"The synergistic Effect of secondary products of locale isolates of Lactic acid bacteria with sesame oil against *Aspergillus niger*

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Abstract

Food is essential for organisms to survive. Food preservation is a necessary process for keeping food safe. There are several difficulties with it, and adopting conventional techniques of food preservation would only exacerbate these issues and their adverse effects. Probiotics have been used as a last resort**.** One of the several types of bacteria used as probiotics is lactic acid bacteria (LAB). It creates a variety of antimicrobial substances, including antifungal ones .Therefore , secondary products of this local LAB isolates (*Pediococcus sp.*, *Lactobacillus sp*., and *Enterococcus sp* .) were extracted and synergistically with sesame oil. *Aspergillus niger* was treated with these extracts. The results of our study showed that there was an inhibitory activity for extracts of different bacterial isolates of LAB according to the type of those isolates, as well as there was a synergistic inhibitory activity of some bacterial extracts with sesame oil and that varies to the species of isolates, while some of isolates of LAB did not show any effectiveness, whether synergistic or otherwise, against *Aspergillus niger*.There was the highest synergy antifungal activity of *Lactobacillus sp* L9 extract with sesame oil at 8000% and a synergistic growth area reached 63.58 mm^2 followed by extract of *Lactobacillus sp* L5 (isolated from milk) synergistic with sesame oil with a synergy inhibitory activity against *A.niger* estimated at 2780% with a synergistic growth area 113.04 mm^2 ,while *Pediococcussp* L3, *Lactobacillus sp* L4 ,*Pediococcussp* L6 and *Enterococcus faecalis* L8 did not show any inhibitory effect . whether synergistic or otherwise , against *A.niger*

Key words : Lactic Acid Bacteria , sesame oil , synergistic activity , antifungal activity
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Introduction

The benefits of microbial species that inhibit in mammals, including humans, have received increased attention from researchers during the past several decades. The explanation is that the animal's largest bacterial reservoir is thought to be in its gut microbiome (Reuben *et al*. 2019).Widespread epidemics and health problems have been brought on by food-borne illnesses connected to the usage of fresh,

minimally processed agricultural products. Since there are now noticeable side effects from improper use of artificial antibiotics and preservatives. High-temperature thermal or chemical sterile treatments are common sterilization methods in the food sectors. However, harmful germs are not entirely organoleptic, and as a result, qualities that have been eliminated suffer. In order to increase shelf life and antibacterial capabilities, various studies attempt to utilize antibacterial compounds obtained from microorganisms (Ren *et al*. 2018).Microorganisms called lactic acid bacteria are employed to stop pathogen development and has been shown to have the ability to create integrated bio refineries .It is Gram-positive rods or cocci that can tolerate acid and are non-sporulating, non-motile, non-respiring but aerotolerant. Numerous antimicrobial metabolites are produced by LAB as it develops. These organic acids and their byproducts, such as phenyllactic, benzoic acids, fatty acids, hydroxy phenyllactic, volatile compounds (eg. diacetyl, acetone), cyclic dipeptides, hydrogen peroxide, hydrogen peroxide, reuterin, and/or proteinaceous substances, have an antifungal effect (Leyva Salas *et al*. 2017).Lactic acid bacteria have a long history of application in the food industry .Some species of LAB are found naturally in human and have favorable impacts on human health, because of their extensive biotechnological potential.. Some organisms are found in the human microbiome naturally, and several techniques have been developed to utilize their engineering advantages due to their extensive biotechnology potential and long history of use (Plavec and Berlec 2020).

Medicinal Plant

Plant substances have been used for decades to treat and prevent disease. Several pharmacologically active medications have been created from natural resources like medicinal plants (Heydari*et al*. 2019). In our study we searched the effect of sesame oil synergistic with secondary products of LAB against *Aspergillus niger*.

Fungus used in experiment

The functioning of habitats is significantly influenced by the diverse collection of organisms known as fungi. through means of symbiotic connections. The communities of fungus may be affected when their environment is contaminated with chemicals or becomes eutrophic because it needs particular ecosystem qualities for a suitable habitat (Newbound, Mccarthy, and Lebel 2010). In our study we used *Aspergillus niger*.*Aspergillus* may be found in a wide range of agricultural goods, including several varieties of fruits, onions, , nuts corn at a range of pH levels (6-47 °C) (1.5–9.8). Therefore, by serving as a model organism, it has significantly advanced our study. *Aspergillus* species' widespread significance propelled it to the forefront of fungus research in the scientific and industrial realms. The availability of *Aspergillus* species' genetic sequences led to an upsurge in *Aspergillus*research(De Vries *et al*. 2017).

