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Antimicrobial and Antibiofilm Activity of Local Wheat Bran Extract and Bacteriocin Combination Against Gastrointestinal Pathogens

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Abstract

Gastrointestinal microbial infections have increased especially between children and represent a community burden. The present experiment was designed to examine the combined impact of local wheat bran bioactive compounds and Bacteriocin over locally isolated pathogens including *S. aureus*, *E. coli* and *C. albicans* and evaluate their antimicrobial and antibiofilm eligibility. Local wheat Ibaa99 bran extract consists of phenolic compounds, phytic acid and inulin, Bacteriocin was purified from *Lacticaseibacillus paracasei* that was identified as a new strain (namely MOIQ35) by the 16S rRNA gene using the PCR technique and after its registration with The National Center for Biotechnology Information (NCBI). The combined antimicrobial activity was examined by well diffusion assay and MIC method at concentrations 1 mg/ ml for wheat bran extract and 0.54 mg/ml for bacteriocin. Meanwhile, antibiofilm dispersal considerations have been completed by the microtiter plate method. The outcomes revealed that the highest inhibition observed over *S. aureus* isolates ranged from 21.5 ±0.1 mm to 40 ±0.1 mm, and the highest inhibition observed over *E. coli* isolates ranged from 18 ±0.1 mm to 38 ±0.1 mm, and 38.5 ±0.1 mm against C. albicans. Moreover, the biofilm suppression tests showed a high reduction in biofilm against *C. albicans* isolate with OD (0.11). In conclusion, the extract of Ibaa99 wheat bran in combination with Bacteriocin has synergistic antibacterial and antifungal effects and is a biofilm repressor.

Keywords: Antibacterial, Antifungal, Bacteriocin, Biofilm, Wheat bran extract.

Introduction

Cereal crops are part of the domain sources of human food consumed, Iraq is one of the developing nations targeted in its economic and food security and expanding in the production of wheat crops[1]. Wheat is an essential food raw product[2], Within the family Poaceae, Wheat is a major grain crop planted worldwide, in Iraq, wheat is grown in the winter season, bread wheat was domesticated since 9500 years BC in this country[3]. Wheat bran (WB) is the main by-product of wheat milling industry, with an estimated yearly production of 150 million metric tons worldwide mostly used in the feed field[4]. Wheat bran has been detailed to display a concentration of phenolic acids such as; Vanillic acid, *p*-coumaric acid, Ferulic acid, Gallic acid, Chlorogenic acid, Caffeic acid in addition to Benzoic acid. Nevertheless, the concentration of phenolic compounds in wheat bran is not well recognized due to the shortage in studies to provide further information[5]. Probiotics are group of

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microorganisms that have beneficial impacts on humans usually achieved by different mechanisms like the production of inhibitory metabolites such as bacteriocins[6]. *Lacticaseibacillus paracasei* (previously termed *Lactobacillus paracasei*) isolated from several sources including dairy products, may be associated with to their health-promoting characteristics mainly antimicrobial and antibiofilm activities[7]. Current studies have revealed that high-volume diarrhea considerably might be caused by *S. aureus* especially with exposure to broad-spectrum antibiotics[8]. Moreover, *E. Coli* causes gastroenteritis and diarrhea and might impair intestine including inflammatory response[9]. *Candida albicans* represents the most common yeast species isolated from human faces, Diarrheal diseases are a major cause of childhood morbidity and mortality globally including Iraq[10]. The target of the current trials is to evaluate the antibacterial, antifungal and antibiofilm effects of new agents extracted from new sources that are cost effective and commonly used as by-product especially wheat bran in combination with bacteriocin purified from new local probiotic strains, might provide a new insight into modern cost-effective metabolites for pharmaceutical purposes.

Materials and Methods

Preparation of wheat bran extract

Samples of local cultivar wheat grains were collected from AL Dourah Silo, Grain Board of Iraq, and belong to the harvest season 2021/2022. The samples were identified for specific variant types through characterizing the physical properties analysis, including kernel dimensions, color, shape, sphericity, thousand kernel weight, and bulk density according to[11]. Then, the chosen wheat sample was subjected to the milling process Fig. 1. Following[12]. Moreover, Wheat Bran active metabolites were extracted with an ethanol solvent ratio (ethanol: water, 80:20, v: v) with percentage (wheat bran: solvent, 1:10) according to[13]. Wheat bran extract (WBE) composition was confirmed by High Performance Liquid Chromatography (HPLC) technique[14]. Finally, the concentration of resulted compounds was calculated according to the equation: C sample = C (St) x A (sample) / A (St) * D.F/ Wt. Were (C: concentration, St: standard, A: area, DF: dilution factor, Wt.: sample weight)[15].

Wheat grains **Wheat Bran**

Figure 1. Ibaa99 wheat grains and its bran after milling process.

Collection, isolation and Identification of *Lacticaseibacillus* spp.

Thirty-six samples from different dairy products were gathered for obtaining *Lacticaseibacillus* species, cultivated in sterile tubes containing de Man, Rogosa and Sharpe (MRS) broth and then incubated at 37ºC for 24hours in microaerophilic conditions in a candle jar, then re-cultured as a single colony and spread in the selective MRS agar medium plate (pH 6.5) at 37ºC for 24hours[16]. Furthermore, Morphological and biochemical tests in the current study were identified according to the morphological tests which included, shape of colonies, size, texture of colonies, production of pigment. Biochemical tests included oxidase, catalase and indole tests[17]. Moreover, *Lacticaseibacillus* isolates was subjected for an advance confirmative molecular identification by the 16 S rRNA[18].

