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Article

The inhibition of selected pathogens by Lactobacillus bulgaricus and Streptococcus thermophiles bacteriocins.

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ABSTRACT

Bacteriocins were produced from Lactobacillus bulgaricus, Streptococcus thermophiles and MIX (Lactobacillus bulgaricus, Streptococcus thermophiles) by two methods: Shaker Fermentation and Static Fermentation (aerobic and anaerobic) through bacterial growth in MRS liquid medium, for each of them and the detection of the inhibitory activity on some types of pathogenic bacteria through the growth of the test bacteria on a solid nutrient medium Agar Nutrient. It was found that the three isolates can produce bacteriocin, and there are significant differences between each of the bacteriocins towards the pathogenic bacteria. It shows that the bacteriocin produced from Lactobacillus bulgaricus has a higher inhibitory activity and can inhibit different types of bacteria from gram-positive and gram-negative **keywords:** Doxazosin, Chloroquine, Synergism, MCF-7, Autophagy.

Keywords: Shaker Fermentation. Static Fermentation. Bacteriocins. Lactic acid bacteria.

INTRODUCTION

Despite modern technologies and safety concepts such as Hazard Analysis and Critical Control Points (HACCP), poisoning and food-borne diseases are still increasing. Foods fermented by lactic acid bacteria possess many antimicrobial activities, including producing lactic acid and other organic acids. Hydrogen peroxide and bacteriocins, and thus many bacteriocins with industrial applications, were isolated and purified that could be used in preserving foodstuffs, especially heat sensitive[7,10], Over the past years, lactic acid bacteria have attracted significant attention for their use as safe food preservatives as it is easily digested by the digestive system of humans[18,11]. Lactic acid bacteria are often active through a range of pH values, resistant to high temperatures and active against a group of pathogenic bacteria [1] *Lactobacillus bulgaricus* and *Streptococcus thermophiles* in the processes of fermenting food and known to man as safe (GRAS) And it can extend the shelf life and enhance the safety of food products [24] Research work focused on the use of LAB to produce bacteriocins, which maintain and prolong the storage life of food by inhibiting disease-causing bacteria, including gram-positive and gram-negative bacteria, and that bacteriocins are a group of compounds of a protein

nature (Active peptides) [8,6] produced by some microorganisms, including lactic acid bacteria, possess antagonistic properties against microorganisms, especially closely related organisms, to create a competitive environment that enables bacteriocin-producing organisms to make maximum use of nutrients in the growth environment, usually creating those Peptides in ribosomes are primary metabolites [15,16] and bacteriocins are sensitive to digestive proteases such as pancreatin complex, trypsin and chymotrypsin, and therefore do not negatively affect the gut microbiota[3].

MATERIALS AND METHOD

Bacterial isolates**:** Bacterial isolates were used in the study, including types of lactic acid and pathogenic bacteria, and were used to detect bacteriocin and estimate the inhibitory activity. Table (1) shows the bacteria used in the research and their sources. Moreover, used types of pathogenic bacteria, which are Bacillus cereus, Staphylococcus aureus, Salmonella typhimurium, and Escherichia Coli.

Source	Bacteria
The standard strain of freeze-dried starter manufacturer DANISCO France SAS Country of manufacture France	**Lactobacillus bulgaricus**
Standard strain of freeze-dried starter manufacturer DANISCO France SAS Country of origin France	**Streptococcus thermophiles**
Standard breed of freeze-dried starter from the manufacturer DANISCO Deutschland GmbH Country of origin Germany	**MIX(Lactobacillus bulgaricus, Streptococcus thermophiles)**

Table 1. Bacteria used in the research with their sources

Activation of bacterial isolates: Each of the three standard bacteria was activated by taking (0.2) g of the initiator and inoculating in [20] ml of MRS liquid medium. *Lactobacillus bulgaricus* was incubated at 37 °C for 24 hours while bacteria were activated *Streptococcus thermophiles* and mixed at a temperature of 42 °C ˚ for 24 hours and was activated on three consecutive days by taking (1) ml from the first activation and inoculating them in the MRS medium and keeping them at the temperatures and time as mentioned above. Temperature and time above [21].
How to prepare the filter: Use method [13] to prepare a bacteriocin filtrate. Centrifugation in culture at 6000 x g to remove bacterial cells for 10 minutes at 4 °C. The initial pH of the filtrate is adjusted to (6.5) using NaOH to exclude the effect of organic acids in inhibition. The filtrate is filtered. Through 0.2 µm porosity,

cellulose acetate membranes were used to examine the inhibitory activity of the selected bacterial isolates.