Methodology

Collectionand Isolationof samples:

LAB were isolated from 50 different sources(canned food , raw milk of sheep, cows and goats, canned cheese, fermented meat , fruits , vegetables and fecal of infant whose his mother did not has any antibiotic and his age ages don't exceed 4 mounths .

. **Identification of lactic acid bacteria**

We performed Gram stain, Catalase, oxidase Reaction, IMVIC tests and Salinity Tolerance Test, after isolating the bacterial strains. Then, we decreased the samples based on similarity of phenotypic, microscopic features, and biochemical tests kept in MRS Agar slant tubes to be employed in the experiment. The isolates were identified as

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Lactobacillus acidophilus, Enterococcus faecalis, and *Pediococcusacidolactici*.

LAB Metabolites extraction

Following an overnight inoculation with 1 ml of active isolates, about 100 ml of MRS broth was fermented. To remove cells and collect the metabolites, we next performed a cooling centrifuge at 10,000 rpm for 10 minutes. The metabolites were then progressively precipitated to saturation with ammonium sulfate (80%), as described in(Vijay Simha*et al.* 2012).After cryogenic centrifugation,the precipitate was obtained and packed in to sterile tubes.

Total proteins estimation

Using a semi-auto analyzer, a tool based on the Biuret reaction, the proteins were quantitatively quantified (copper salt in an alkaline medium), when a protein sample is exposed to cupric ions in an alkaline solution, a blue colored complex results. The ratio of the protein content to the blue color's intensity. According to the equation:

Total protein con.(g/dl) =Absorbance of sample / Absorbance of standered * con. Of standered.

Where Wave lenghth = 546 nm Con . of standered was 6 g/dl

Getting sesame oil

Sesame oil (SO) was brought from Heet Mill after making sure that it was extracted by the cold method**.**

Getting Fungus

The biological sciences department of the college of science's fungal laboratory provided the *Apergillusniger*. diagnosed with biology.

Inhibitory Potency of sesame oil and synergistic bacterial and sesame oil extracts:

Discs made of filter paper with a diameter of 6 ml were immersed in sesame oil for 30 minutes to achieve saturation, which allowed the effect of sesame oil on *Aspergillus niger* to be measured. The same process was also used to bacterial extracts. *A.niger*was placed in the center of plate by cork borer , at 1 cm from fungus disc saturated with sesame oil was placed and at the same distance a disc saturated by bacterial extract was placed .On the third side with the same distance 1 cm two discs were placed adjacent . One for sesame oil and the other for bacterial extract synergistically , they were incubated at 25 ^{0}C and monitored for 5 consecutive days. The inhibitory ratio were calculated by measuring the diameter of discs. According to the equation(Al-Ani 2021):

I% = T- C/ T*100

Where I% is inhibition percentage , T is fungus diameter in comparation.

C is fungus diameter in the treatment .

The experiments were repeated as three times.

Results and discussion:

A total of 141 samples were collected in the city of Al-Ramadi from various sources. These samples were then cultured on MRS agar supplemented with Tween 80. The isolates were determined to be Gram-positive by microscopic investigations because they appeared under the microscope in red and in a variety of forms, including rods, Figure 1 and cocci Figure 2

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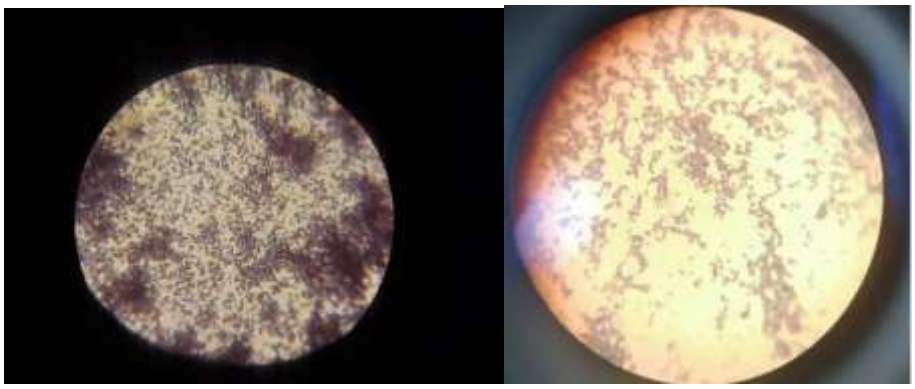