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Partial purification of bacteriocin

Precipitation of bacteriocin was carried out by using ammonium sulphate[19]. The cell free supernatant (CFS) of *lacticaseibacillus paracasei* culture was prepared from 400 ml of 24hours grown culture of the probiotic. The CFS was transferred into a beaker placed in an ice bath on a magnetic stirrer. Additionally, according to the specific addition standard, an exact amount of ammonium sulphate (NH4)2SO4 was added gradually to the CFS to achieve 30, 40, 50, 60, 70, and 80 percent (w/v) saturations, respectively, through slow and constant stirring at 4ºC. Notably, the stirring was continued for an additional 30min. furthermore, the precipitate formed in every saturation level was separated by centrifugation for 30min at 10000 rpm. Then, supernatant was decanted and the precipitates were redissolved in phosphate buffer (0.1M, pH 7.2) in an appropriate volume. Further purification was carried out by gel filtration chromatography via Sephadex G-150 gel. The gel was loaded carefully in the column to obtain 1.6 × 21 centimeters (diameter: height). The column was equilibrated with 0.1M phosphate buffer (pH 7.2). The dialyzed protein sample (3 ml) was then applied to the column and proteins were eluted using 0.1M phosphate buffer (pH 7.2) at room temperature. The flow rate was adjusted to 20 ml/hour and fractions of 3 ml were collected. forty fractions were collected and the absorption of these fractions was measured at 280 nanometers by UV-spectrophotometer. The fractions were tested for antimicrobial activity against indicator strains by well diffusion assay. Fractions showing antimicrobial activity were mixed in one tube and protein concentration and bacteriocin activity were determined[20].

Collection and isolation of pathogenic samples

Exactly 203 clinical samples (*Staphylococcus aureus, Escherichia coli* and *Candida albicans*) were obtained from children's patients of both genders (males and females) aged between 2- 10 years old suffering from gastrointestinal infections and diarrhea who settled in Al-Yarmouk Teaching Hospital, Baghdad, Iraq. All collected clinical specimens that represent three pathogenic indicators were grown under each required condition, *S. aureus* specimen was incubated aerobically on Blood agar at 37°C for 24hours, *E. coli* was at 37°C for 24 hours on MacConkey agar and *C. albicans* cultured on Sabouraud dextrose agar at 37°C for 24 hours. Furthermore, after incubation and, isolates suspected to belong to *E. coli, S. aureus as* well as *C. albicans* were taken for primary identification when subjected for macroscopic tests depending on colony morphology (shape, size, color and texture), microscopic and biochemical tests[5], also isolates were subjected for VITEK 2 system identification[21].

Antimicrobial assays

The agar well diffusion method[22] was applied to screen the antimicrobial activity of both the crude wheat bran extract, the plant material was dissolved in 2 percent of Dimethyl sulfoxide and bacteriocin concentration was obtained after purification process, the concentration was 1 mg/ ml for WBE and 0.54 mg/ml for bacteriocin in combination status (v: v, 50:50) against three pathogenic microbes (*S. aureus, E. coli* and *C. albicans.* The inhibition zones measurements[23] were measured in millimeters (mm) as: (Inhibition zone (mm)= Diameter of growth inhibited zone- Diameter of the well). Additionally, The Minimum Inhibitory Concentration (MIC) has been completed as a second trial to investigate the combined efficiency of both agents over used indicator microbes when the bioactive compound in merge status has been quantified as subjected to the twofold dilution trial by utilizing a microtiter plate[24].

Detection of biofilm formation

The Congo Red Agar (CRA) assay is a qualitative method for detection of biofilm producing microorganisms which depends on colonies color change grown on the medium. Moreover, Microtiter plate (MtP) method is a quantitative assay used to determine biofilm through the microplate reader[25].

Antibiofilm assay

Microtiter plate (MtP) method[24] is used to determine the antibiofilm efficiency of wheat bran extract and Bacteriocin combination, after incubation, part of a grown colony of each isolate was suspended in normal saline, then the concentration of all isolate's suspensions was equilibrated with 0.5 McFarland standards. Furthermore, exactly 180 µl of Mueller-Hinton broth was added to each of the 96 wells, and then, only 20 µl of the combined agents was added to it. Notably, each well was washed twice with 200 µl of phosphate buffered saline and dried for 30 min at 60°C before staining with 150 µl of crystal violet for only 15 min. After staining, the plates were again washed twice with PBS. After that, the microplate was kept at room temperature for drying; the dye bound to the cells was re-solubilized by eluting the

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attached cells with exactly 150 µl of 96% ethanol per well. Thereafter, the microtiter plate was covered with the lid and left still for at least 10 min at room temperature. Stained biofilms were measured at 560 nm using microtiter reader. Each strain was examined in triplicate and mean was taken.

Results and Discussion

Identification of wheat bran active compounds

The results of wheat grains sample identification were Ibaa 99 variety type, the identification of the bioactive metabolites of this variety concluded within Fig. 2 into phenolic compounds with total concentration 5059 ppm as shown within Fig. 3 containing (Vanillic Acid 2162 ppm, Ferulic acid 973 ppm, Rutin 919 ppm, p-coumaric acid 560 ppm and apigenin 445 ppm) and inulin with 598 ppm in addition to phytic acid with 3.12 ppm. In a similar pattern[25], obtained much close results.

