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Antagonistic Activity of Bacteriocin-producing *Lactobacillus* Against *Candida* spp

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Abstract

In order to screen antifungal activity of lactobacilli that produce bacteriocin against the yeast *Candida*, ninety-two food and clinical samples were collected. Also, several commercial brands of *Lactobacillus* probiotics and ready-made isolates of *Lactobacillus* were used. The isolated lactobacilli were subjected to microscopic, macroscopic, and biochemical tests. Moreover, molecular identification was performed for the best producer isolate. For *Candida* spp. isolation, seventy- two samples were collected from different clinical sources; in addition, nine of ready-made isolates were also used. All the isolated *Candida* spp. were subjected to microscopic and macroscopic examinations which were confirmed by VITEK® 2 YST card automated system. Detection of bacteriocin production from *Lactobacillus* was investigated by primary and secondary screening techniques and the results showed that the agar wells diffusion method was the best. The most efficient isolate to produce bacteriocin was *L. plantarum* WZD3, in which antifungal activity of bacteriocin was at the level of 80 and 40 AU/ml against the most sensitive *Candida* isolates; *C. albicans* CA and *C. albicans* CB, respectively. After partial purification of bacteriocin by n-butanol extraction method, bacteriocin activity was increased to 320 AU/ml against both yeast isolates. In conclusion, *L. plantarum* WZD3 or its bacteriocin could be a good candidate as antifungal agent to treat *Candida* infections and more studies are required to evaluate its acivity against other types of medical fungi.

Keywords: Antifungal, bacteriocin, *Candida*, *Lactobacillus*.

الفعالية التضادية للفطريات من العصيات اللبنية المنتجة للبكتريوسين ضد خميرة الكانديدا

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الخلاصة

لغرض التحري عن الفعالية المضادة للفطريات من العصيات اللبنية المنتجة للبكتريوسين ضد خميرة المبيضات ، تم جمع اثنين وتسعين عينة غذائية وسريرية ، كما تم استخدام بعض العلامات التجارية من المعززات الحيوية للعصيات اللبنية وبعض عزلات العصيات اللبنية الجاهزة. خضعت العصيات اللبنية المعزولة للاختبارات المجهرية والمزرعية والكيموحيوية. علاوة على ذلك ، تم تشخيص افضل عزلة منتجة جزيئيا. لعزل المبيضات ، تم جمع 72 عينة من مصادر سريرية مختلفة. كما تم استخدام تسع عزلات جاهزة. خضعت جميع المبيضات المعزولة للاختبارات المجهرية والمزرعية وتم تأكيد التشخيص بواسطة الفايتك

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(VITEK® 2 YST). تم التحري عن إنتاج البكتريوسين من العصيات اللبنية بواسطة تقنيات الغربلة الأولية والثانوية وأظهرت النتائج أن طريقة الانتشار في الحفر هي الأفضل. سميت العزلة الأكثر كفاءة في انتاج البكتريوسين بـ *Lactobacillus plantarum* WZD3 ، وكانت فعالية البكتريوسين المضادة للفطريات (40 و 80) وحدة/ مل ضد أكثر عزلات المبيضات تحسسا وهما *C. albicans* CA و *C. albicans* CB ، على التوالي. بعد التنقية الجزئية للبكتريوسين بطريقة الاستخلاص بالبيوتانول ، ازدادت فعالية البكتريوسين إلى 320 وحدة / مل ضد عزلتي الخميرة كلتيهما. في الختام، يمكن أن يكون *L. plantarum* WZD3 أو البكتريوسين مرشحًا جيدًا كعامل مضاد للفطريات لعلاج عدوى المبيضات ، وهناك حاجة إلى مزيد من الدراسات لتقييم فعاليته ضد الأنواع الأخرى من الفطريات الطبية.