Figure (1) Bacilli shape of LAB under (100X) Figure (2)cocci shape of LAB under (100X)

Biochemical tests were carried out since the isolates were positive of Indole, Methyl Red, but negative of Citrate tests and VogaseProskaure . All isolates could grow on medium containing NaCl at levels of 4%, 6%, and 8%. Depending on the closeness of phenotypic and microscopic traits and the outcomes of biochemical tests, the number of isolates was first decreased to 37 and then to 10. then picked at random and diagnosed with a Vitek 2 system *Pediococcusacidolactici, Lactobacillus acidophilus* and *Enterococcus faecalis* were the isolates identified. All of these isolates were centrifuged for 15 minutes at a cooling rate of 1000 rpm after being precipitated with ammonium sulfate at saturation rates of 80% to 90%, which explains why the precipitates had a low molecular weight. The precipitate was recovered with 3 ml of distilled water while the filtrate was neglected.The sedimentation process did not correspond the to the study of (Islam et al. 2020) ,because the isolates were deposited at an ammonium sulfate saturation rate of 70%.Each isolate's precipitate was collected, and the amount of protein was quantified using an automated analyzer which its mode of operation relies on the Biurate reaction to determine total protein concentration. According to equation:

Total protein con.(g/dl)=Ab. of sample/ Ab. of standard * con. of standard

Where Ab. = 546 nm. , standard con. = 6 g/ dl

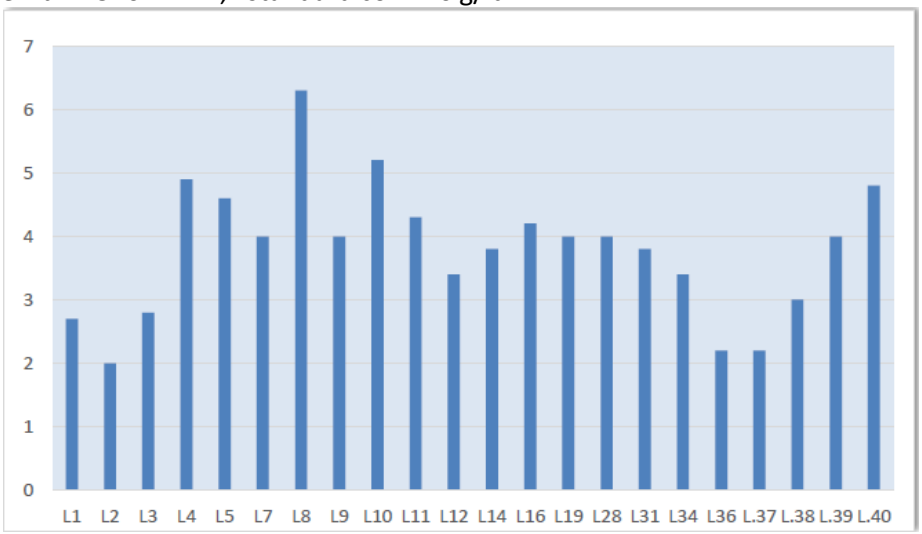

Figure (3) concentration of Proteins

Where L is a symbol of isolates

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The results showed that bacterial and sesame oil extracts have synergistic inhibitory effect. As in Table 1

	A.niger		
Samples	**Net growth area (2 mm)**	**Net Synergy area (2mm)**	**Inhibtion. %**
Sesame oil	0.785	-	-
L.acidophilus(L1)	0	7.065	800
Lactobacillus sp.(L2)	3.14	78.5	1900
Pediococcus sp.(L3)	0	0.785	0
Lactobacillus sp. (L4)	0	0.785	0
Lactobacillus sp. (L5)	3.14	113.04	2780
Pediococcus sp.(L6)	0	0.785	0
P.acidolactici(L7)	12.56	113.04	747.05
E.faecalis(L8)	0	0.785	0
Lactobacillus sp.(L9)	0	63.585	8000
Lactobacillus sp.(L10)	0	3.14	300