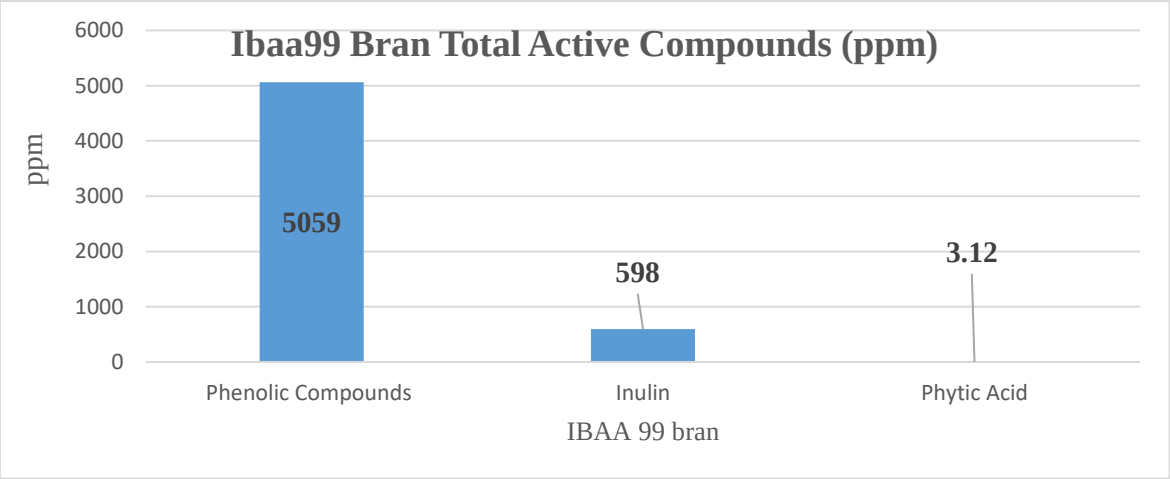

Figure 2. The total content of bioactive metabolites for Ibaa99 wheat bran.

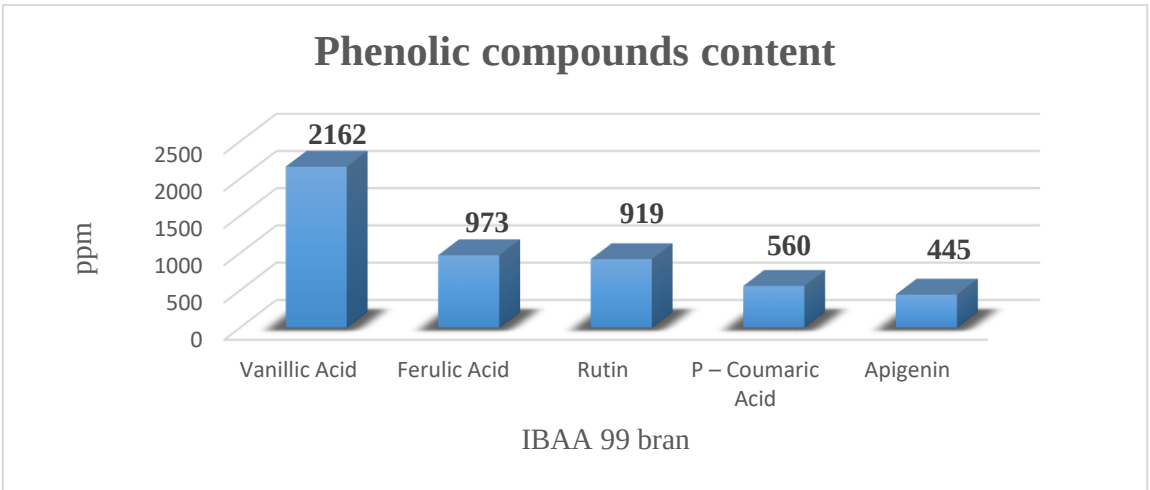

Figure 3. The phenolic compounds content for Ibaa99 wheat bran.

Identification of *Lacticaseibacillus* spp.

All colonies grown on De Man–Rogosa–Sharpe agar were rounded in shape, and ranged in consistency from creamy white to glossy white with a moist-mucoid colony appearance on the surface. The results of microscopic examination showed that the isolates were Gram positive with long or short they were negative to catalase and oxidase biochemical tests. Depending on the 16S rRNA-based molecular identification of one capable isolate of

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Lacticaseibacillus paracasei to produce bacteriocin and to confirm its identification, the 16S rRNA gene amplification was performed using PCR technique to detect the positive result as imaged in Fig. 4, a new strain of *Lacticaseibacillus paracasei* namely (MOIQ35) with identity percentage 99% were obtained from local homemade yoghurt sample has been detected and registered in The National Center for Biotechnology Information (NCBI), as[26] registered a new strain of *L. paracasei* by the same fashion.

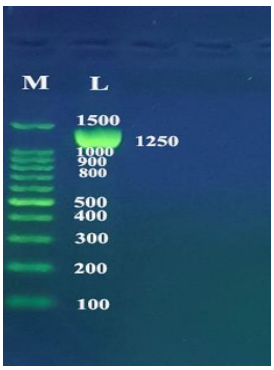

Figure 4. The agarose gel electrophoresis of amplified 16S rRNA gene using *Lacticaseibacillus paracasei* (L) primers.

Purification of Bacteriocin

Based on the results, maximum Bacteriocin precipitation was obtained at 80 percent saturation level. As indicator strains, *S. aureus, E. coli and C. albicans* were used to confirm the effectiveness of the formula, 15 ml of bacteriocin with 0.54 mg/ ml of concentration have been yielded as calculated within Table 1. The results matched [27] results when partially purified bacteriocin at 80% saturation.

