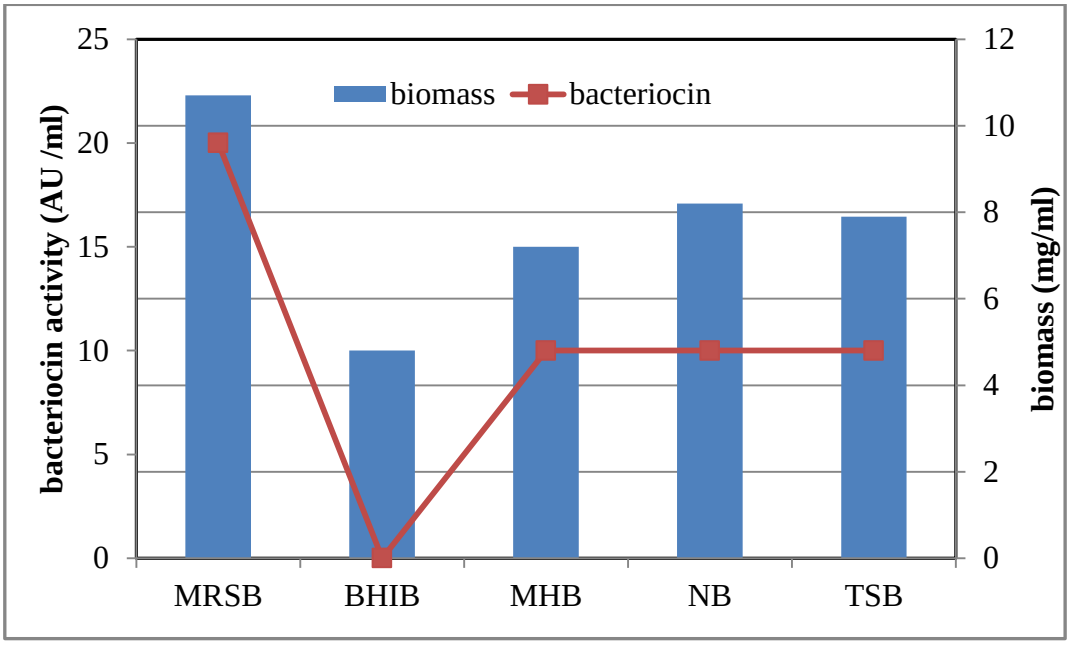

Figure 2-The effect of different media on biomass and bacteriocin production by *Lactobacillus crispatus* IS30 isolate.

In addition, different carbon sources were investigated to find the best carbon source for biomass and bacteriocin production. As can be seen in Figure 3, *Lactobacillus crispatus* IS30 was able to consume all types of carbon sourcesused in this study with maximum biomass of 13.8 mg/ml obtained in culture contained glucose. However, bacteriocin production was only observed in media supplemented with glucose and fructose with 40 Au/ml and hence, glucose was selected as the best carbon source. Ahn, (2017) used MRS medium supplemented with 2.0% of various carbon sources to investigate the effect of the carbon source on growth and bacteriocin production. They found that maximum production of bacteriocin (320 AU/ml) was obtained in medium with glucose as a carbon source followed by fructose (80 AU/ml). the results also showed that no bacteriocin production was found in media supplemented with lactose, sucrose, mannose, dextrin, and mannitol [43]. MRS medium contains peptone, yeast extract and meat extract which were subjected to an optimization strategy in order to investigate their effect on biomass and bacteriocin production. Figure 4 shows that most nitrogen sources used supported biomass and bacteriocin production, however, maximum production was observed in culture contained yeast extract as a sole nitrogen source with 15.9 mg/mL and 40 AU/mL for biomass and bacteriocin production respectively. Previous studies have reported that yeast extract is already containing growth factors and relatively more proportion of free amino acids as well as short-peptides of 2 or 3 amino acids which might be the reason for the increase observed in the production of biomass and bacteriocin [44,45]. These results provide the possibility to eliminate peptone and meat extract from the original composition of MRS medium without affecting the growth and bacteriocin production