Introduction

One of the most difficult diseases to control in human beings are fungal diseases [1], with mortality rate of 45% [2]. The mortality rates are still high in spite of the availability of many effective antifungal medicines [3]. Fungal species that belong to *Cryptococcus*, *Candida*, *Aspergillus*, *Histoplasma*, and *Pneumocystis* are responsible for more than 90% of all reported fungal-related deaths [4]. *Candida* spp. is one of the most common causes of invasive candidiasis and the most important one is *C. albicans* [5]. Development of resistant fungi and therapy failures following long-term use of antifungal drugs have been increased in immunocompromised patients [6]. Therefore, finding an alternative of some useful compounds, such as bacteriocin, for the better control and treatment of microbial infections has been suggested [7, 8, 9]. The fungicidal effect of plantaricins (Pln E/F and J/K) against *C. albicans* was investigated by Sharma and Srivastava [10]. Also, the investigation of Santos and co-authors [11] showed that bacteriocin produced from *Lactobacillus plantarum* has an anti-inflammatory effect against *C. albicans*. While, Graham *et al*.[12] demonstrated that bacteriocin EntV from *Enterococcus faecalis* has potential as a novel antifungal agent against *C. albicans*.

Bacteriocins of lactic acid bacteria (LAB) are simple to produce, constant at low pH, non-toxic to humans, and susceptible to proteases [13]. In general, the antimicrobial activity of LAB species is well known [14]. Various investigators demonstrated anticandidal effects of different *Lactobacillus* species [15-19] and other clinical isolates of *Lactobacillus*. The genus *Lactobacillus* that comprises 261 species [20] is part of the normal human microbiota that colonizes the mouth, gastrointestinal (GI) tract, and female genitourinary tract [21]. *Lactobacillus* comprises a high number of species which are generally recognized as safe [22]. Members of this genus contribute to the health status of the gastrointestinal tract and vagina. They also produce a wide variety of bacteriocins that demonstrate a wider range of activities towards bacteria or fungi [23]. This study aims to detect bacteriocin production by *Lactobacillus* spp. isolates and evaluate its antimicrobial effects against *Candida* spp., with the final aim of applying it therapeutically against this pathogenic yeast.

Materials and Methods

Lactobacillus Ready-made Isolates

Ten isolates of *Lactobacillus* spp. were obtained from the Department of Biotechnology, College of Science, University of Baghdad. In addition, six types of commercial brands of *Lactobacillus* probiotics including *L. acidophilus* capsules (Natrol ,USA) , *L. acidophilus* powder (HoneyCombs,USA), *L. acidophilus* tablet (Puritan's Pride, Inc. Holbrook , USA), *L. fermentum* sachets (Aptalis, Italy), *L. plantarum* (Swanson health products Fargo, USA), and *L. acidophilus* and *L. plantarum* capsules (Vitane pharma ,Germany) were obtained from pharmacies in Baghdad.

Candida Ready-made Isolates

A total of 9 *Candida* isolates, including 5 of *C. albicans*, one of *C. kefir*, and one of *C. parapsilosis*, were obtained from the Department of Biotechnology, College of Science, University of Baghdad. Two isolates of *C. albicans* were obtained from the Department of Biology, College of Science, Soran University in Erbil.

Collection of Samples and Isolation of *Lactobacillus*

A total of 92 samples were collected from different sources; 48 samples from food sources such as commercially unsweetened yogurt, house made yogurt, and fermented vegetables, and 44 samples from clinical sources (mouth, feces, and vagina) obtained from Al- Alwiya Hospital for Childbirth and Al-Imamain Al-Kadhimain Medical Hospital in Baghdad. For the isolation from clinical samples, the

swabs were immersed in 9 ml of de Man, Rogosa and Sharpe (MRS) broth medium tubes and transferred to the laboratory in ice box. For the isolation from food samples, each tube containing 9 ml of MRS broth was inoculated with 1 ml of liquid sample or 1 g of solid sample and mixed gently to get a uniform sample, separately. All the inoculated tubes were incubated anaerobically at 37°C for 48 hours. After that, serial decimal dilutions were made for each culture by using physiological saline solution and then they were streaked on MRS agar medium supplemented with 1% calcium carbonate. The plates were incubated anaerobically at 37°C for 48 hours. After incubation, large white colonies with intensity were selected, transferred, and purified by the streaking method on MRS agar and SL agar plates. The plates were incubated under the same incubation conditions above before being subjected to identification tests [24, 25].