Detection of the ability of bacteria to produce bacteriocins: The disc method and the etching method were used to detect the ability of the isolates to produce bacteriocin.

Disc diffusion method: The method shown in [26] was used to detect the ability of bacteria to produce bacteriocin, as shown below: Dilutions were made from the test bacteria *B.cereus S. aureus, E. coli, Salmonella typhimurium* in the medium with peptone water and 100 micrometers of the bacterial suspension were taken from the fourth dilution and wiped superficially with L-Shape on the solid nutrient Media separately 50 µl of the filtrate was taken from each of the isolates and the 6 Mm diameter Whatman NO3 filter paper discs were soaked in the bacteriocin filter. The discs were placed on the surface of the solid feeder plate on which the test bacteria were grown, using sterile forceps, 3 discs per plate. The dishes were incubated at 37°C for 24 hours. The diameters of the damping areas around the discs were measured.

Diffusion method Well: The method shown in [26] was used as follows:

Dilutions were made of the test bacteria *B.cereus S. aureus, E. coli, and Salmonella typhimurium* in the medium with peptone water and 100 micrometers of the bacterial suspension were taken and wiped superficially with L-Shape on the solid nutrient media separately. Four Pit with a diameter of 6 mm were made in the dish in which the test bacteria were cultured, one standard and three for isolates. 50 µl of each filtrate was transferred to the pits and incubated at 37 °C for 24 hours. The diameters of the damping areas around the Pit were measured.

Study of the ability of bacteria to produce bacteriocin: To find out if the filtrate was bacteriocin or another substance, the filtrate was heated to a temperature of 70°C for 30 minutes, and the test bacteria were grown on the solid nutrient medium plate by making dilutions of the test bacteria *B.cereus, S. aureus, E. coli, Salmonella typhimurium* in the medium peptone water and 100 micrometers of suspended bacteria were taken and wiped superficially by L-Shape on the solid nutrient media separately. The Pit method was used to test the inhibitory activity of the filtrate against the test bacteria, and then the dishes were incubated at a temperature of 37 °C. For 24 hours, the inhibition zones were observed, the presence of which indicates that the filtrate is bacteriocin since the bacteriocin is not affected by temperature. However, if the zone of inhibition is not formed, the filtrate is not bacteriocin [5].

Bacteriocin production methods: Two methods are used to produce bacteriocin:

Shaker Fermentation Method: The method shown from [22] was adopted for the production of bacteriocin, where 2 ml of bacteriocin-producing cells were grown under aerobic conditions in 200 ml of MRS medium at pH 6.5 and a temperature of 37 °C ˚ 150 rpm for 24 hours in the vibrating incubator. The filter was used to examine the inhibitory activity.

Static Fermentation Method: The mentioned method from [22] was used at a temperature of 37 °C for 24 hours in aerobic and anaerobic conditions, where 2 ml of bacteriocin-producing cells were grown in aerobic and anaerobic conditions in 200 ml of MRS medium with pH 6.5 at a temperature. 37 C ˚ for 24 hours.

Estimation of bacteriocin efficacy: The efficacy of bacteriocin was estimated according to the method of [5].

By taking 100 microliters of the test bacteria suspension of *B.cereus, St.aureus, E. coli, and Salmonella typhimurium*, after dilution in the peptone water medium and wiping superficially with L-Shape on the solid feed medium, each separately. The dilutions were prepared by taking (100) µl of crude bacteriocin and diluted by adding (100) µl of peptone water previously prepared to make the dilutions and supplement the dilutions to (1/2, 1,/4, 1/8.1/16, 1/32, 1/64). Where 6 mL diameter pits

were made, as well as using discs, and each dilution was placed on the surface of the solid feeder planted with the test bacteria. The dishes were incubated at 37°C for 24 hours. It expressed the activity of bacteriocin (units/ml), and it was calculated according to what was stated in [4]
from the highest dilution that shows a clear inhibition area from the following equation:
Bacteriocin activity (unit/ml) = diameter of the zone of inhibition for highest dilution' reciprocal of dilution $\times\frac{\mathbf{1000}}{\mathbf{50}}$
Note that 50 microliters represent the amount of bacteriocin preparation in one hole.
Statistical analysis: All laboratory experiments were conducted using a fully randomized design (Two-way ANOVA in CRD). The averages were compared using the LSD test [2]. The analysis was done using Excel and Genstat V. 12.1 software.