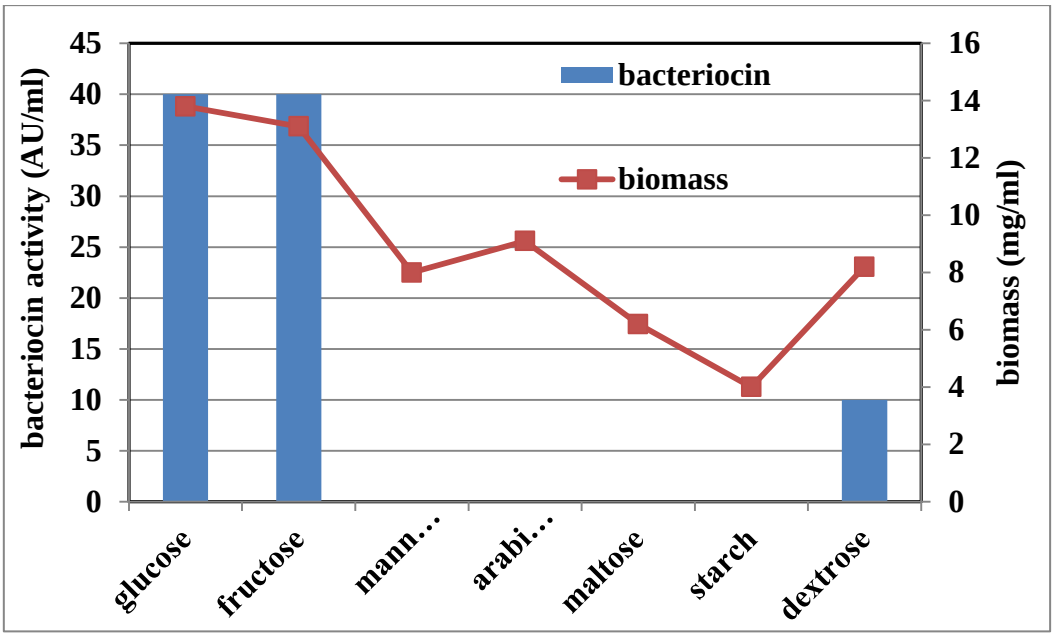

Figure 3-The effect of carbon sources (glucose, starch, fruct: fructose, man: mannose, arab: arabinose, malt: maltose and dext: dextrose) on biomass and bacteriocin production by *Lactobacillus crispatus* IS30 in MRS medium.