The results showed a synergistic inhibitory activity of *Lactobacillus acidophilus* L1with sesame oil against *A.niger* estimated at 800% with a synergistic growth area of 7.06 mm^2. *Lactobacillus sp.*L2 showed synergetic inhibitory by its extract with sesame oil estimated at 1900% and a synergistic growth area 78.5 mm^2. The extract of *Lactobacillus sp* L5 which isolated from milk showed a synergistic inhibitory effect with sesame oil was about 2780% with growth area at 113.04 mm^2 . Our study showed that the extract of *Lactobacillus sp* L9 with sesame oil had a synergistic activity against *A.niger* estimated at 8000% with a synergistic growth area of 63.58 mm^2, in addition to a synergistic inhibitory of *Lactobacillus sp* L10 which isolated from goat raw milk with sesame oil and it was 300% with growth area of 3.14 mm*2* .The experiment demonstrated the existence of a synergy inhibitory activity of *Pediococuusacidolactici* L7 with sesame oil reached at 747% and a synergistic growth area of 113.04 mm^2.The isolates *Lactobacillus sp.* L4 which isolated from carrots ,*Pediococcussp* L3 which isolated from kiwi , *Pediococcussp* L6 which isolated from carrot and *Enterococcus faecalis* L8 had no synergistic activity and did not effect on the growth of *A.niger* .From the foregoing ,it is clear that the results obtained for *Pediococcus sp*. are consistent with what was reached by (Vaitkevičienė et al. 2019) , ,where he proved that *Pediococcusacidolactici*did not show any activity against *Aspergillus niger* and *A.terreus*, while it showed medium and low effectiveness against *A.verciolor* and *A.fumigatus* ,as well as a

low and medium effect against *Pancillium sp*. Also the results of our study are parallel to whatYanina obtained at (Yanina*et al*. 2018) (Yanina*et al.* 2018),where he abled to isolate phanyllactic acid from *Pediococcusacidolacti*i and tested it on many fungi that grew on bread , including *Aspergillus niger ,A.japonicus ,Pancillium sp.* And some yeast like *S. cervisiae*. Yaninaproved the inability of *Pediococcusacidolacti*i to inhibit *A. niger*CH1and *A. niger* CH3, while it was able to inhibit *A. niger* CH2, *A. japonicus* and all *Pancillium*, while it did not inhibit *S. cerevisiae*. Nevertheless, it was found that *Pediococcusacidolactici* LAB5 showed a wide range of inhibition against fungi. This is what Chandra proved(Chandra 2013) this isolate was able to prevent the growth of all types of fungi, including the phytopathogenic (*Cladosporium herbarum),Colletotirchumacutatum ,Fusarium sp*. And *Aspergillus sp*., but it failed to inhibit *Alternaria alternate* and *Alternaria solani*. The fact that sesame oil contains phenolic compounds and alcohol groups are important in biological activity , making sesame oil appear antagonistic and synergistic effect. Where sesame oil interferes with the bilipid layer of the fungal cell wall and causes vacuoles to occur (Arasu*et al*. 2019) and thus allows the biological active bacterial metabolites to reach and destroy the contents of the cell and this supports the results obtained in our current study.Ion Exchange Chromatography was used to purify the *Pediococcusacidolactici*(L7) extract in order to find and demonstrate the presence of active compounds in bacterial extracts that showed antifungal activity. CMC is an ion exchanger that is used to purify cationic proteins. Using a CMC column, 28 fractions were produced. When the absorbance of each fraction was measured at 280 nm, one peak was seen to extend from fraction 14 to fraction 17As in figure 4:

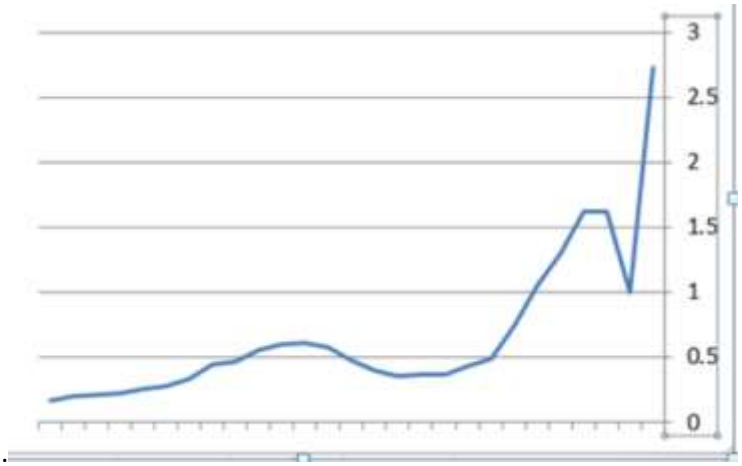

Figure (4) : Concentration of **protein**

The outcome from the CMC ion exchanger shows that this exchanger is distinctive in that proteins are separated well. Its capacity to separate proteins produced by a precipitation procedure at a saturation limit of 80%–90% may be what proves this. This suggests that the separated proteins have a relatively low molecular weight. The concept of charge-dependent linking on the osmotic force of the recovery solution is the key feature of CMC ion exchangers, which makes it easier to get various types of linked proteins.The study's results on protein separation agree with those of Sunying Zhou, a scientist.(Zhou et al. 2010).The primary peak of xylanase activity that was eluted at 0.2 M (NaCl) may be used to distinguish between the enzymes that interact with Avicel, CMC, and barley b-glucan, according to Bronnenmeier and his team (Bronnenmeier et al. 1995). As in Figure5 :