Identification of *Lactobacillus* Isolates

The initial identification of the achieved pure cultures was based on Gram staining, the ability to grow on a selective MRS and SL agar [26], the ability to grow on nutrient agar, and biochemical tests, including catalase, oxidase, and gelatinase tests [27]. Molecular identification was performed by sequencing of 16S ribosomal RNA (16S-rRNA) for *Lactobacillus* isolates. Genomic DNA was extracted using ABIOpure™ Total DNA purification kit (ABIOpure, USA). The extracted DNA was estimated using Quantus (Promega, USA) with the Quantiflour dye. The primers (27F; AGAGTTTGATCCTGGCTCAG and 1492R; TACGGTTACCTTGTTACGACTT) [28] were supplied by Macrogen Company (Korea) in a lyophilized form. PCR products were sent for Sanger sequencing, using ABI3730XL automated DNA sequencer, by Macrogen Corporation (Korea) and the results were analyzed by using genious software.

Isolation and Identification of *Candida*

In this study, 72 samples were collected from different sources (skin, mouth and vagina) of patients (children and women) from Al- Alwiya Hospital for Childbirth and the Central Children's Teaching Hospital in Baghdad. The taken swabs were cultured on Sabouraud Dextrose agar (SDA), then incubated aerobically at 37°C for 24-48 hours [29]. All isolates were identified macroscopically and microscopically [30], and the identification was confirmed by VITEK® 2 YST card automated system (BioMérieux ,France).

Detection of Bacteriocin Production from *Lactobacillus*

Primary Screening

A. Agar-Plug Diffusion Method

A volume of 0.1 ml of 1.5×10^8 CFU/ml (according to McFarland tube No. 0.5) of fresh Lactobacillus broth cultures was spread on MRS agar plates and incubated anaerobically at 37°C for 24-48 hours. After incubation, sterile cork borer (6 mm) was used to cut plugs of cultured MRS agar plates. A volume of 0.1 ml of 10^6 cell/ml [31] of the activated yeast suspension was spread on SDA agar plates. After that, the plugs of cultured MRS agar were placed on the cultured SDA agar plates and incubated aerobically at 37°C for 18-24 hours. The formed inhibition zone around each plug was measured and recorded. Uninoculated MRS agar was used as a negative control [32].

B. Agar-Wells Diffusion Method

Lactobacilli (1.5×10^8 CFU/ml) were inoculated in MRS broth under anaerobic conditions at 37 °C for 24-48 hours. The cultures were centrifuged at 6000 rpm for 15 minutes. A volume of 0.1 ml of 10^6 cell/ml of the activated yeast suspension was transferred and spread on SDA agar. Wells cut into the pour plates by using a 6 mm sterile cork borer were filled with 100 µl of the cell-free supernatant (CFS). The plates were kept at room temperature for 2 hours and then incubated at 37°C for 18-24 hours. Finally, the inhibition zones formed around the wells were measured in mm and compared with that of control which contained MRS broth only [16].

Secondary Screening By Filter Paper Disc Method and Agar-Wells Diffusion Method

Lactobacilli (1.5×10^8 CFU/ml) were inoculated in MRS broth and incubated anaerobically at 37°C for 24-48 hours. Then, the CFS was obtained by centrifuging at 6000 rpm for 15 minutes. The CFS was neutralized to pH 6.5 with 1N NaOH and sterilized by filtration through 0.45 µm membranes. A volume of 0.1 ml of 10^6 cell/ml of the activated yeast suspension was transferred and spread on SDA agar. In the filter paper disc method, sterile filter paper discs, measuring 5 mm diameter and saturated with 100 µl of CFS, were placed on the seeded SDA agar plates [33]. While in the agar-wells diffusion method, wells cut into the pour plates with 6 mm sterile cork borer were filled with 100 µl of the CFS [16]. All the plates were left in laboratory temperature for 2 hours and then incubated aerobically at

37°C for 18-24 hours. The inhibition zones formed around the paper discs or wells were measured in mm and recorded.

According to the results of secondary screening, the most efficient isolate in the bacteriocin production of *Lactobacillus* spp. and the most sensitive isolates of *Candida* spp. were selected and used in the subsequent experiments.