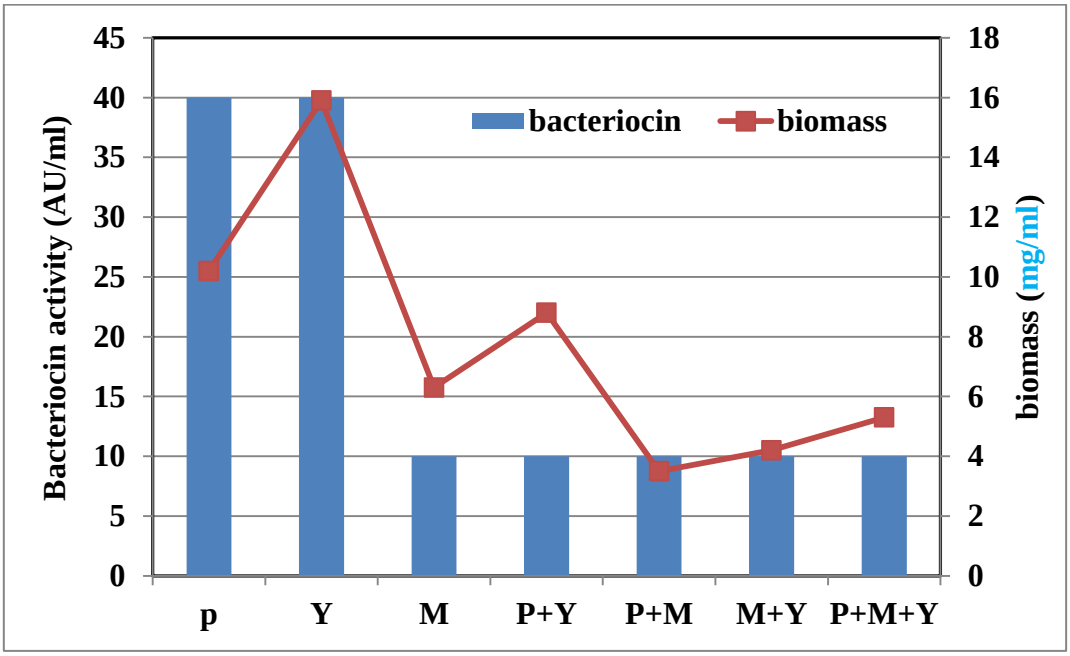

Figure 4-The effect nitrogen sources (Peptone (P), Yeast extract (Y), Meat extract (M)) on biomass and bacteriocin production by *Lactobacillus crispatus* IS30 in MRS medium.

Probiotic properties of *Lactobacillus crispatus* IS30

In order to evaluate the possibility of using *L. crispatus* IS30 as a vaginal probiotic, the following tests were performed: antibiotic susceptibility, biofilm formation, acid tolerance, adhesion to vaginal epithelial cells (VECs), autoaggregation and coaggregation assay. In fact, these testes are important to address the safety of the selected isolate as well as its ability to colonize in the vaginal environment. eighteen different antimicrobial drugs were used to test the antibiotic susceptibility of *L. crispatus* IS30. The results showed that *L. crispatus* IS30 was resistance to antibiotics inhibiting protein synthesis, such as erythromycin, clindamycin and azithromycin, whereas, it was sensitive to tetracycline and chloramphenicol. This result is

agreed with Ammor et al ., [46] who mentioned that *Lactobacillus* was commonly susceptible to antimicrobials that inhibit protein synthesis such as chloramphenicol and tetracycline. In addition, *L crispatus* IS30 was resistant to cephalosporin members such as Cefixime and Ceftazidim as well as to the β-lactams group such as penicillin, however, it was susceptible to amoxicillin/clavunic acid, piperacillin and meropenem because the piperacillin and meropenem are highly resistant to degradation by β-lactamases or cephalosporinases, while amoxicillin/clavunic acid binding with β-lactamase inhibitor [47]. Furthermore, *L. crispatus* IS30 showed higher resistance to aminoglycosides members such as Amikacin and Gentamicin. In addition, *L. crispatus* IS30 displayed higher resistance toward Ciprofloxacin and norfloxacin which is a fluoroquinolone member. Whereas, it was susceptible to Rifampicin antibiotic.

pH of the vagina is usually at 3.0 to 4.5 resulting in inhibiting the growth of a wide range bacterial species [48-50]. Therefore, investigate the tolerance to vaginal acid is an important selection criterion for probiotic strain that asses its ability to survive in such acidic conditions. Cells from *L. crispatus* IS30 was tested for its sensitivity to low pH. As can be seen in Figure 5, 71.6% of *L. crispatus* IS30 cells were survived in acid culture (pH 2) after 2 h of incubation indicating the ability of this isolate to tolerate the vaginal acidity [51].