Table 1. Summary of purification of Bacteriocin from crude culture filtrate of *Lacticaseibacillus paracasei.*

Purification Step	Volume (ml)	bacteriocin activity (AU/ml)	Protein Conc. (mg/ml)	Specific activity (AU/mg)	Total activity (AU)	Purification (folds)	Yield %
Crude Extract	40	40	0.23	173.9	1300	1	100
Concertation with sucrose	12	40	0.25	160	900	0.92	30
Sephadex gel G-150	15	40	0.54	740.7	600	4.3	37.5

Identification of pathogenic microbes

According to microscopic, microscopic and biochemical tests (catalase, oxidase and VITIK 2 system) isolates of pathogenic microorganisms proved to be *S. aureus, E. coli* and *C. albicans,* as [28] did.

Antimicrobial effect of Wheat Bran Extract-Bacteriocin combination

The inhibitory effect of WBE- Bacteriocin with the concentration of 1 mg/ ml for WBE and 0.54 mg/ml for bacteriocin in combination status (v: v, 50:50) against *S. aureus* isolates explicated that all isolates were affected as shown there in Fig. 5, the highest inhibition zone (40 mm) in dimeter average was recorded for the isolate (symbolled St 17), while the lowest inhibition zone diameter was (21.5 mm) for isolate (symbolled St 44). The outcomes were close to[29]. Moreover, the inhibitory effect towards the three *E. coli* isolates depending on the well diffusion method illustrated that all isolates were affected based on Fig. 6, the highest inhibition zone (38 mm) in dimeter was recorded for isolate (symbolled Ec 67) and much close diameter for the isolate (symbolled Ec 28) when recorded (21.5 mm), while the lowest inhibition zone diameter was (18 mm) for isolate symbolled Ec 51. The inhibitory impact over *C. albicans* isolates (symbolled Ca 37) leaded to

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inhibition circle measured exactly (38.5 mm) diameter as Showed by Fig. 7. In a parallel pattern[30] obtained similar results.

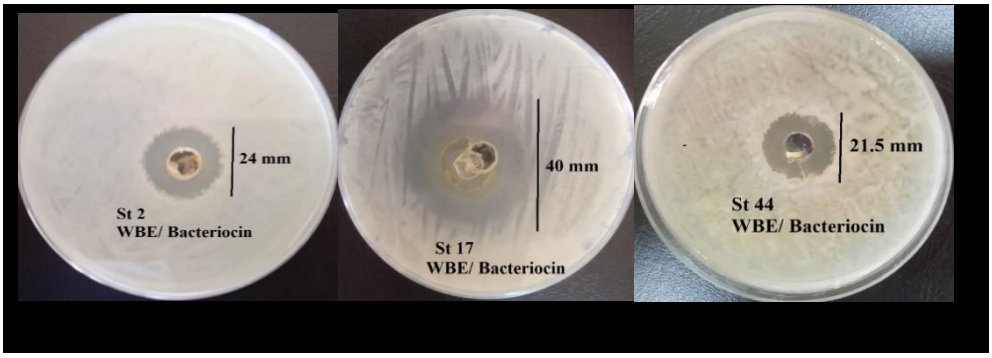

Figure 5. Antibacterial activity of WBE/ Bacteriocin by well diffusion assay against *Staphylococcus aureus* isolates (symbolled St 2, St 17 and St 44) at 37ºC for 24hrs.

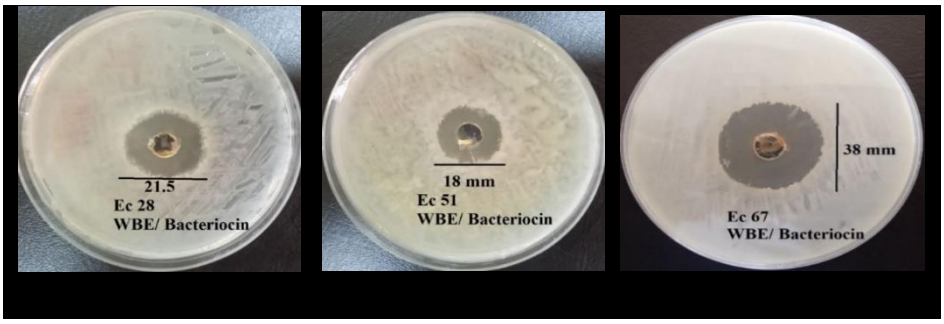

Figure 6. The antibacterial activity of WBE/ Bacteriocin against *Escherichia coli* isolates symbolled (Ec 28, Ec 51 and Ec 67) by well diffusion technique at 37ºC for 24hrs.

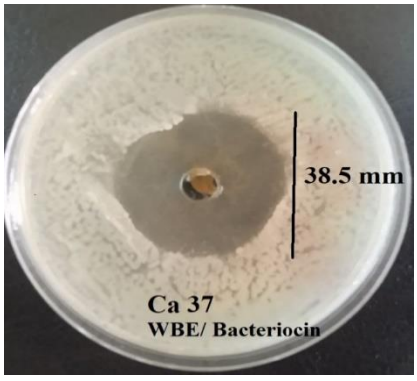

Figure 7. Antibacterial activity of WBE/ Bacteriocin by well diffusion process *Candida albicans* isolate (symbolled Ca 37) at 37ºC for 24hrs.