RESULTS

Detection of the ability of isolates to produce bacteriocin: Detection of the ability of isolates to produce bacteriocin

Lactobacillus bulgaricus, Streptococcus thermophiles, and *MIX* for testing To detect their ability to produce bacteriocin by growing it in MRS liquid medium and to determine the best isolate for the production of bacteriocin using the Pit and discs method, and the best method for producing bacteriocin by the fixed and vibrating method by measuring the inhibition area around the pits and discs. *Escherichia Coli* and *Salmonella typhimurium* are gram-negative bacteria, as well as Bacillus cereus and *Staphylococcus aureus*, which are gram-positive bacteria.
It was noted from Table (2) the diameters of the inhibition zones by drilling method and using the vibrating production method that the highest activity of bacteriocin was for *Lactobacillus bulgaricus* against *Bacillus cereus* and *Staphylococcus aureus*, which gave the highest inhibition area with a diameter of (12.33, 14Mm) respectively, and the highest activity of bacteriocin was observed for *Streptococcus thermophiles* were against the test bacteria *Bacillus cereus* and Staphylococcus aureus, which gave an inhibition area with a diameter of (10.67 Mm).
In comparison, *MIX* bacteria gave the highest inhibition area for bacteriocin activity against Bacillus cereus and *Staphylococcus aureus* with a diameter of (12.00 and 11.00 Mm), respectively, for each of them. In contrast, the lowest area of Bacteriocin inhibition of *Streptococcus thermophiles* was late against *Salmonella typhimurium*, with a diameter of 7 Mm. Bacteriocin produced from Streptococcus thermophiles against the test bacteria was observed in Salmonella typhimurium with a diameter of 7 Mm.
As indicated in the statistical analysis, there were significant differences at the probability level of $P \geq 0.05$; the values of the inhibition diameters differed, and it showed that the bacteriocin produced by Lactobacillus

average producing bacteria	test bacteria				Bacteriocin-producing bacteria
	Salmonella typhimurium	*Staphylococcus aureus*	*Bacillus cereus*	*E. Coli*	
11.25[a]	8.33	12.33	14.00	10.33	***Lactobacillus bulgaricus***
9.08[c]	7.00	10.67	10.67	8.00	***Streptococcus thermophiles***
10.00[b]	8.00	11.00	12.00	9.00	***MIX***
0.628	1.256				**LSD**
	7.78[d]	11.33[b]	12.22[a]	9.11[c]	**average test bacterium**
	0.725				**LSD**

Table 2. Diameters of the inhibition zones for pit growthShaker Fermentation Method

average producing bacteria	test bacteria				Bacteriocin-producing bacteria
	Salmonella typhimurium	***Staphylococcus aureus***	***Bacillus cereus***	***E. Coli***	
[a]11.08	**9.00**	**12.00**	**13.00**	**10.33**	***Lactobacillus bulgaricus***
[c]8.25	**7.00**	**9.00**	**10.00**	**7.00**	***Streptococcus thermophiles***
[b]9.58	**8.00**	**11.00**	**10.33**	**9.00**	***MIX***
0.794	**1.589**				**LSD**
	8.00[b]	**10.67[a]**	**11.11[a]**	**8.78[b]**	**average test bac-**
	0.917				**LSD**

Table 3. The diameters of the discs inhibition zones Shaker Fermentation Method

Table (4) shows that the drilling method used fixed production, as the three isolates were incubated in aerobic and anaerobic conditions. The results showed that the highest inhibition zone was for bacteriocin produced by *Lactobacillus bulgaricus* against *Bacillus cereus* with a diameter of 15 Mm in aerobic conditions. In comparison, the bacteriocin produced by *Streptococcus thermophiles* and *MIX* had the highest inhibition zone towards the gram-positive bacteria of the test bacteria *Staphylococcus aureus* with a diameter of 12 and 13 mm, respectively, in aerobic conditions. At the same time, there was no inhibition zone for *Streptococcus thermophiles* and *MIX* isolates against the test bacteria *E. Coli* when grown in anaerobic conditions.