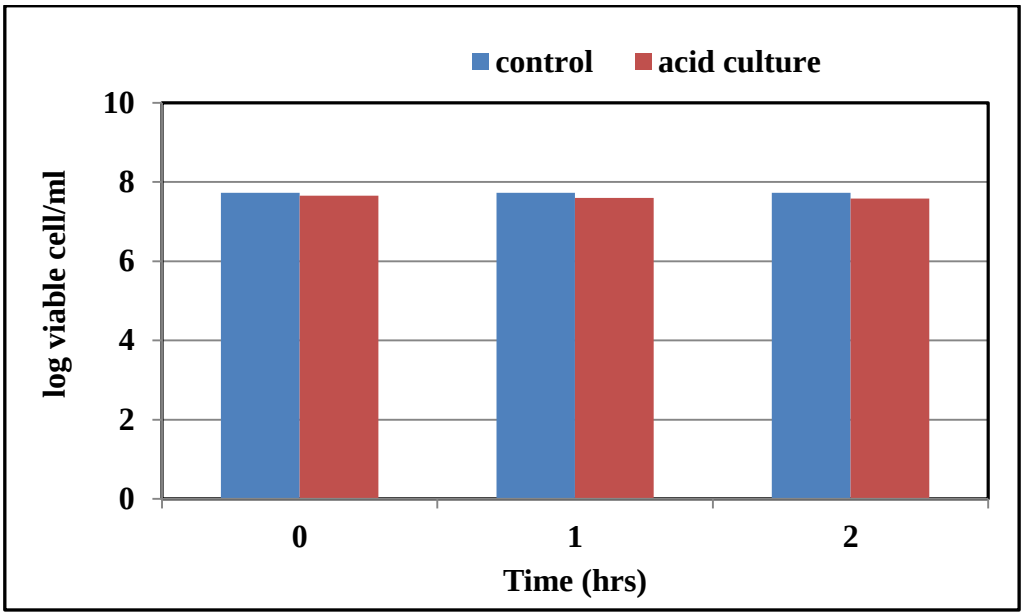

Figure 5- The effect of acid on *Lactobacillus crispatus* IS30 cells

In addition, the ability of *L. crispatus* IS30 cells to adhere to vaginal epithelial cells was investigated. In fact, *Lactobacillus* adhesion to vaginal epithelial cells is the first step for the colonization capability of this microorganism. The adhesion ability and colonization on tissues by the probiotic bacteria may prevent pathogen to access via steric interactions or specific blockage on cell receptors. Results presented in Figure 6 shows that *L. crispatus* IS30 had the ability to adhere to vaginal epithelial cells with more than 190 bacteria per VEC. This finding is in agreement with several previous findings [51,52], for example, Strus *et al* [51] showed that from111 isolates, 50% have a high adherence to vaginal mucus.