The Minimum Inhibitory Concentrations (MICs)
According to the series of dilution on microtiter plate method, WBE/ bacteriocin combination had minimum inhibitory concentration for all tested microorganisms (*S. aureus*, *E. coli* and *C. albicans* isolates) color of agents inside the plate comparing to the control had transformed from blue to pink or pale pink and the MIC data calculated to (1/0.05) of WBE (mg/ml)/Bacteriocin (mg/ml) for all pathogenic isolates indicated in the trial. Although the MIC of the combination of wheat bran extract/Bacteriocin has been determined in the present scientific trial, may be no clue for the availability of other studies in the same pattern to be compared[31] estimated by their tests for bacteriocin minimum inhibitory concentration against *E. coli* and *S. aureus* was 0.45 mg/ml only.

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Detection of biofilm formation

After incubation of *S. aureus*, *E. coli* and *C. albicans* isolates were found can form biofilm when black colonies were observed for the biofilm formation via Congo red agar, which matches[32] results in other experiments. Additionally, the results of Microtiter plate (MtP) method for biofilm production by *S. aureus* charted in Fig. 8, *E. coli* as numerated by Fig. 9 and *C. albicans* is shown within Fig. 10 illustrated the strong and moderate formation of biofilm for the chosen isolates.

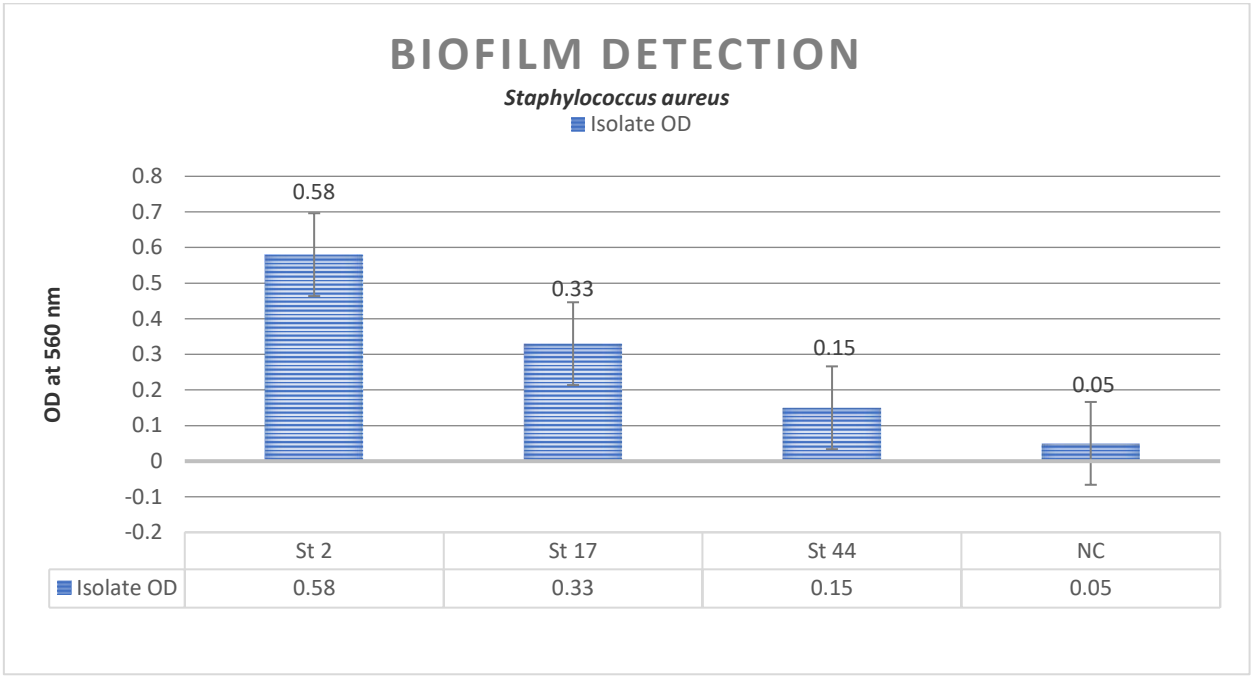

Figure 8. Detection of biofilm formation by *Staphylococcus aureus* isolates (symbolled St 2, St 17 and St 44) depending on microtiter plate method, NC: Negative Control.