Bacteriocin Activity Assay

To quantify the bacteriocin activity, CFS or crude bacteriocin was serially diluted two-fold with physiological saline solution. These dilutions were used to examine the antifungal activity of bacteriocin against the indicator yeast by agar well diffusion assay (as previously described). Bacteriocin activity was expressed as AU/ml and defined as the reciprocal of the highest dilution showing a distinct inhibition zone of the indicator yeast. AU was calculated as: (1000 / 100) × D, where 1000: constant, 100: volume of supernatant in a well (µl), and D : the dilution factor [34].

Partial Purification of Bacteriocin

MRS broth was inoculated with the bacterial isolate (D) and incubated at 37°C for 48 hours. Cells were harvested by centrifugation at 6000 rpm for 15 minutes. CFS was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15 minutes [35]. The supernatant was mixed thoroughly with n-butanol at a ratio of 1:1.The mixture was centrifuged at 4000 rpm for 10 minutes to achieve phase separation. The organic phase was evaporated at 65°C by rotary evaporator, then the sediment was re-suspended in 1.0 mM sodium phosphate buffer (pH 6) and referred to as partially purified bacteriocin (PPB) [36] . The antimicrobial activity of bacteriocin was determined, as previously described, by the agar well diffusion method.

Results and Discussion

Identification of Bacterial Isolates

The results showed that 22 of the bacterial isolates appeared as having white colored, soft, large or small, convex, creamy, smooth, and circular colonies with entire margins and surrounded by clear zones after being cultured on MRS agar containing calcium carbonate, as a result of dissolving it by the acid produced by the bacteria. *Lactobacillus* showed white colored, convex, large, slightly mucoid, smooth, and round forms with entire margins on SL agar, whereas no colonies on nutrient agar were developed. Under microscope, they were purple rods, singly, in pairs, or in short chains, and non-spore forming. They were catalase, oxidase, and gelatinase negative [26, 37] . Moreover, the most efficient isolates (D and L1) in the bacteriocin production were selected for molecular identification to confirm the diagnosis. According to16S rRNA analysis, the two isolates were identified as *L. plantarum* (Table -1).

Table 1-Identification of *Lactobacillus* isolates using 16S rRNA gene sequencing.

Isolate	Source of isolation	The nearest matched species from GenBank	Identity (%)*	Accession Number
D	Yogurt	*Lactobacillus plantarum* strain 2.28.10	100%	MK611396.1
L1	Yogurt	*Lactobacillus plantarum* strain *ASAL A.DH.Z*	100%	MT126340.1

*Identity values were determined using the basic local alignment search tool (BLAST) of the GenBank.

Identification of Yeast Isolates

Under microscopic examination, the shape of yeast cells was spherical to oval, with the presence of budding, and they were much larger than bacterial cells. On SDA, yeasts appeared as raised, glossy, smooth, glabrous yeast-like, with distinctly smelling, white to cream colored, and circular to oval colonies [38, 39]. Moreover, the identification of these isolates was confirmed by Vitek2 YST card automated system. The results showed that 24 isolates were identified as *Candida* spp., 12 isolates as *C. albicans* (10 from mouth and 2 from vagina), 6 isolates as *C. famata* (one from vagina and 5 from mouth), *C. dubliniensis* and *C. parapsilosis* from vagina, and *C. guilliermondii*, *C. tropicalis*, C. *ciferrii* and *C. catenula* from mouth.

Detection of Bacteriocin Production from *Lactobacillus*

All isolates which were confirmed through the identification process, as well as the commercial brands of *Lactobacillus* probiotics, were subjected to primary and secondary screening techniques in order to select the most efficient isolate as bacteriocin producer.

Primary Screening

The results showed that the agar-plug diffusion method did not demonstrate any antifungal activity, while the agar well diffusion method demonstrated this activity. Among 38 isolates of *Lactobacillus*, only 20 isolates recorded inhibition zones against 17 isolates of *Candida* (Table- 2). While 18 isolates of *Lactobacillus* did not show any antifungal activity, since no inhibition zones were recoded. Thus, the selection of isolates for the secondary screening was relied on the results of this method. The ability of *Lactobacillus* isolates to inhibit yeast growth is clearly strain and culture condition-dependent, with antimicrobial substances such as bacteriocins and mechanisms being involved in the inhibition process *in vitro*, as reported by [40, 41].