It is noted from Table (4) that the diameters of the inhibition zones for bacteriocin produced from the three isolates against the test bacteria by the disc method and by using the fixed production method in aerobic and anaerobic conditions. The highest inhibitory activity of *Streptococcus thermophiles* in aerobic and anaerobic conditions was against

The test bacteria Bacillus cereus and *Staphylococcus aureus* with a diameter of 10 Mm, and the highest inhibitory activity of bacteriocin produced from isolate *MIX* in aerobic conditions was against the test bacteria *Staphylococcus aureus* with a diameter of 12 Mm. In contrast, in anaerobic conditions, it was the activity. The test bacteria *Bacillus cereus* and *Staphylococcus aureus* had a diameter of 11 Mm. In contrast, the inhibitory activity of bacteriocin produced by *Streptococcus thermophiles* and *MIX* against the test bacteria *E. Coli* was harmful, and there was no inhibition zone.

It is noted from Table (5) the diameters of the inhibition zones for the bacteriocin produced from the three isolates against the test bacteria. The results obtained showed that the three isolates were capable of producing bacteriocin. The type that showed the highest inhibition by the rocking method and the fixed method in aerobic conditions by the method of discs and pits is *Lactobacillus. bulgaricus*, where the results of the statistical analysis at the probability level of P≥ 0.05 showed that bacteriocin produced from *Lactobacillus bulgaricus* has a higher inhibitory activity than bacteriocin produced from *Streptococcus thermophiles* and *MIX*.

Bacteriocin-producing bacteria	**test bacteria**				**average producing bacteria**
	E. Coli	*Bacillus cereus*	*Staphylococcus aureus*	*Salmonella typhimurium*	
Lactobacillus bulgaricus	10	15	14	11	12.5[a]
incubated in aerobic conditions					
Streptococcus thermo-	9	11	12	9	10.25[c]
incubated in aerobic con-					
MIX	10	12	13	10	11.25[b]
incubated in aerobic con-					
Lactobacillus bulgaricus	12	13	12	12	12.25[a]
Incubated in anaerobic conditions					
Streptococcus thermo-	0	11	10	9	7.50[e]
Incubated in anaerobic conditions					
MIX	0	12	11	11	8.50[d]
Incubated in anaerobic					
LSD	1.572				0.786
average test bacterium	6.83[c]	12.33[a]	12.00[a]	10.33[b]	
LSD	0.642				

Table 4. Diameters of the inhibition zones for digging by the Static method

Bacteriocin-producing bacteria	**test bacteria**				**average producing bacteria**
	E. Coli	*Bacillus cereus*	*Staphylococcus aureus*	*Salmonella typhimurium*	
Lactobacillus bulgaricus **incubated in aerobic conditions**	11	14	13	9	11.75[a]
Streptococcus thermophiles **incubated in aerobic conditions**	8	10	10	7	8.75[c]
MIX **incubated in aerobic conditions**	10	12	12	9	10.75[b]
Lactobacillus bulgaricus **Incubated in anaerobic conditions**	11	13	13	8	11.25[ab]
Streptococcus thermophiles **Incubated in anaerobic conditions**	0	10	10	7	6.75[d]
MIX **Incubated in anaerobic conditions**	0	11	11	8	7.50[d]
LSD	1.572				0.786
average test bacterium	6.67[c]	11.67[a]	11.50[a]	8.00[b]	
LSD	0.642				

Table 4.1 Diameters of the inhibition zones for digging by the Static method

These results agreed with what was indicated by [12], who stated that *Lactobacillus bulgaricus* has an inhibitory spectrum towards gram-positive and gram-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas Synsantha*. In contrast, he does not agree with him that it does not inhibit *E. coli* bacteria. The results also agreed that [17] *Streptococcus thermophiles* have inhibitory activity against many Gram-positive bacteria but do not inhibit *E. coli*.