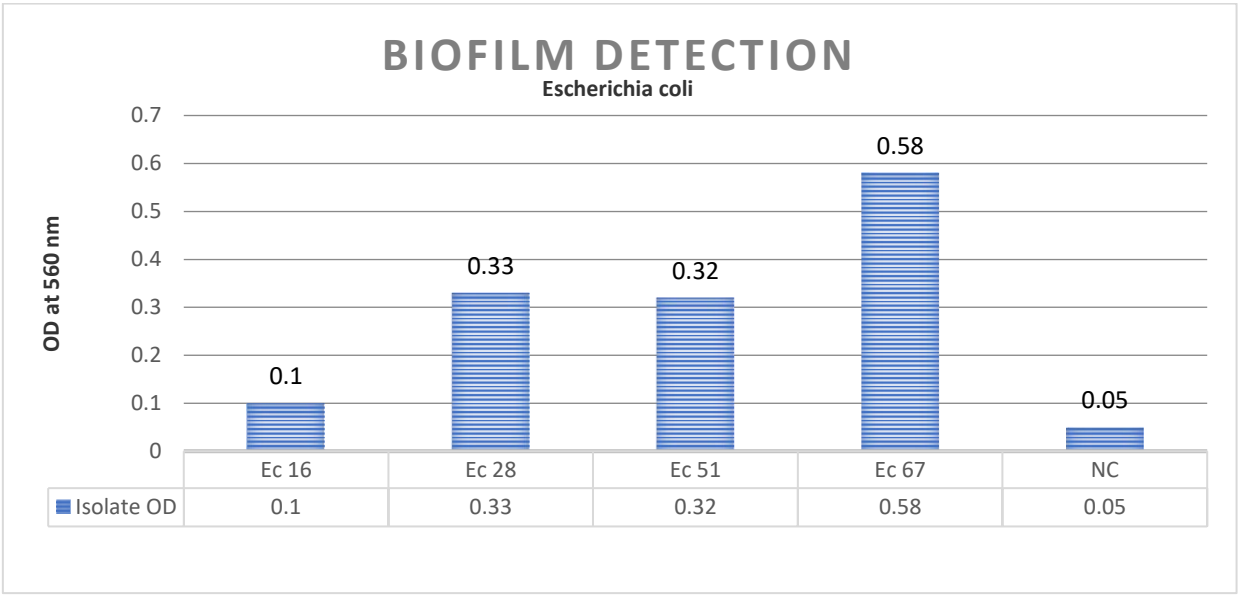

Figure 9. Detection of biofilm formation by *Escherichia coli* isolates (symbolled Ec 16, Ec 28, Ec 51 and Ec 67) depending on microtiter plate method, NC: Negative Control.

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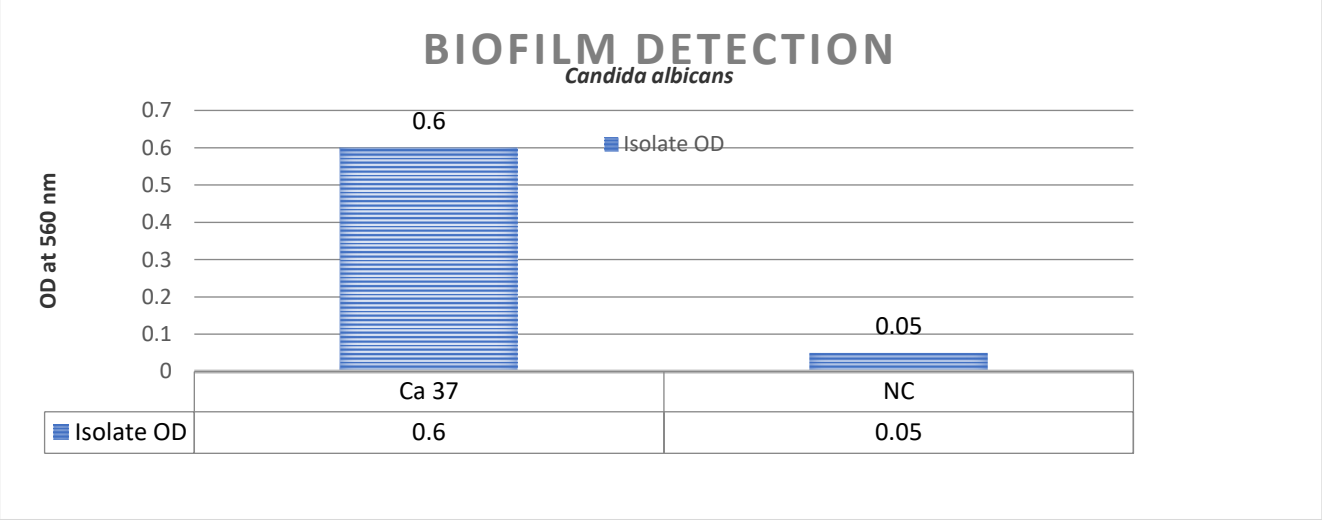

Figure 10. Detection of biofilm formation by *Candida albicans* isolate (symbolled Ca 37) depending on microtiter plate method, NC: Negative Control.

Antibiofilm activity of Wheat Bran Extract-Bacteriocin combination

Biofilm produced by *S. aureus* has been eradicated by WBE- Bacteriocin when the recorded averages of biofilm OD values were reduced from averages of each isolate control as shown in Fig. 11. However, the highest reduction in biofilm of OD (0.3) was registered in the isolate (symbolled St 2). Moreover, the highest reduction in *E. coli* isolates biofilm OD (0.08) was registered in the isolate (symbolled Ec 28) charted by Fig. 12. Also, Biofilm produced by *C. albicans* isolates (symbolled Ca 37) has been eradicated in the same manner when recorded an average of biofilm OD value (0.11) was reduced from the average of the isolated control (0.58) according to Fig. 13. For comparative studies, perhaps there is no evidence for articles handling the exact combination of wheat bran extract and bacteriocin. Gallic acid as plant extract when combined to bacteriocin suppressed *L. monocytogenes* biofilm[33].