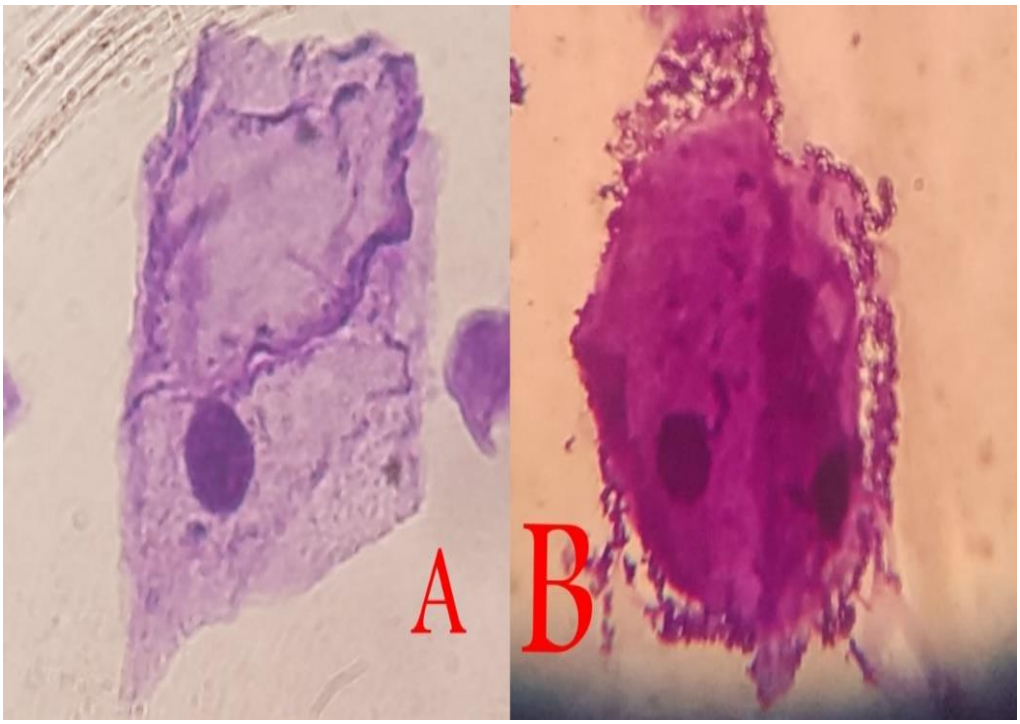

Figure 6-Adhesion of *Lactobacillus crispatus* IS30 cells to vaginal epithelial cells A: Control, B: Test

Auto and co-aggregation of bacteria is an important phenomenon in several ecological places where probiotics are vital and is considered as one of the important defense mechanisms against infection of UGT [**53**]. Several studies have reported that adhesion and co-aggregation of lactobacilli probiotic may inhibit the adherence of pathogens to tissue receptors on the vaginal epithelial cells and hence prevent their colonization which provide a protection for the vaginal ecosystem [54,55]. Therefore, it was suggested that using lactobacilli as a vaginal probiotic may participate in the reduction of pathogens growth and facilitate their removal via preventing their adhesion to the tissue receptors on the vaginal epithelial cells [54,55]. In this context, Reid *et al.* [**56**] reported that most *Lactobacillus* strains undergo co-aggregation with uro-pathogens. They suggested that this phenomenon is an important factor in the establishment and maintenance of healthy urogenital flora. Therefore, co-aggregation ability is considered as an important parameter to select probiotic strains for vaginal use. Based on the results obtained in this study, *L. crispatus* IS30 showed high auto-aggregation percentage (72.66%) after 5h of incubation and 58% of *Pseudomonas* co-aggregation percentage after 5h of incubation. Therefore, it can be said that *L. crispatus* IS30 have the possibility to use as a probiotic to protect the vaginal environment based on their ability to adhere to the epithelium tissue, auto-aggregation and co-aggregation with potential pathogens. [57]. This result agrees with Jessica *et al*., **[58]** who reported that *L. crispatus* and *L. jensenii* had the ability to adhere and co-aggregate with pathogenic bacteria.

Pharmaceutical formula including lived *Lactobacillus crispatusIS30*

Topical delivery drugs are a good method for systemic and local treatments and generally used in the treatment of inflammatory conditions [59]. Vaginal drug delivery systems are usually used to deliver contraceptive and drugs to treat the vaginal infections. Though, vaginal drug delivery is not limited to these drugs as the vagina has potential as a site to topically deliver drugs which will be absorbed systemically because of the dense network of blood vessels in the vaginal wall **[60]**.

The first drug delivery systems for utilize in the vagina were established in 1970, when the first vaginal ring was used for the delivery of medroxy progesterone acetate for contraception. Nowadays, tablets, creams and counter (OTC) vaginal medications as well as vaginal rings are the most common long-term drug delivery systems currently used. The greatest advantage of such dosage forms is the possibility of maintaining them in the vagina for an extended period of time including day hours and night, thereby enabling lower dosing frequencies. The idea of controlled-release drug delivery has also been effectively used to the intra-vaginal administration of a systemic prostaglandin derivative for abortion indication. Intra-vaginal controlled release drug delivery system is an effective means of continuing delivery of therapeutically active agents such as a contraceptive steroids and prostaglandins. The physical characteristic of prepared gel formula has a smooth texture and homogeneous with odorless [61].