Secondary Screening

In the secondary screening, only three isolates of *L. plantarum* (D and L1) and *Lactobacillus* sp.(L2) had antifungal activity against only 11 yeast isolates; 7 isolates of *C. albicans* (C1,C3, C4,C8, C40, CA and CB), *C. famata* (C15), *C. guilliermondii* (C17), *C. parapsilosis* (C30), and *C. dubliniensis* (C33) . The isolate D was the most active one. Also, the agar wells diffusion method was more efficient than the other method (Tables- 3 and 4). Thus, depending on the results of the secondary screening, the most efficient *Lactobacillus* isolate (D) was selected as a bacteriocin producer and called *L. plantarum* WZD3. Also, the most sensitive *Candida* isolates, namely *C. albicans* CA and *C. albicans* CB, were selected as indicator yeasts due to heir higher sensitivity toward *Lactobacillus* isolates, where the diameters of the inhibition zones of *L. plantarum* WZD3 against *C. albicans* CA and *C. albicans* CB were 20 and 30 mm, respectively. The activity of bacteriocin against *C. albicans* CA was at the value of 80 AU/ml, whereas that against *C. albicans* CB was 40 AU/ml (Figure-1).

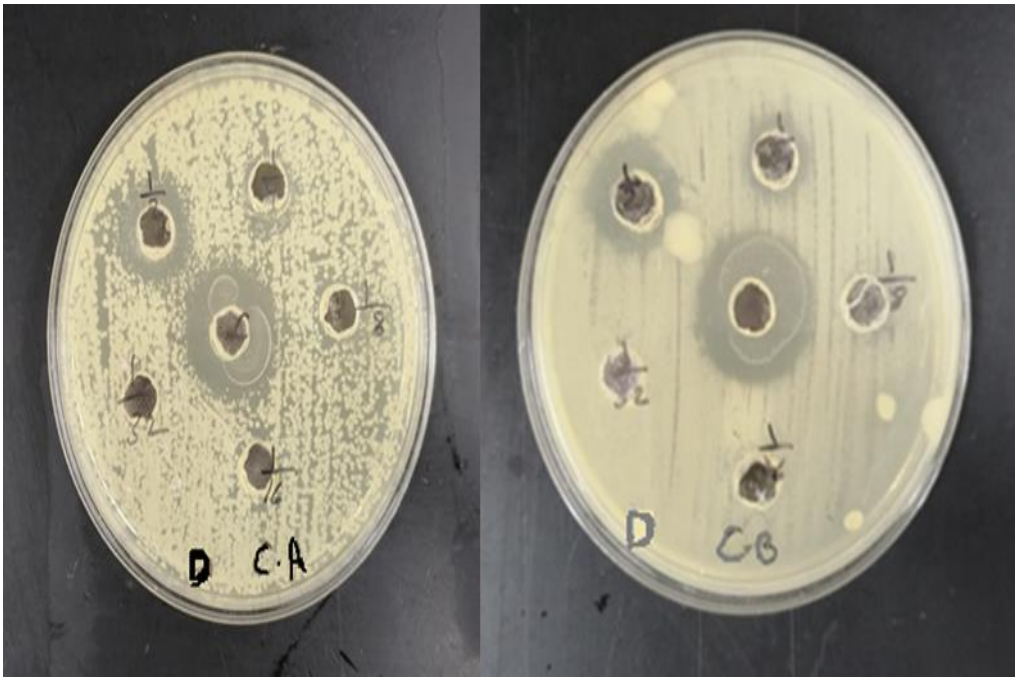

Figure 1- Antifungal activity of bacteriocin produced by *Lactobacillus plantarum* WZD3 against *Candida albicans* CA and *Candida albicans* CB.

Table 2-Antifungal activity of *Lactobacillus* isolates against *Candida* isolates tested by agar-wells diffusion method in the primary screening.