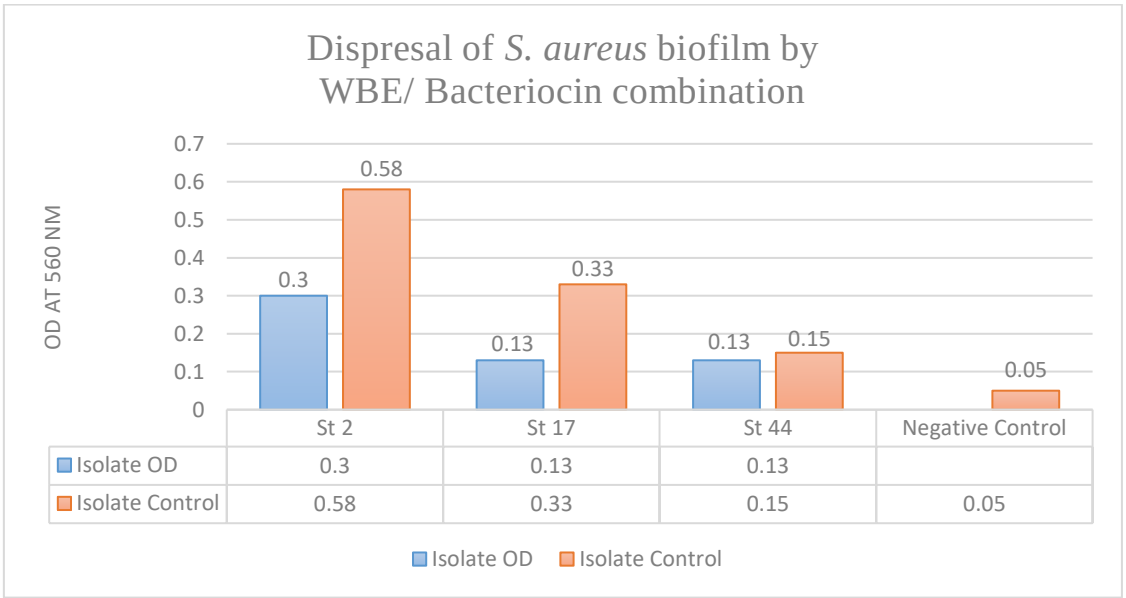

Figure 11. The antibiofilm effect of Ibaa99 bran extract (WBE)/ Bacteriocin derived from *Lacticaseibacillus paracasei* on *Staphylococcus aureus* isolates biofilm using microtiter plate assay.

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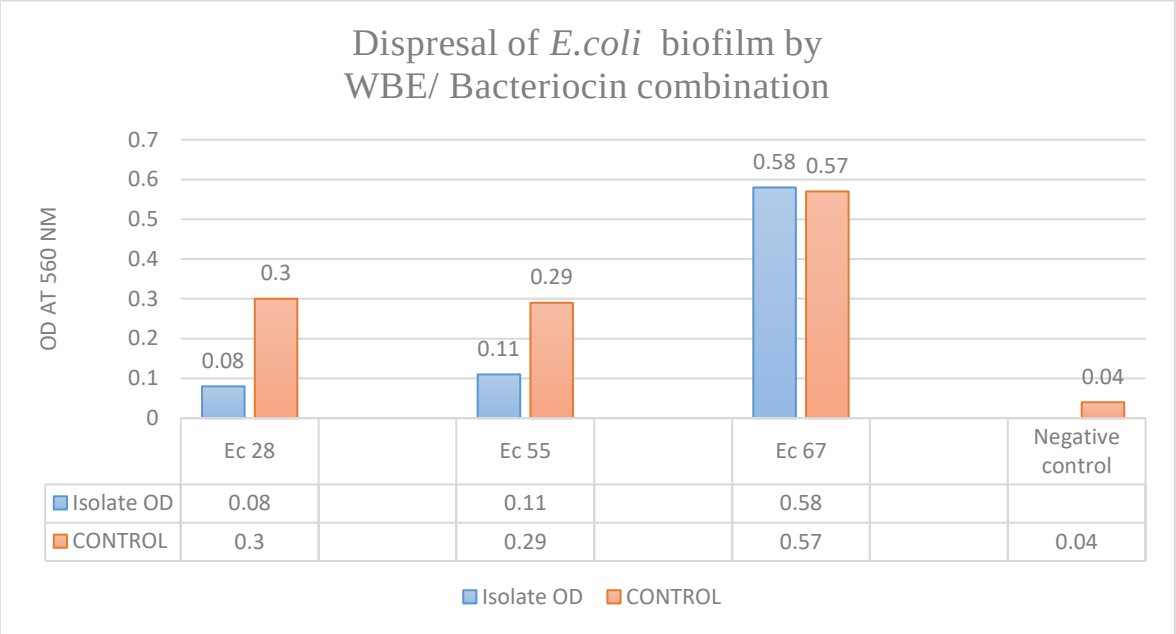

Figure 12. The antibiofilm effect of Ibaa99 bran extract (WBE)/ Bacteriocin derived from *Lacticaseibacillus paracasei* on *Escherichia coli* isolates biofilm using microtiter plate assay.