In this study, *L. crispatus* IS30 cells was successfully incorporated into a gel bases formula. The viability of *L. crispatus* IS30 cells was checked by daily culturing on MRS agar, in addition to the homogeneity of formula components to ensure the activity of formula. Based on results, cells have kept their viability over 14 days, the findings of the current study are consistent with those of Hiba and coworkers [61] who found that after 14 days, *L. acidophilus* HT1 cells was viable in the cream formula prepared for the treatment of some skin pathogens.

The antibacterial activity of *L. crispatus* IS30 cells within gel formula preparation was investigated against *Pseudomonas aeruginosa* and *E. coli* according to the method described by Hiba *et al*., **[61].** Hiba *et al* showed that 10^8 CFU/ml was the best concentration that led to inhibit the indicator pathogenic bacteria. As shown in Figure 7, formula contain *L.crispatus* IS30 cells inhibited the growth of *P. aeruginosa* and *E. coli* with 10 and 18mm inhibition zone diameter respectively. These results confirm the efficiency of the formula through several points included: the viability of *L.crispatus* IS30 cells, its ability to produce bacteriocin within the formula, as well as its releasing from the formula to the external environment (tested media), without trapping or interaction among bacteriocin and any component of formula that may affect bacteriocin ability to inhibit tested bacteria.

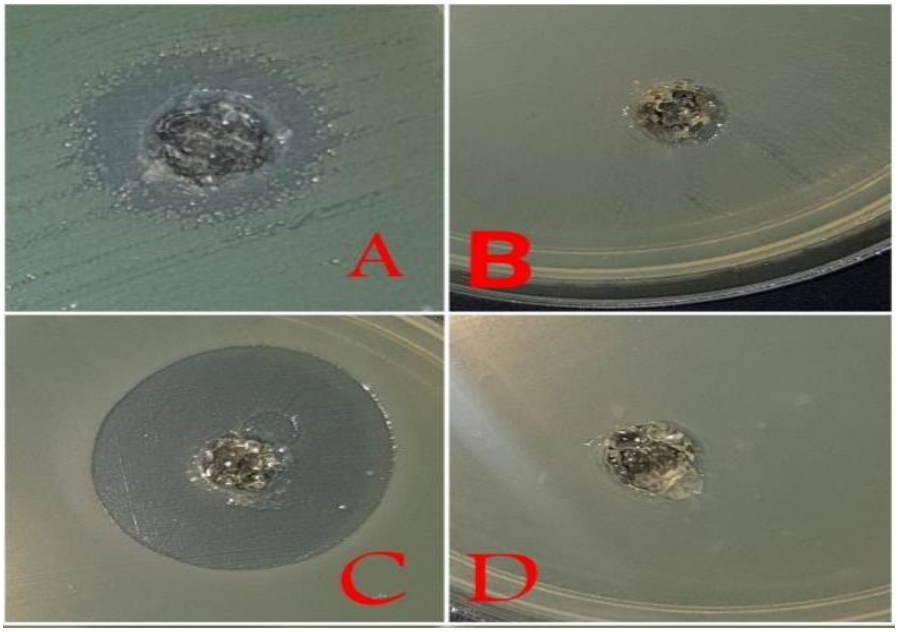

Figure 7-The antibacterial activity of L. crispatus IS30 cells in gel formula against A: P. aeruginosa, B: control for P. aeruginosa, C: E. coli, D: Control for E. coli

Conclusion

In conclusion, as a result of increasing the resistance to common antibiotics, it is necessary to find new strategies to compete pathogens. This study produced results which corroborate the findings of a great deal of the previous work on using bacteriocins as a good alternative to antibiotics. A cream formula contains *L .crispatus* IS30 cells is effective against some common vaginal pathogens. The results of this study confirm and supports previous research into the effectiveness of the cells-containing formula in terms of the viability of *L.crispatus* IS30 cells within the formula and its ability to produce and release bacteriocin without interaction formula components.

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