Lb. isolates	Antifungal activity against *Candida* isolates (diameters of inhibition zones, mm)																
	C1	C3	C4	C8	C15	C16	C17	C21	C30	C33	C39	C40	C51	C53	C27s	CA	CB
B	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	15	18	14	15	20	14.5	14	-	14	-	-	-	-	-	-	22	24
F	15	-	13	10	-	-	13	-	10.5	9	11	10	-	-	-	-	-
J	15	-	17	-	-	-	-	-	12	-	-	-	-	-	-	-	-
K	14	-	16	-	-	-	12	-	10.5	-	-	-	-	-	-	-	-
1	12	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lb1	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-
Lb2	10	-	10	-	-	10	-	-	11	-	-	-	-	-	11	-	-
Lb3	10	-	-	-	-	-	10	-	10	-	-	-	-	-	-	-	-
Lb5	-	-	-	-	-	10	11	-	-	-	-	-	-	-	-	-	-
Lb6	9	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
Lb7	10	-	-	-	-	-	-	12	-	-	-	-	10	-	-	-	-
Lb8	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lb9	10	-	-	-	-	-	-	12	-	-	-	8	-	11	-	-	-
Lb10	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L1	18	-	14	-	-	-	13	-	14	-	-	-	-	-	-	20	17
L2	13	-	14	-	-	-	-	-	12.5	14	-	10	-	-	-	-	-
L4	-	-	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L5	12	-	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L9	18	-	16	-	-	-	-	-	13	-	-	-	-	-	-	-	-

(-) No inhibition zone , (C1, C3, C4, C8, C40, C51, C53, CA, CB) *Candida albicans*, (C15and C21) *Candida famata*, (C16) *Candida tropicalis*, (C17) *Candida guilliermondii*, (C30) *Candida parapsilosis*, (C33) *Candida dubliniensis*, (C39) *Candida ciferii* ; (Lb.) *Lactobacillus* (F,J,K, L2, 1,Lb1,Lb2,Lb3,Lb5 and Lb6) *Lactobacillus spp.* isolated from different sources; (Lb7,Lb8,Lb9,Lb10,) *Lactobacillus spp.* ready-made; (B,D and L1) *Lactobacillus plantarum*, (L4) *Lactobacillus acidophilus* (Capsules), (L5) *Lactobacillus fermentum* (Sachets), (L9) *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Capsules).

Table 3- Antifungal activity of *Lactobacillus* isolates against *Candida* isolates tested by filter paper disc method in the secondary screening.

Lactcobacillus isolates	Antifungal activity against *Candida* isolates (diameters of inhibition zones, mm)										
	C1	C3	C4	C8	C15	C17	C30	C33	C40	CA	CB
D	9.5	10	11	-	9	11	-	-	-	14	14
L1	8	10	10	-	9	8	-	-	-	12	12
L2	-	-	9	-	-	-	-	-	-	8	10

Table 4- Antifungal activity of *Lactobacillus* isolates against *Candida* isolates tested by agar-wells diffusion method in the secondary screening.

***Lactcobacillus* isolates**	**Antifungal activity against *Candida* isolates (diameters of inhibition zones, mm)**										
	C1	**C3**	**C4**	**C8**	**C15**	**C17**	**C30**	**C33**	**C40**	**CA**	**CB**
D	10	18	16	15	15	18	16	15	10	20	30
L1	10	-	-	-	-	-	-	-	-	15	29
L2	12	-	-	-	-	-	12.5	-	-	-	-

(-) No inhibition zone, (C1, C3, C4, C8, CA and CB) *Candida albicans*, (C15) *Candida famata*,(C16) *Candida tropicalis*,(C17) *Candida guilliermondii*, (C30) *Candida parapsilosis*, (D and L1) *Lactobacillus plantarum*, and (L2) *Lactobacillus* spp.

In antimicrobial activity researches, the agar plug method is considered as a practical and suitable technique and the filter paper disc method is regarded as appropriate and easy to use. Nevertheless, in the present investigation, it was observed that agar wells diffusion method was more appropriate and more effective than the filter paper disc method and the agar plug method. It is mainly based on forming a zone of inhibition around the well where the indicator has not grown, and the size of the zone is based on how much the antimicrobial compound is effective. Therefore, this method was used in this study to define the antimicrobial activity because of its sensitivity to determine the antifungal activity in next steps, as reported previously [42, 43].