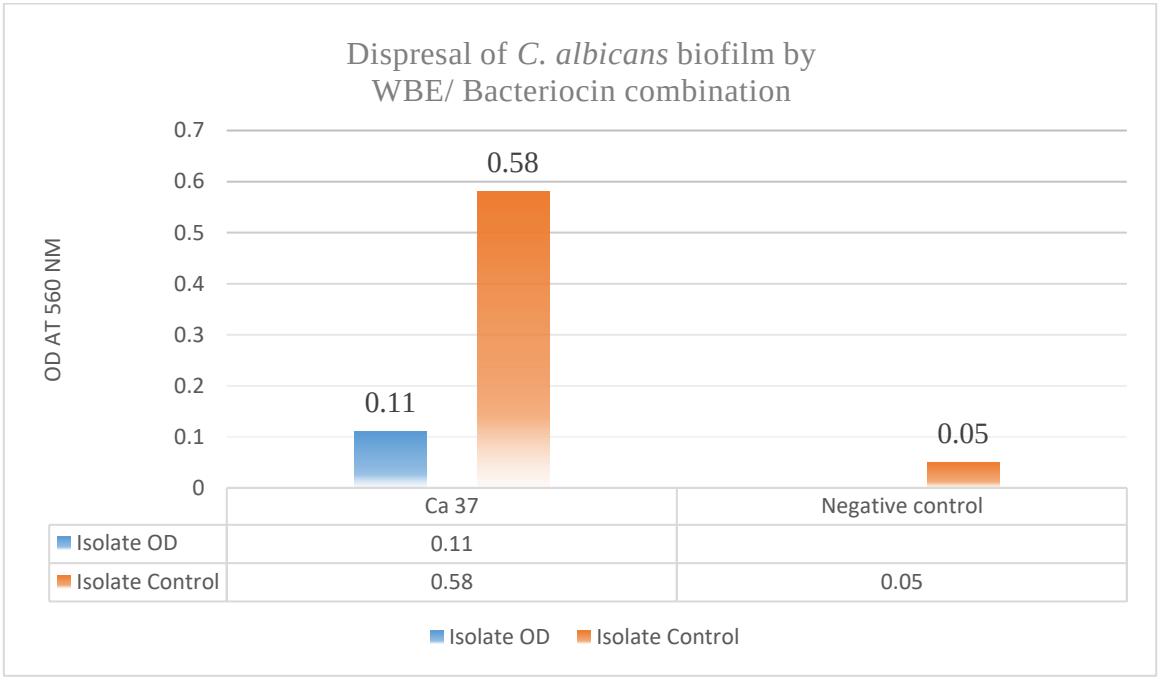

Figure 13. The antibiofilm effect of Ibaa99 bran extract (WBE)/ Bacteriocin purified from *Lacticaseibacillus paracasei* against *Candida albicans* isolate biofilm using the microtiter plate method.

Conclusion

Considering the outcomes of the present work, wheat bran, as a cheap by-product achieved from the milling process is rich in bioactive compounds, on the other hand, Bacteriocin from probiotics in combination showed a significant efficiency of synergistic combination as antimicrobial and antibiofilm metabolites over Gram negative, Gram positive, and fungal microorganisms as well, and could successfully be an alternative for more resistant microbes. Notably, pathogens gained more

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capability of resistance to several commonly used antibiotics due to numerous reasons updated over the years, such as the miss use, incorrect prescription, and the shortness in developing new antibiotics. Moreover, the combination status of wheat bran extract and bacteriocin might represent a new sight for developing a new antibacterial and antifungal compound, as previously the extraction of phenolic metabolites, inulin, and phytic acid from exclusively wheat bran could be considered rare and never reached; usually, such compounds have been extracted from other different types of plant.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

O. N. A. and M. R. M. Contributed to the design and implementation of the research, to the analysis of the results and to the writing and translation of the manuscript.

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الفعالية المشتركة لمستخلص نخالة حنطة محلية و البكتريوسين المضادة لفعالية بكتريا وخمائر الجهاز الهضمي و لتشكيل غشائها الحيوي

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الخلاصة

تسبب العديد من البكتريا والفطريات التهابات للجهاز الهضمي وتسبب اصابات حادة خاصة للاطفال, ويعد البحث عن مركبات جديدة تستعمل مضاد بكتيري وفطري ومشتت للاغشية الحيوية المايكروبية ضرورة اساسية في ضوء تزايد المقاومة المايكروبية لاسباب متنوعة, لذلك هدفت الدراسة الحالية الى التحقق من فعالية مستخلص نخالة حنطة محلية والبكتريوسين بحالة مزجهما معا المضادة للمايكروبات و المضادة لتشكيل الغشاء الحيوي لعدد من مايكروبات الجهاز الهضمي المعزولة محليا من اطفال مصابين بالمكورات العنقودية الذهبية الموجبة لصبغة غرام و بكتريا الايشيريشيا القولونية السالبة لصبغة غرام بالاضافة الى خمائر المبيضة البيضاء. درس المضاد البكتيري بطريقتي إخْتِبار إنتِشار الحفر وطريقة التركيز المثبط الأدنى و كمضاد للغشاء الحيوي بطريقة صفيحة المعايرة الدقيقة, و أظهرت النتائج ان للمزج المشترك لمركبي مستخلص نخالة حنطة محلية صنف اباء99 بتركيز 1 ملغم/ مل و البكتريوسين المنقى من بكتريا *Lacticaseibacillus paracasei* والمشخصة جينيا بتقنية 16S rRNA وبتركيز 0.54 ملغم /مل فعالية مضادة مشتركة للبكتريا والفطريات بالطريقتين ، وكان اعلى معدل قطر تثبيط ضد عزلات بكتريا المكورات العنقودية الذهبية وكان معدل قطر التثبيط يتراوح بين 21.5 الى 40 ملم. كما بينت نتائج فعالية تشتيت عالية ضد تشكيل الغشاء الحيوي حيث دون اعلى تشتيت بنسبة امتصاصية بلغت (0.11) لبكتريا المكورات العنقودية الذهبية . بالامكان الاستنتاج الى ان المزج التآزري لمركبي مستخلص نخالة حنطة نوع اباء99 والبكتريوسين المنقى من بكتريا معزولة من مصادر البان محلية الصنع اشارة الى رؤية جديدة من خلال مركبات طبيعية تمثل بدائل متوفرة ورخيصة كمضاد مايكروبي لعدد من البكتريا والفطريات ومضاد هام حين تكوينها للاغشية الحيوية.

الكلمات المفتاحية: المضاد البكتيري، المضاد الفطري، بكتريوسين، الغشاء الحيوي., مستخلص نخالة الحنطة.