Also, these results agree with what was indicated by [14] and [19] that lactic acid bacteria filtrates grown in MRS liquid medium have higher inhibitory activity against Gram-positive and Gram-negative test bacteria. He explained [9] that the test produces the antibacterial effect against certain types of bacteriocin with different physical and chemical properties and different genes and that the lethal activity is due to its lack of receptors for its transport, or it may be produced in small quantities that are unable to kill sensitive cells, and this shows that the inhibition of bacteriocin towards Some types of test bacteria. The results agree with what was indicated by [25], showing that bacteriocin produced by *Lactobacillus bulgaricus* can

inhibit *E. coli*. 23 indicated that many bacteriocins have a high inhibitory spectrum towards Gram-positive bacteria compared to Gram-negative bacteria due to the cell wall of Gram-positive bacteria containing only a peptidoglycan layer, which is an ineffective permeable barrier, which makes it very sensitive. The thin film of peptidoglycan on the outer membrane consists of phospholipids that carry the structural components of the lipopolysaccharide (LPS) layer, so the wall is more complex.

Figure (1) shows the inhibition zones for bacteriocin produced from the three isolates against the test bacteria *Salmonella typhimurium* by the disc method, fixed and aerobic conditions.

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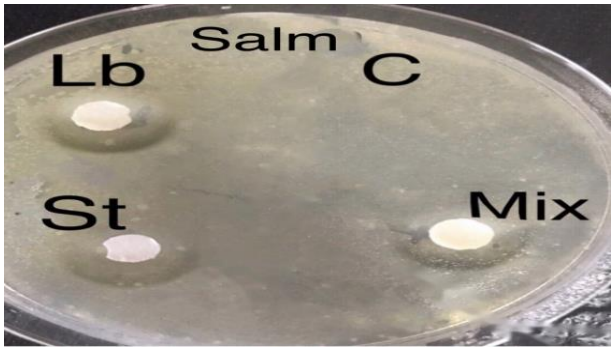

Figure (1) shows the inhibition zones for bacteriocin produced from the three isolates against the test bacteria *Salmonella typhimurium* by tablet and fixed method and aerobic conditions.

Study of the ability of the three bacterial isolates to produce bacteriocin: It can be seen from Figure (2) when exposing bacteriocin filtrates produced from the three isolates to heat and in aerobic conditions to ascertain whether the filtrate is bacteriocin or another substance. The isolate *Lactobacillus bulgaricus* showed a large spectrum of inhibition against Gram-positive and harmful test bacteria. In contrast, the filters of *Streptococcus thermophiles* and *MIX* showed inhibition towards *Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus cereus,* but *E.Coli* did not show inhibition against Accordingly, the three isolates produced bacteriocins with different inhibitory activity shown by each of the bacteriocins.

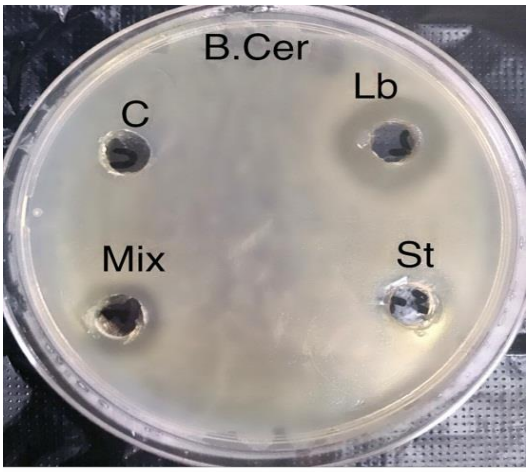

Figure (2) shows the inhibition zones for bacteriocin produced from the three isolates exposed to a temperature of 70 °C against the test bacteria Bacillus cereus under aerobic conditions4.

DISCUSSION

Bacteriocins were produced from Lactobacillus bulgaricus, Streptococcus thermophiles and MIX (Lactobacillus bulgaricus, Streptococcus thermophiles) by two methods: Shaker Fermentation and Static Fermentation (aerobic and anaerobic) through bacterial growth in MRS liquid medium. For each of them and the detection of the inhibitory activity on some types of pathogenic bacteria through the growth of the test bacteria on a solid nutrient medium Agar Nutrient. It was found that the three isolates can produce bacteriocin, and there are significant differences between each of the bacteriocins towards the pathogenic bacteria. **Keywords:** Doxazosin, Chloroquine, Synergism, MCF-7, Autophagy

CONCLUSIONS

It shows that the bacteriocin produced from Lactobacillus bulgaricus has a higher inhibitory activity and can inhibit different types of bacteria from gram-positive and gram-negative

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