Partial Purification of Bacteriocin

Crude bacteriocin extract was heated before starting purification to denaturize proteases and any heat-labil proteins, as investigated by Powell *et al*. [35]. The heating step did not affect bacteriocin activity, as reported earlier [44]. Bacteriocin partially purified by extraction with n-butanol was named plantaricin WZD3, according to the producer isolate, *L. plantarum* WZD3. By using this method for purification, the antifungal activity of bacteriocin reached to 320 AU/ml against both of *C. albicans* CA and *C. albicans* CB, compared with the antifungal activity results of crude bacteriocin extract, which was only 80 AU/ml against *C. albicans* CA and 40 AU/ml against *C. albicans* CB, respectively (Figure -2). Butanol extraction exhibited complete recovery of bacteriocin activity, suggesting that at least part of the bacteriocin molecule has a hydrophobic character and shares this property with other bacteriocins [45, 46]. Researchers such as ten Brink *et al*. [47] showed that butanol extraction yields almost pure (≥ 90%) bacteriocin, and that this simple procedure could be adopted for partial purification of bacteriocins. Extraction of bacteriocins using n-butanol in a 1:1 ratio was reported for several plantaricins [36, 44, 48].

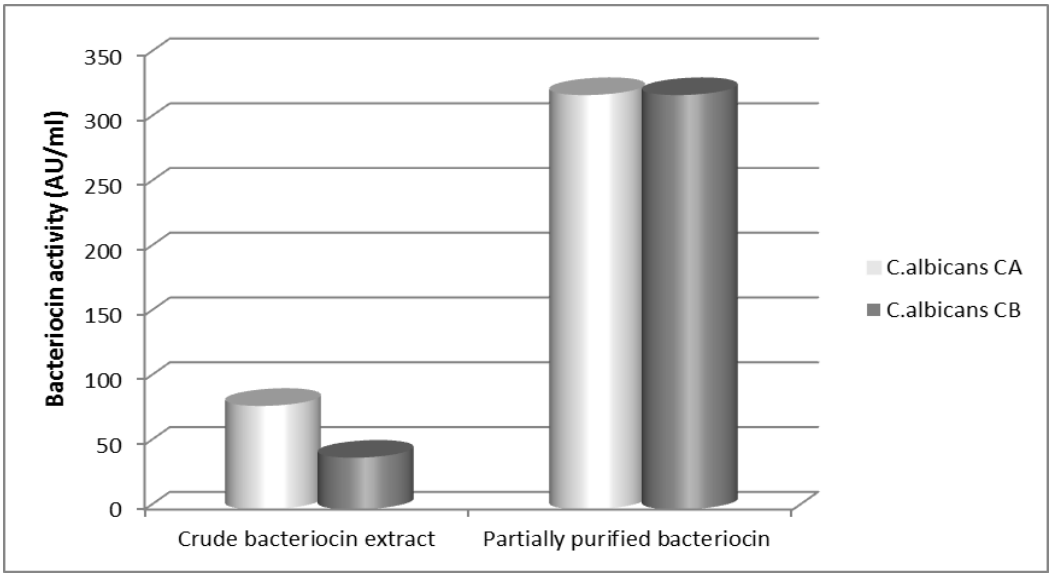

Figure 2-Antifungal activity of partially purified bacteriocin produced from *Lactobacillus plantarum* WZD3 by n-butanol extraction method in comparison with crude bacteriocin extract against indicator yeast

Conclusion

In the present study, *Lactobacillus* isolates exhibited variations in their antagonistic activity and bacteriocin production against *Candida* spp. It is clear that the capability of bacterial isolates to inhibit the growth of yeast *in vitro* is related to the isolation and culturing conditions, as well as, the used method for detection of antifungal activity and also mechanisms which involved in the inhibition process as mentioned by Siro [49]. *L. plantarum* WZD3 and its bacteriocin could be used as antifungal agents against *Candida* infections instead of antibiotics, but more investigations are actually needed to evaluate the antifungal activities of lactobacilli and their bacteriocins.

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