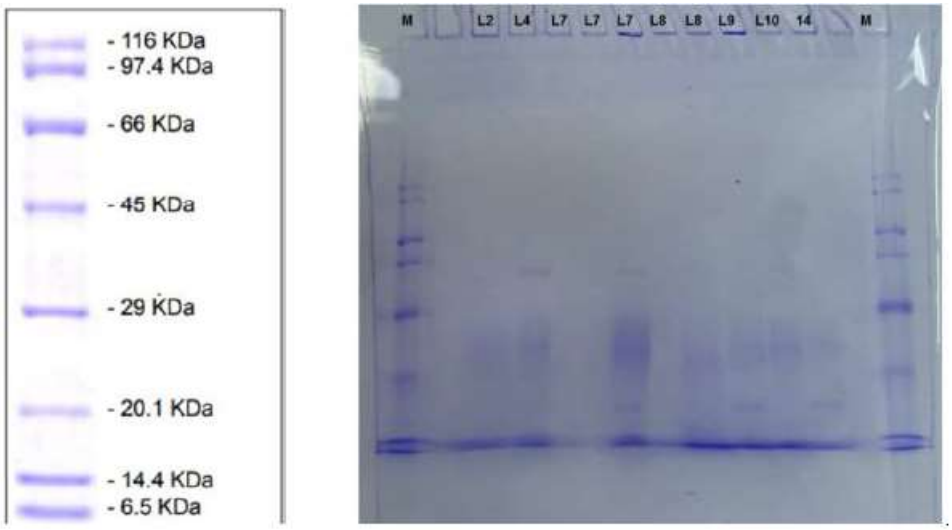

Figure (5): Electrophoresis in polyacrylamide gel in the presence of SDS PAG for calculation of molecular weight of bacterial extract. Where (A) leader proteins. (B)Electrophoresis for estimated the molecular weight of LAB extracts, where L2=L2extract, L4=L4extract, L7a = primary supernatant of L7 cells removed, L7b = proteins purified by ion exchange, L7C= L7extract, L8a=primary supernatant of L8extract, L8b=L8 extract, L9=L9 extract, L10=L10 extract, L14=L1extract.

The results of the electrophoresis were consistent with Salah's work(Saleh and El-Sayed 2004) , which was able to estimate the biggest and smallest molecular weight spectra of bacteriocin isolated from Bifidobacterium lactis at 89 KDa and 25 KDa, respectively.Our results were in line with those of Islam(Islam et al. 2020), who was able to extract and determine the bacteriocin generated by *Lactobacillus sp*. He calculated their molecular weights to be between 40 and 30 KDa for three different *Lactobacillus species*. Additionally, the findings of our investigation were in agreement with Chu's work (Chu et al. 2019) , who determined that the molecular weight of alkaline phosphatase released by Lactic Acid Bacteria was around 43 KDa. In a separation column with dimensions of 3*16 cm, the flow rate was 1 ml per minute, and the CMC column included the use of a buffer potassium phosphate solution (0.05 M) free on EDTA at a concentration of (0.0001M) with PH= 6 was recovered using NaCl at (0.5 M).This study is comparable to that of Rhee and Park(Rhee and Park 2001), who found anti-mutagenic activity in glycoproteins extracted from the fungus L. plantarum. They extracted the proteins using ammonium sulfate, purified them using DEAE-cellulose, divided the purified proteins into three fractions, and then put them through an electrophoresis gel. The three fractions each had a single band as a consequence, indicating that all three fractions contained the same glycoprotein.

Conclusion

LAB isolates which was collected from variety local sources has varying antifungal activity , according to the isolates species . The most LAB isolates which have antifungal activity were belong to *Lactobacillus* sp. And this effect was increased when synergistic with sesame oil .Some isolates have no antifungal activity but when synergistic with sesame oil , there were a noticeable

antifungal efficacy. This means that there is a successful synergistic relationship between LAB bacterial extracts and sesame oil against fungi.

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